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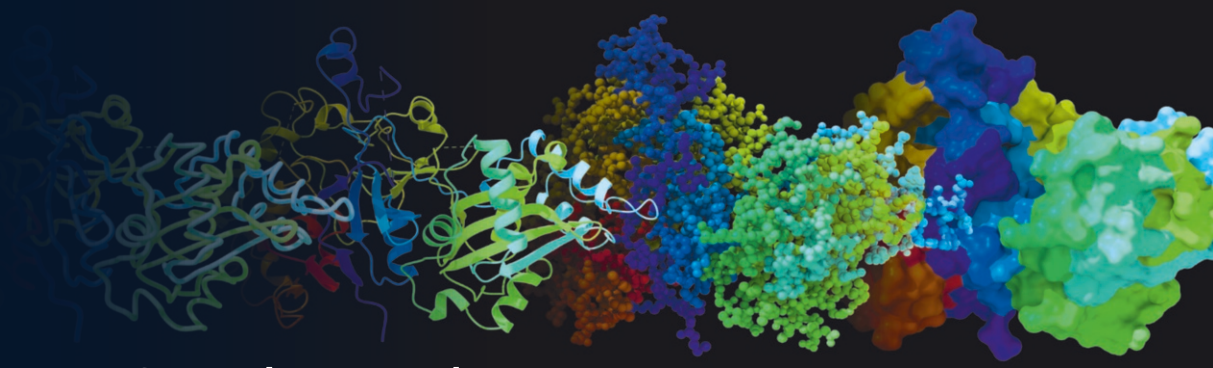

Anna S. Kashina *Editor*

Protein Arginylation

Methods and Protocols

Second Edition

Humana Press

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Second Edition

Edited by

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Preface

Posttranslational modifications are pivotal regulators of virtually every biological process and the major contributors to the complexity of functions in every physiological system. Arginylation – tRNA-mediated posttranslational addition of arginine to proteins – is a posttranslational modification of emerging importance that affects an estimated 25% of the cellular proteome and is critical for multiple aspects of embryogenesis and the functioning of an adult organism. The intent of this book is to present a comprehensive overview of all the existing methods on analyzing protein arginylation, from the early methods utilizing crude protein preparations and whole-cell assays to the latest advanced methods involving recombinant protein techniques, antibodies, high precision mass spectrometry, and chemical probes. The book also includes essays from the founders of the field, who originally discovered arginylation in early 1960s and brought it to international recognition. We hope that this book would be of interest not only to the emerging body of scientists involved in the studies of posttranslational arginylation but also to the rapidly growing community of researchers working on a broad range of posttranslational modifications, analysis of which often meets similar challenges and utilizes similar principles as posttranslational arginylation.

In this second edition, we preserved most of the original chapters, including the introductory chapter summarizing the first 50 years of the field and the concluding chapter outlining methodological approaches to arginylation studies. We also added a number of new chapters, describing methods that have been developed since the publication of the first edition. While new methods are always emerging in the field, this volume is as complete as possible to date and should serve as an essential reference to any researcher working on arginylation.

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Chapter 1

Protein Arginylation: Milestones of Discovery

Anna S. Kashina

Abstract

Posttranslational modifications have emerged in recent years as the major biological regulators responsible for the orders of magnitude increase in complexity during gene expression and regulation. These "molecular switches" affect nearly every protein in vivo by modulating their structure, activity, molecular interactions, and homeostasis ultimately regulating their functions. While over 350 posttranslational modifications have been described, only a handful of them have been characterized. Until recently, protein arginylation has belonged to the list of obscure, poorly understood posttranslational modifications, before the recent explosion of studies has put arginylation on the map of intracellular metabolic pathways and biological functions. This chapter contains an overview of all the major milestones in the protein arginylation field, from its original discovery in 1963 to this day.

Key words Posttranslational modifications, Biological complexity, Regulation, Protein arginylation

Genomes of higher mammals encode an estimated 30,000 proteins; however, the complexity of the functions performed by these proteins in vivo is orders of magnitude higher. This complexity is achieved in a large part by posttranslational modifications that modulate structure and functions of proteins after synthesis, thus increasing the variety of forms in which the proteins encoded by the same gene can exist in vivo (Fig. 1).

Evidence suggests that posttranslational modifications constitute a major mechanism for the regulation of virtually every biological process. To date, over 350 possible protein modifications have been described, and proteins in vivo have been found modified on virtually every reactive residue or group; however, only a handful of these modifications have been characterized to the extent that sheds major light on their biological functions. From this handful, it is already evident that understanding of posttranslational modifications will transform our knowledge of biology and that this direction is one of the most important – and complex – in modern-day research.

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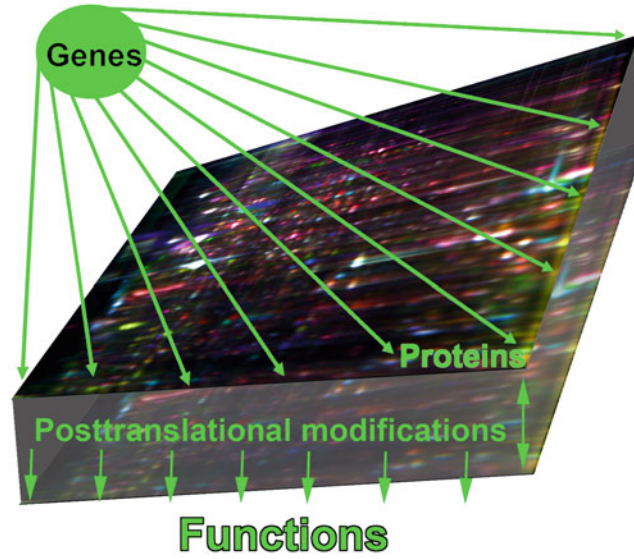

Fig. 1 Levels of biological complexity. A limited number of genes can generate a larger number of proteins through such events as differential expression and alternative splicing, but this complexity reaches another dimension through posttranslational protein regulation, achieved to a large extent by posttranslational modifications

Past and recent studies increasingly demonstrate that posttranslational enzymatic addition of various chemical groups can modulate protein structure, activity, molecular interactions, and homeostasis and thus regulate the functions performed by these proteins in vivo. Posttranslational modifications usually act as "molecular switches," regulating the on- and off-state of proteins and protein assemblies or driving their differential stability and degradation. More recently, finer aspects of this regulation are being identified, when one or multiple modifications can fine-tune the activity of a protein by attracting or repelling binding partners or modifying its active site. Discovery and understanding of new posttranslational modifications and novel forms of post-translational protein regulation is a pivotal, rapidly emerging field that ultimately holds a key to understanding biological complexity.

Until recently, protein arginylation has belonged to the list of obscure, poorly understood posttranslational modifications, before the recent explosion of studies has literally put arginylation on the map of intracellular metabolic pathways and biological functions. Notably, this year, at least two groups have independently solved the structure of Ate1, enabling new levels of critical insights into this enigmatic enzyme.

This volume constitutes a comprehensive collection of all the methods that have been employed in the studies of arginylation, since its original discovery in 1963, until the modern days. A special

introductory section in this book is devoted to recollections by those scientists who have originally discovered arginylation and shaped its early studies.

The timeline below lists all the major milestones in the protein arginylation field, from 1963 to 2023, marking the 60th year since its original discovery.

1 Early Milestones in Protein Arginylation Studies

1963: The Discovery of Arginylation In 1963, a group of researchers discovered that ribosome-free extracts from cells and tissues can exhibit prominent incorporation of specific radioactive amino acids into proteins. This was first observed in prokaryotes using Leu and Phe [1–4] and then was also seen in liver extracts using Arg [5, 6]. In both systems, the amino acid-incorporating activity was shown to depend on "small" RNA (s-RNA, the fraction later found to contain tRNA) (Fig. 2), leading to the belief that these studies constituted a discovery of "non-ribosomal protein synthesis." However, follow-up work has subsequently confirmed that this process of amino acid incorporation is independent of protein synthesis and that it depends on a new enzymatic activity that modifies the existing proteins by the addition of Arg using tRNA [1–3, 6, 7].

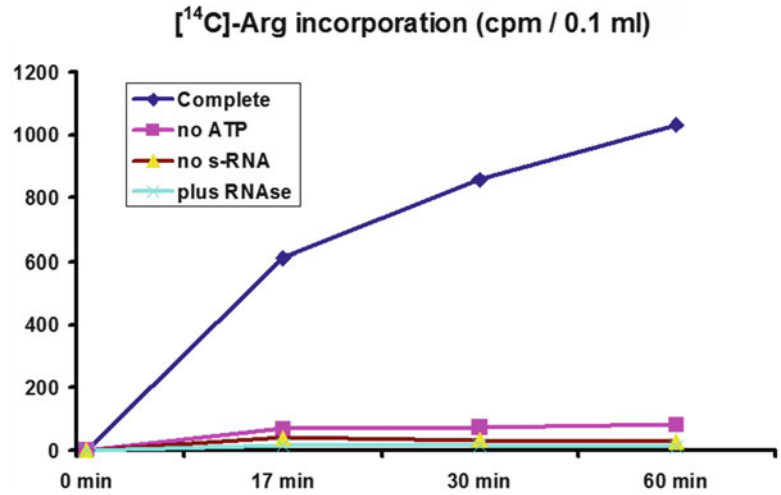

Fig. 2 The original discovery of protein arginylation as an activity in ribosome-free cell extracts that adds Arg to proteins in "small RNA" (s-RNA)-dependent manner. In the figure, selected data from the original publication on arginylation [5] is plotted to show the levels of [^{3}H-Arg] incorporation into proteins by the complete arginylation system (including the ATE1 activity-containing fraction from liver extract, ATP, s-RNA, and [^{3}H-Arg]) or the same system with the omission of ATP or s-RNA or addition of RNAse. This discovery, made in 1963, stemmed all the subsequent studies that spanned over 50 years and are still emerging with new breakthrough findings

1970s–1990s: Identification of Arginyltransferase During the next three decades, several groups actively continued arginylation studies. In addition to bacteria and mammalian tissues, a similar activity was described in plants [8] and guinea pig hair follicles [9]. Changes in arginylation have been implicated in aging [10], nerve regeneration after injury [11, 12], and oxidative stress [13]. Partial biochemical purification and characterization of the arginylation enzyme has been performed [14]. In 1990, this enzyme has been cloned and characterized in yeast, receiving its current name ATE1 (for Arginyl Transfer Enzyme [15]). Yeast ATE1 knockout proved to be viable, somewhat reducing further interest to arginylation studies at the time.

1970s–2000s: Characterization of Arginylation Specificity and Early Attempts at Identification of Arginylated Proteins Since its early discovery, many of the arginylation studies have been focusing on the attempts to identify arginylated proteins in vivo and characterize the target sites for protein arginylation. It was found early on that Arg can be incorporated into membranes and chromatin [6, 7, 16], as well as into dozens of unidentified gel bands in preparations from different tissues and/or different species [10, 17–23]. Different approaches showed that ornithine decarboxylase [24–26], BSA, alpha-lactalbumin, and thyroglobulin [14, 27] can be arginylated, the latter three – after the removal of the N-terminal signal peptides to expose the N-terminally arginylatable residues. In addition, studies revealed arginylation of regulatory peptides and hormones, including neurotensin (identified in vivo, [28]), as well as in vitro-tested beta-melanocyte-stimulating hormone [29], insulin [13], and angiotensin II [29]. The major commonality discovered among all these arginylation target involved N-terminally exposed acidic residues, Asp or Glu, leading to an early conclusion that arginylation is exclusively N-terminal and specifically modifies N-terminally exposed acidic residues by the addition of Arg via a conventional peptide bond.

1980s–2000s: Arginylation and Ub-Dependent Protein Degradation The discovery of the N-end rule pathway of protein degradation showed that a protein's half-life can depend on the identity of its N-terminal residue [30]. In the original study, the researchers systematically tested artificially engineered recombinant beta-Gal-derived proteins, with different amino acids in the N-terminal position, in a yeast expression system. While the "wild-type" beta-Gal containing N-terminal Met proved to be highly stable in this system, substitution of Met to several other N-terminal residues, including Arg, led to its rapid degradation, resulting in the near abolishment of the beta-Gal signal [30, 31]. Moreover, N-terminally placed Asp and Glu – also destabilizing in this system – were later proved to

undergo arginylation, and it was shown that arginylation, not the Asp/Glu per se, is responsible for these substrates' degradation [31–33]. Further tests showed that in mammalian systems, unlike yeast, N-terminal Cys can also become arginylated and subsequently destabilized and that in the case of Cys, this likely occurs after its in vivo oxidation to cysteic acid, which yields a side chain structure reminiscent of Asp and Glu. Thus, N-terminal arginylation has been claimed as a universal signal for protein degradation targeting N-terminally modified proteins and protein fragments containing N-terminally exposed Asp, Glu, or Cys. In support of this idea, it was found that N-terminal Arg attracts Ub conjugation machinery, which can lead to protein ubiquitination and degradation [34].

Starting with these discoveries, many papers cite arginylation as a branch of the N-end rule pathway and name ATE1 as a component of protein degradation machinery. However, follow-up studies suggested that this label may be premature and that the biological effects of ATE1 may reach further than protein degradation. Despite extensive screens, very few proteins in vivo were found to be destabilized upon arginylation. To date, only one class of naturally occurring targets of this pathway have been proven to exist: several members of the regulators of G-protein signaling (RGS) family [35, 36], containing Cys in the second position of their coding sequence. It is believed that such RGS proteins undergo removal of N-terminal Met, followed by Cys oxidation and arginylation that leads to their decreased metabolic stability. It has been proposed that such Cys oxidation-dependent degradation participates in oxygen sensing and protection of cells from oxidative stress and that this mechanism may ultimately underlie many RGS-dependent regulatory processes, but overall this mechanism has not been widely studied. At the same time, no other in vivo arginylation targets that follow the N-end rule and massively degrade after arginylation have been identified.

2000s: ATE1 Plays an Essential Biological Role After the initial finding that ATE1 is not essential for cell viability in yeast, Ate1 gene has been labeled as "non-essential," and most of the functional studies of this enzyme have been abundant. However, in the course of characterization of the N-end rule pathway, Ate1 gene has been identified by small stretch homology searches in multiple eukaryotic species [8, 15, 37, 38]. It became clear that while Ate1 in these species share very little overall sequence homology, this gene is present in all eukaryotes and appears to be functionally conserved. In mice and human (and possibly in some of the lower eukaryotic species), Ate1 gene encodes multiple isoforms generated by alternative splicing [37–39].

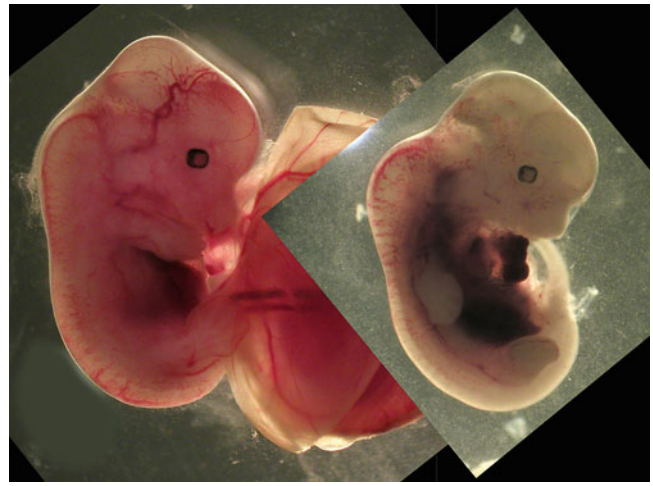

Fig. 3 Mouse knockout of arginyltransferase ATE1 leads to embryonic lethality. Littermate wild-type (left) and Ate1 knockout (right) E12.5 mouse embryos exhibit marked differences in shape and blood vessel prominence and patterning, due to the underlying defects in cardiovascular development, angiogenesis, and craniofacial morphogenesis. This discovery, originally published in 2002 [40], for the first time, demonstrated ATE1's essential biological role

In 2002, the first mouse knockout of Ate1 has been generated. Strikingly, this knockout led to embryonic lethality and severe defects in cardiovascular development and angiogenesis [40] (Fig. 3). This finding constitutes the first proof that ATE1 plays an essential biological role.

2000s–2010s: Physiological Role of ATE1 The first publication on the severe consequences of ATE1 deletion in mice [40] led to the re-emergence of the field. In the subsequent years, a series of studies from different groups demonstrated the essential role of ATE1 in different physiological systems using tissue-specific and complete Ate1 knockout mouse models. It was shown that ATE1 regulates neural crest-dependent craniofacial morphogenesis [41], the formation and contractility of the cardiac muscle [42], and gametogenesis [43]. Postnatal deletion of ATE1 was found to affect multiple physiological systems, leading to weight loss, mental retardation, and infertility [44]. ATE1 deletion in *Arabidopsis thaliana* led to delayed leaf senescence [45, 46], defective shoot and leaf development [47], and abnormal seed germination [48]. Genomic screens showed that while ATE1 did not affect viability of *C. elegans*, it led to embryonic lethality in *Drosophila*, suggesting that the key physiological systems that depend on arginylation for survival have evolved somewhere between these species [49].

After 2004: Arginylation Affects Multiple Proteins on Different Sites In 2007, the first mass spectrometry-based approaches enabled the high-throughput identification of proteins arginylated in vivo [50, 51]. Notably, this analysis utilized samples from wild-type tissues and thus was biased toward proteins that remained abundant after arginylation, likely omitting those that depend on arginylation for ubiquitin-dependent degradation via N-end rule pathway. To date, over 100 arginylated proteins were found in different tissues, revealing several interesting trends. First, while most of these proteins appeared to be arginylated on a very limited set of sites (mostly 1 site per protein), many of these proteins were not modified N-terminally and often affected residues different from Asp/Glu, which have been found previous studies to constitute preferential targets for ATE1. It is possible that ATE1's specificity in vivo can be modulated by protein or non-protein cofactors to target other amino acid residues. It is also possible that other arginyltransferase(s) with different substrate specificity also exist in vivo.

Some of the in vivo arginylated proteins have been found either mono- or dimethylated on added Arg, suggesting that these added Arg are long-lived and bringing up a possibility that this double modification may play a new, previously undiscovered, biological function [52].

More recently, it has been found that proteins can be arginylated in vivo on side chains of Asp/Glu, rather than their N-terminus (Fig. 4), and identified a number of such "intact"

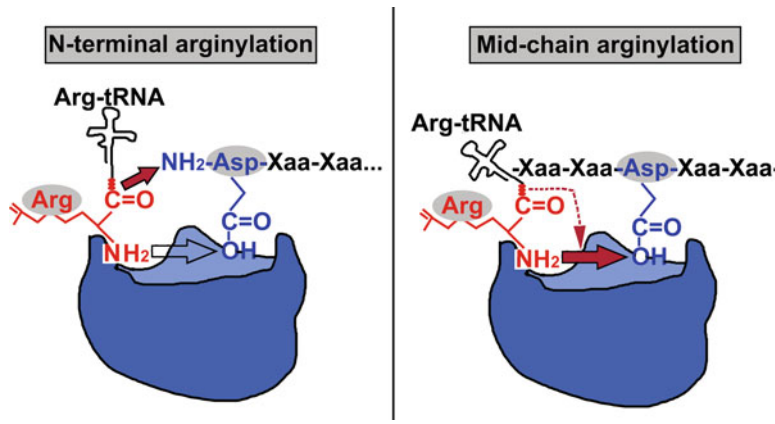

Fig. 4 Arginylation can affect a protein's N-terminus or side chains of acidic residues (Asp or Glu). In 2014, it has been found that in addition to the previously demonstrated ATE1-mediated addition of Arg to the proteins' N-termini, the same enzyme can also mediate addition of Arg to the acidic side chains of Asp or Glu within the intact protein chain [28]. This discovery expands the potential scope of the functional consequences of arginylation and demonstrates its ability to target intact proteins in vivo, in the manner reminiscent of protein phosphorylation

proteins arginylated in vivo [28]. This discovery significantly broadens the scope of potential in vivo effects of ATE1, since the fact that protein side chains can be modified points to a spectrum of intact protein targets, which do not need to be primed for arginylation by proteolytic pre-cleavage or the action of aminopeptidases to expose target sites. Similar to protein phosphorylation, and other regulatory posttranslational modifications, it is now known that arginylation can potentially serve as a molecular switch that modulates transient regulation of biological processes.

2006–Present: Functional Consequences of Arginylation on Individual Proteins The discovery of intact proteins that are modified in vivo by arginylation and do not subsequently degrade by the N-end rule pathway led to an emerging view of this modification as a potential global biological regulator parallel to such modifications as protein phosphorylation. The molecular effects of arginylation on individual proteins proved to be quite diverse. It was found that N-terminal arginylation of beta-actin facilitates cell migration by driving lamellipodia formation [53] and actin polymerization in vivo [54]. Arginylation of calreticulin during the ER stress facilitates its role in stress granules [55–58]. Arginylation of a proteolytic fragment of the cell adhesion protein talin mediates its novel role in cell-cell adhesion [59]. Myosin arginylation affects its regulation by phosphorylation and its contractility in platelets [60] and directly controls its contractile strength in the skeletal muscle [61]. Recent studies identified the role of arginylation-dependent degradation in oxygen sensing in plants through targeting of ERF-VII transcription factors RAP2.12 and RAP2.2, the primary activators of the anaerobic response genes [62]. Even more strikingly, arginylation was recently proposed as a key regulator of the cellular *autophagic* flux and clearance of proteotoxic proteins as a master switch between these two major protein degradation pathways [63–67]. Studies of other arginylated proteins will eventually enable assessing the broad spectrum of functions of this emerging regulatory pathway.

2010: Arginylation Can Be Regulated Co-translationally Through Coding Sequence-Dependent Changes in Translation Speed An unexpected discovery in the arginylation studies stemmed from the fact that one of the first well-characterized arginylated proteins, beta-actin, is selectively arginylated on the N-terminus unlike its close homolog, gamma-actin, which is nearly identical in the amino acid sequence and is equally abundant in non-muscle cells. The mechanism for such selectivity could not be explained by the minor differences in the amino acid sequence of these two proteins and initially presented a puzzle of its own. Remarkably, in-depth studies demonstrated that this selective arginylation of beta- but not gamma-actin is regulated by differences in their mRNA coding sequence, rather than in their amino

acid sequence. Indeed, these two actin isoforms are over 99% identical at the amino acid level but are encoded by different genes and contain a number of synonymous substitutions, accounting for ~13% difference in their coding sequence. This difference leads to different secondary structure of beta- and gamma-actin mRNA, resulting in faster translation of beta-actin compared to gamma. As a result, co-translationally arginylated gamma-actin remains in a nascent state for a longer period of time and becomes accessible to arginylation-dependent ubiquitination and degradation, while beta-actin completes its translation and folding much faster, escaping such degradation [68]. This finding constituted the first demonstration of the regulation of protein's posttranslationally modified state by coding sequence and unexpectedly bridged the studies of arginylation-dependent N-end rule degradation and functional modification by arginylation. In the case of actin isoforms, N-end rule degradation appears to be a regulatory mechanism that ensures the specificity of arginylation toward the faster-translated, functionally unique beta-actin isoforms. It is possible that, rather than being a major consequence of arginylation, ubiquitin-dependent N-end rule degradation actually constitutes one of the mechanisms of its regulation. It is also likely that other closely homologous protein isoforms with vastly different coding sequences are regulated by the same mechanisms.

2022: Structure of Arginyltransferase ATE1 Most recently, two independent groups solved yeast Ate1 structure using X-ray crystallography. One group solved the structure of Ate1 from *S. cerevisiae* [69]. Almost at the same time, the other group crystallized Ate1 from another budding yeast, *Kluyveromyces lactis*, a thermostable species more amenable to structural studies [70]. Both groups characterized ATE1 as a bilobed protein containing a GNAT fold, greatly expanding on early structural predictions that proposed a "dupli-GNAT" fold of ATE1 [71], and gained major insights into the mechanisms of ATE1-mediated enzymatic catalysis. These studies represent a major milestone in the field that will enable utilization of this newly obtained structural knowledge to understanding ATE1's mechanisms of substrate recognition, tRNA specificity, and Arg transfer to protein and peptide targets.

In summary, protein arginylation studies have experienced many phases of growth during the last six decades. At present, these studies are rapidly expanding, and multiple researchers are joining these studies and uncovering different pathways that involve protein regulation by arginylation. It seems likely that in the near future, we may see arginylation added to the commonly known major processes that regulate protein function in vivo and constitute an overwhelming driving force for increasing biological complexity.

This book includes a comprehensive overview of the available methods, tools, and techniques that have driven the discoveries in this exciting emerging field from the early pioneering studies to the present day.

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Chapter 2

Recollection of How We Came Across the Protein Modification with Amino Acids by Aminoacyl tRNA-Protein Transferase

Hideko Kaji and Akira Kaji

Abstract

Protein arginylation has been discovered in 1963 as a soluble activity in cell extracts that mediates the addition of amino acids to proteins. This discovery was nearly accidental, but due to the persistence of the research team, it has been followed through and led to the emergence of a new field of research. This chapter describes the original discovery of arginylation and the first methods used to demonstrate the existence of this important biological process.

Key words Discovery of arginylation, Soluble amino acid-incorporating system, Assaying arginylation in cell extracts

In 1962, we were delighted to join Dr. David Novelli's laboratory at Oak Ridge National Laboratories. Dr. Novelli was well known at that time for his work on protein biosynthesis. Before his work in protein synthesis, he was known for his work on the discovery of CoA together with Dr. Fritz Lipmann. The field of protein biosynthesis was one of the hottest areas at that time. In 1955, Crick announced his adaptor hypothesis for the transfer of genetic messages stored in DNA to protein [1]. In essence, this proposes that it is the tRNA and not the amino acid which determines where that amino acid will be incorporated into the protein according to the information stored in DNA. The hypothesis that RNA called messenger RNA is an intermediate of the information transfer between DNA and protein was well accepted, and the proof of existence of such RNA was just published [2]. Furthermore, the elucidation of the control of gene expression by a molecule called repressor has just been announced in a well-known paper describing the so-called pajama (meaning Pardee, Jacob, and Monod) experiment [3].

The Novelli lab was prosperous, and we were surprised to see more than 20 post-docs and staff members working on the

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mechanism of protein synthesis. At that time, only in vitro de novo protein synthesis was that of beta-galactosidase [4], and most of the assay was performed by measuring the ^{14}C amino acid incorporation into hot trichloroacetic acid (TCA)-insoluble fraction. We were instructed to follow this method to study in vitro beta-galactosidase synthesis. One of us (HK) was given a parallel project on the in vitro synthesis of permease and another on the ORF of the lactose operon. One odd thing that struck us was that almost everyone in the lab was studying protein synthesis by following the incorporation of either ^{14}C leucine or ^{14}C phenylalanine. We asked Dr. Novelli why these two amino acids are used mainly as a marker of protein synthesis. Dr. Novelli said "because they are incorporated into hot TCA-insoluble fraction best." As described above, ^{14}C amino acid incorporation into hot TCA-insoluble fraction was a synonym of protein synthesis at that time. We both felt a bit strange because we knew that proteins consist of 20 amino acids and phenylalanine and leucine are only 2 of them. However, no further discussion ensued.

As a preliminary step for studying de novo protein (beta-galactosidase and permease) synthesis, we felt strongly that we have to have a system which is stimulated by the production of mRNA freshly transcribed by RNA polymerase from added DNA. To accomplish this, we used crude DNA-dependent RNA polymerase described by Chamberlin and Berg [5] and were delighted to see a nice stimulation of ^{14}C phenylalanine and leucine incorporation into hot TCA-insoluble fraction. This was consistent with the dogma that mRNA freshly made from DNA is directing beta-galactosidase or permease synthesis. However, we quickly noticed that this nice stimulation of "protein synthesis" was not much dependent on added DNA. Despite this, we kept working on this system for about 1 month trying to convert the amino acid incorporation into de novo synthesis of the enzymes.

The big surprise came when we accidentally forgot to add the ribosome fraction into our reaction mixtures. Since protein synthesis was strictly dependent on the ribosome, it was kind of foolish to conduct a control without ribosomes. Therefore, we did not intentionally conduct the control. It was an "absentminded" oversight. Surprisingly, the incorporation of phenylalanine and leucine was only reduced by 10–20%. We wondered: "Protein synthesis without ribosomes?" We of course did not believe our result in the beginning, until it was repeated many times. We then subjected the entire ribosome-less extract to sucrose density centrifugation and asked where the ^{14}C phenylalanine or ^{14}C leucine incorporation activity resided. The activity peak was far smaller than the ribosome fraction! At this point, we walked into Dr. Novelli's office and told him: "We believe that 'protein synthesis' occurs without ribosomes in our system." The immediate response from our post-doc mentor was "forget it." But we didn't. We spent the next 2 months proving

that what we observed was true and that certain amino acids are incorporated into preexisting proteins. We will not list the evidence here because it is well documented [6–10]. Although we did not publish this, we even took electron microscopic picture to prove that ribosomes do not exist in our extract.

At this point, Dr. Novelli gave us permission to present the data at the Biochemistry meeting.

The question remains, why was it that so many people who studied "protein synthesis" by following radioactive amino acid incorporation did not realize the existence of this protein modification system. There are a number of strange coincidences, which misled many scientists – including us – in the beginning. First of all, the incorporation of radioactive amino acids into hot TCA-insoluble fraction was the only way of studying protein synthesis at that time. The first claim of in vitro de novo protein synthesis [4] was confirmed and extended only by one publication [11], and no further work was reported. The stimulation of the amino acid incorporation "by the ribosomes" for such in vitro "protein synthesis" was observed because the ribosome fraction contained the acceptor protein for the transfer of phenylalanine and leucine (see references above) and the omission of the ribosomes indeed reduced such "incorporation" to some extent. Our finding that the ribosomal fraction contains such acceptor proteins was later confirmed [12]. To make the matters worse, the crude RNA polymerase fraction prepared according to the Berg method [5] (protamine precipitate) had very high concentration of unknown acceptor proteins. The addition of this "RNA polymerase" gave a significant stimulation of "protein synthesis" measured by the incorporation of ^{14}C phenylalanine or leucine. This observation gave a false credence to the "protein synthesis" measured by the incorporation of these amino acids because the interpretation was that mRNA made by the polymerase in the presence of added DNA boosted the "protein synthesis."

One additional feature of the soluble system which mimics the ordinary ribosome-dependent protein synthesis is that it is very efficiently inhibited by puromycin [9]. This is quite understandable in retrospect, because the well-known effect of puromycin on the ribosome-dependent protein synthesis is due to its partial structural similarity to aminoacyl tRNA, which binds to the A-site. The product is peptidyl puromycin. In the leucine and phenylalanine transfer reaction, the transferase recognizes aminoacyl tRNA and transfers the amino acid moiety to the acceptor protein. Puromycin binds to the site where aminoacyl tRNA binds to the transferase. The product would be aminoacyl puromycin. In the regular ribosome-dependent protein synthesis system, the product is peptidyl puromycin. These features fooled many researchers and made them miss the existence of the soluble system. Although the deletion of the gene coding for the phenylalanyl- and/or leucyl-tRNA

synthetase was not lethal to *E. coli* [13], it appears to play an important role for the maximum growth rate of bacteria. Together with the ribosome recycling factor (RRF) [14–16], these enzymes and factors could be important targets of new antibiotics because they are bacteria-specific and higher eukaryotes do not have these factors for the cytoplasmic protein synthesis.

Upon the discovery of the soluble system for the incorporation of ^{14}C phenylalanine and leucine, we immediately turned our attention to the question of whether a similar system exists in eukaryotes, namely, rat liver. It should be noted that in the early 1960s, most biochemists were using rat liver as the source of enzymes. The use of the *E. coli* system for protein synthesis was only introduced a few years before 1963 by the pioneering work of Lamborg and Zamecnik [17]. For the reasons that the ribosome-less system for amino acid incorporation from higher eukaryotes turned out to have an important role with an important biological significance, as the rest of this book indicates, brief description of how we prepared the system is given below with historical recollections.

Male rats (Sprague-Dawley strain) weighing 250–300 g were decapitated, and their livers were placed in ice-cold 0.3 M sucrose solution. In 1962, there were no rules about handling live animals for experimental use, and we were quite free to sacrifice them according to our experimental needs. We are not sure if decapitation is allowed to kill rats nowadays. After excess blood was washed off in the ice-cold sucrose solution, 100 g of liver was minced by scissors in 15 mL of Medium A containing 4 mM Mg-acetate, 12.5 mM KCl, 0.01 M beta-mercaptoethanol, 0.05 M Tris–HCl (PH 7.8), and 0.3 M sucrose. Minced liver (5 g) was then homogenized with the Potter-Elvehjem glass homogenizer. The total volume of Medium A added was 250 mL (2.5 times the tissue weight), and cellular debris were removed by centrifugation at 20,000 g for 20 min. The top lipid layer was removed and discarded. This was because it was believed that lipid can accept amino acids from aminoacyl tRNA as has later been reported [18]. In fact, this tradition of removing lipid is continued even now, since most of the protein preparations for amino acid incorporation are washed through the ether-alcohol mixture (50/50) to remove lipid [19]. The supernatant after the removal of the debris was re-centrifuged at 32,000 g for 20 min after dilution with 1.5 volume of Medium B (the same as Medium A without Tris–HCl). The lipid layer was again aspirated and discarded. The supernatant (S32) thus obtained was centrifuged at 85,000 g for 2.5 h, and the resulting supernatant (S-85) was fractionated as follows. First, protein concentration was adjusted to 12 mg/mL using Medium A. Then, 0.05 vol. of freshly prepared 10% streptomycin sulfate was added. The precipitate (the complex of nucleic acid and streptomycin) was removed by centrifugation, and to the supernatant,

0.05 volume of 1% protamine in Medium A was added. The protamine precipitate was eluted with 50 mL of 0.3 M ammonium sulfate in Medium A. The protamine eluate was centrifuged to remove insoluble material. The fractionation by ammonium sulfate was performed on this solution. Briefly, 50 ml of the protamine eluate was mixed with 23.5 mL saturated $(NH)_4SO_4$, the precipitate was removed, and additional 23.7 mL of saturated $(NH_4)_2\ SO_4$ was added to the supernatant, to obtain the precipitate. The precipitate was dissolved in 10 mL of Medium A and dialyzed against Medium A. In retrospect, this fraction contained arginyl-tRNA protein transferase (ATE1), arginyl-tRNA synthetase, and the acceptor proteins. We added crude rat liver s-RNA prepared according to Brunngraber [20]. We should hasten to add that in 1963 the word tRNA did not exist. It was called s-RNA meaning "soluble RNA" [21]. This means tRNA. Therefore, in the original description of the preparation of the rat liver system as well as the *E. coli* system, it states that "s-RNA" was added. We did not know, of course, what were the acceptor proteins in this fraction. The incorporation of arginine was 40–1000 times higher than that of other amino acids [10, 22]. We spent the major part of our effort to establish the fact that the ribosomes were not involved. It is noted that we used protamine to precipitate the rat liver system simply because of the analogy to the *E. coli* system. Purification and further characterization of this enzyme from rat liver were performed [22]. Recent studies identified one of the acceptor proteins of ATE1 in rat hepatocytes to be ornithine decarboxylase (ODC). The arginylation of ODC appears to facilitate its degradation [23]. Perhaps one of the most significant developments in the field of arginyl-tRNA protein transferase in higher eukaryotes is the establishment that it is an essential protein for higher eukaryotes [24]. Remarkable series of papers followed this paper as summarized in this book. We are certain that this is only a beginning of our understanding of the important functions of the ATE1 in higher eukaryotes. As we described in a separate section of this book, the arginyl-tRNA protein transferase appears to play a vital role in the process of aging (or anti-aging?) [25, 26]. We feel that this is just a small tip of the iceberg. Further exploration of this field will be promising as it was with phosphorylation of proteins [27, 28].

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Chapter 3

Arginyltransferase: A Personal and Historical Perspective

Richard L. Soffer

Abstract

In the late 1960s and early 1970s, characterization of arginylation has been spearheaded via biochemical studies that enabled the first characterization of ATE1 and its substrate specificity. This chapter summarized the recollections and insights from the era of research that followed from the original discovery of arginylation and led up to the identification of the arginylation enzyme.

Key words Arginyltransferase, Specificity of arginylation, Early studies of arginylation

My first research grant proposal in 1965 necessitated that I prepare a cell-free system for protein synthesis from sheep thyroid glands. When I did so, I noticed that arginine, in addition to its participation in protein synthesis, was also incorporated into protein by a second route that did not require ribosomes but did proceed through tRNA [1]. This transfer of arginine from tRNA to proteins also differed from protein synthesis in not requiring magnesium ions, and the incorporated arginine was reactive with dinitrofluorobenzene. A similar type of incorporation of arginine had previously been reported with soluble fractions from the liver by Kaji, Novelli, and Kaji [2] and from plasma cell tumors by Weinstein and Osserman [3].

At this point, I had to decide whether I should continue to investigate these findings and, if so, how I should go about it. There were two basic questions about this atypical incorporation of arginine that intrigued me. First, what is the biochemical reaction; and second, what are the physiologic consequences. I suspected that there was (an) "arginine-transferring enzyme(s)" [1]. An important mentor had instilled in me the notion that significant research was often associated with purification of enzymes and characterization of the reactions they catalyzed. Moreover, I felt that if I could assay and purify a responsible enzyme, I could see a roadmap for addressing two basic questions. If there were such an enzyme, then there

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must be a gene for this enzyme. If there were a gene, then I could look for mutants, particularly if the enzyme were present in an organism that could be manipulated easily. And if I found such mutants, they might have obvious, even lethal, defects, or perhaps I could provide proper conditions to elicit their altered phenotype. In addition, characterization of the purified enzyme might enable the preparation of useful specific antibodies against it, and understanding the reaction might suggest specific chemical inhibitors.

These early dreams depended upon my willingness to commit myself fully to isolating a novel enzyme, always a formidable task, and never one certain of success. The first step in the purification of an enzyme is the development of a simple assay, and I already had one, namely, the transfer of radioactive arginine from arginyl-tRNA to material insoluble in hot 5% trichloroacetic acid (i.e., "protein"). Furthermore, in my initial attempts at fractionating the enzyme, I noticed that almost any step was associated with a large loss of activity, which could be restored by inclusion in the reaction mixture of boiled, enzymatically inert soluble fraction, by albumin, or by thyroglobulin [4]. This early finding of what were probably acceptor proteins in the reaction solidified my commitment to the project.

At about this time, I was joined by Hiroo Horinishi, and we decided to use rabbit liver rather than thyroid gland as the source of the enzyme. We felt that it was imperative to establish, with certainty, precisely where arginine was attached to an acceptor protein. To do so required transfer of sufficient arginine for chemical analyses of the resulting peptides, This posed a problem because the amount of arginine that could be directly transferred from tRNA was ultimately limited by the concentration and instability of arginyl-tRNA and because our fractionated enzyme preparation contained some endogenous acceptor and could not be used at high concentration. Therefore, to prepare the necessary amount of radioarginylated bovine serum albumin, we coupled the transfer reaction with an arginyl-tRNA-generating system that permitted long-term linear incorporation of arginine, and we used a 15-hour, large-scale incubation with a low concentration of enzyme activity [5]. We digested the protein product with chymotrypsin and identified the principal radioactive product as a tetrapeptide in which the radioactive arginine was attached to the known aminoterminal aspartyl residue of the albumin. Since we had chemically established transfer to the acceptor protein, we felt justified in naming the responsible enzyme "arginyl-tRNA-protein transferase." The enzyme commission subsequently decided that "arginyltransferase" was the "accepted name" although our designation was retained as an "other" name.

Identification of *bona fide* acceptor proteins was generally problematic because most protein preparations displayed some acceptance, but it was usually miniscule on a molar basis. An enzyme

preparation was needed that could be used at a sufficient concentration so that near stoichiometric arginylation of true acceptors would be feasible. I finally developed a method [6] that resulted in a nearly 10,000-fold purification and that largely eliminated endogenous acceptors. This procedure exploited a change in the properties of the enzyme during purification such that it was not retained by CM cellulose before fractionation on DEAE cellulose but was retained afterward. Thus, the final CM cellulose step alone provided an enormous (about 250-fold) purification. Using this preparation, I found that virtually all arginyl residues on arginyl-tRNA could be transferred to protein and that bovine albumin and thyroglobulin accepted, respectively, nearly one and two molar equivalents of arginine. Acceptance by many other protein preparations remained very small on a molar basis and was presumably due to contaminants. Bovine thyroglobulin was known to contain two NH_2-terminal aspartyl residues, and when the labeled protein was digested with pepsin, the two major resulting radioactive peptides were isolated and shown to contain arginine linked to aspartic acid at their NH_2-termini [7].

I examined 38 defined proteins among which I found 7 acceptors [8]. All of these contained aspartic or glutamic acid as their NH_2-terminal residue. However, their presence at the amino terminus does not guarantee that a protein will be an acceptor. Donald Capra and I [9] studied three of his well-characterized myeloma proteins and found that the native molecules with acidic NH_2-terminal residues failed to accept arginine, whereas their denatured separated chains with appropriate termini were acceptors. One of these chains contained pyrrolidone carboxylic acid as its terminal residue and, as anticipated, failed to accept arginine. These results demonstrated that tertiary structure can be an important determinant of acceptor specificity.

I had wondered for some time whether small peptides could function as acceptors before I realized that there was a simple assay to determine this. Since they are soluble in hot 5% trichloroacetic acid, small peptides, if they competed with albumin in the transfer reaction, should competitively inhibit the albumin-dependent incorporation of arginine into material insoluble in hot trichloroacetic acid ("protein"). I used two criteria to determine acceptance by small molecules [10]: first, the ability to inhibit transfer to albumin in the reaction and second, the determination of whether inclusion of the small molecule in reaction mixtures resulted in a new radioactive product as determined by paper electrophoresis. In one case, namely, that of glutamylalanine, I prepared sufficient product for analysis and established that the new, peptide-dependent radioactive product was indeed the expected Arg-Glu-Ala. Among 17 dipeptides containing different residues linked to alanine, only those with NH_2-terminyl aspartic acid, glutamic acid, or, with lower affinity, cystine were inhibitory and yielded new

radioactive products. There was a 15-fold variation in K_i values among seven peptides with NH_2-terminyl aspartic acid linked to different residues. Thus, whereas the NH_2-terminal residue is an absolute determinant of acceptor structure, other nearby residues, at least, are relative determinants. Also of interest was acceptance by isoglutamine and isoasparagine and, to a lesser extent, by free aspartic acid.

The importance of stoichiometry in assessing acceptance by a protein preparation emerged dramatically in work that Mike Leibowitz and I were doing [11] on leucyltransferase, an enzyme of gram-negative bacteria that resembles arginyltransferase but has different donor and acceptor aminoacyl specificities. Early on, we had noted that albumin was an acceptor, and we had used it in our standard assay for the enzyme. However, when we needed near stoichiometric acylation with leucine or phenylalanine to determine chemically exactly where they were added, we found that we could transfer amounts equivalent to only about 5% of the albumin molecules. We then prepared arginylated albumin enzymatically and determined that it accepted almost molar amounts of either of the two donor amino acids. Tryptic digestion of arginylated albumin that had been subsequently acylated with phenylalanine or leucine yielded leucyl or phenylalanylarginine establishing that addition was to the NH_2-terminus. Much to our astonishment, however, we found the same dipeptides when we digested the acylated preparations of albumin that we had not arginylated. The molecules that accepted leucine or phenylalanine in the albumin preparation were thus a small fraction that contained NH_2-terminal arginine. This was the first indication that acceptor specificity of leucyltransferase might depend on a basic NH_2-terminal residue. That lysine and, to a smaller extent, histidine could also function in this role was subsequently established with defined peptides [12].

Since it was clear that we were dealing with an enzyme, and since we had a relatively simple assay for its activity, it seemed feasible to find a mutant lacking the enzyme which might prove useful in determining its physiological role(s). We felt that the best organism would be one that could be easily genetically and physiologically manipulated, and we identified the activity in *Saccharomyces cerevisiae* [13], a much-studied and well-characterized unicellular eukaryote that seemed well suited to our aims. After mutagenesis, we were able to isolate a mutant [14] whose residual transferase activity was thermosensitive suggesting that the responsible mutation, ate1, probably involved the structural gene. However, the mutant cells grew normally under a variety of circumstances, and we were unable to find any physiological deficit that might suggest the identity of a responsible protein, perhaps an acceptor substrate of the transferase.

I am gratified, but not surprised, that so much excellent research has been done in this field since our original work on

enzymatic arginylation.. It remains an interesting and challenging area. I am pleased that our own emphasis on an enzyme perspective has proven useful.

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I was fortunate to have very able students and fellows who shared my interest in the aminoacyl transferases. They were, in alphabetical order, Charles E. Deutch, Hiroo Horinishi, Michael J. Leibowitz, Margaret Savage, and Richard C. Scarpulla.

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Chapter 4

Arginylation in a Partially Purified Fraction of 150 k xg Supernatants of Axoplasm and Injured Vertebrate Nerves

Nicholas A. Ingoglia

Abstract

Transfer RNA-mediated posttranslational protein modification by arginine has been demonstrated in vitro in axoplasm extruded from the giant axons of squid and in injured and regenerating vertebrate nerves. In nerve and axoplasm, the highest activity is found in a fraction of a 150,000 g supernatant containing high molecular weight protein/RNA complexes but lacking molecules of <5 kDa. Arginylation (and protein modification by other amino acids) is not found in more purified, reconstituted fractions. The data are interpreted as indicating that it is critical to recover the reaction components in high molecular weight protein/RNA complexes in order to maintain maximum physiological activity. The level of arginylation is greatest in injured and growing vertebrate nerves compared with intact nerves, suggesting a role for these reactions in nerve injury/repair and during axonal growth.

Key words Arginylation, Posttranslational protein modification, Axoplasm, Injured nerves, Protein/RNA complexes

1 Introduction

Axoplasm extruded from the giant axon of squid contains relatively large quantities of a 76 nt (4S) RNA, some of which has been identified as transfer RNA [1–3]. In vertebrate nerves, the biochemical components of axons are more difficult to identify because of the close association of axons with surrounding glia/Schwann cells. Nevertheless, a series of experiments has shown that following injury and during regeneration of goldfish optic and rat sciatic nerves, vertebrate axons also contain large concentrations of a 76 nt RNA [4, 5]. Some axonal tRNA is likely involved in the recently described axonal protein synthesis (reviewed in [6]). However, the disproportionately large amount of axonal 76 nt RNA (see, e.g., [1, 4]) suggests other roles for this RNA in axons.

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In an attempt to ascribe function to the bulk of the 76 nt RNA in axons, we tested the hypothesis that these tRNAs serve as amino acid donors in the posttranslational modification of proteins. In other vertebrate systems, only $tRNA^{Arg}$ has been shown to be able to posttranslationally modify proteins. While our experiments focused on $tRNA^{Arg}$, we have found that several other amino acids also could be incorporated posttranslationally into endogenous nerve proteins [3, 7]. These reactions can be demonstrated in vitro only in partially purified, high molecular weight fractions of high-speed supernatants from which molecules of <5.0 kDa have been removed. This activity is dependent on the physiological state of the tissue; i.e., nerve injury and nerve elongation in vivo result in large increases in these reactions when measured in vitro. Based on these data, we suggest critical roles for arginylation and other amino acid modifications of endogenous proteins in nerve injury/repair and during growth of vertebrate axons.

The experiments described below were performed in the late 1970s through the mid-1990s. The materials described are those that were available at the time.

2 Materials

2.1 Tissues

1. Axoplasm. Pure axoplasm, void of contamination from surrounding tissue, can be isolated from the giant axons of the stellate nerves of squid. Squid (*Loligo pealeii*) were obtained at the Marine Biological Laboratories, Woods Hole, MA. Axoplasm (about 5 μL per axon) is pooled and stored in 1.2 mL Eppendorf tubes at 0 °C in 10 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 30 mM 2-mercaptoethanol, and 10 mM KCl brought to 50% glycerol (to prevent samples from freezing). Based on squid availability from the daily catch, it is reasonable to dissect six to ten axons in a day.
2. Sciatic and optic nerves of rats. Male, Sprague-Dawley rats (~150 gms) are used in all experiments. Rats are anesthetized with ketamine/xylazine (100 mg/kg body weight) and sacrificed by decapitation. Isolated nerve segments are placed in chilled buffer (100 mM Tris–HCl, pH 7.4, containing 250 mM sucrose and 6.0 mM dithiothreitol).

2.2 Sample Preparation

1. Squid axoplasm. On the day of the experiment, remove 100 μL of axoplasm (approx. 1.0 ug protein/μL) from −20 °C storage, and transfer to a 1.2 mL conical Eppendorf tube. Keep samples at 4 °C during the entire procedure.
2. Rat sciatic and optic nerves. Remove a 1 cm portion of the nerve from (a) the site of injury (include nerve proximal to the cell body) and (b) a segment distal to the injury. Place nerves

on chilled glass slides. Dissect the nerve segments as described in Methods. Place the desheathed, minced nerve segment in 0.35 mL of ice-cold buffer (100 mM Tris–HCl, pH 7.4, containing 250 mM sucrose and 6.0 mM dithiothreitol) in a 1.2 mL conical Eppendorf tube.

2.3 Reactions

Reaction components contain approximately 0.5–1.0 μg protein/μL of partially purified axoplasm or rat nerve extracts, an equal volume of buffer (5 mM $MgCl_2$, 5 mM ATP, 100 mM KCl, in 30 mM Tris–HCl (pH 7.4)), and 5 μL (approx. 5 μCi) of ^{3}H-Arg or other labeled amino acids.

3 Methods

3.1 Preparation of Squid Axoplasm

Live squid are used immediately after capture or kept in tanks with running sea water for no longer than 2–3 days. Decapitate the squid with sharp scissors, and with the aid of a dissecting microscope, identify the stellate nerves containing the paired giant axons. The axons, still connected to their cell bodies in the stellate ganglion, should be removed intact. Cut the distal end of the axon using dissecting scissors and extrude the axoplasm from the sheath by pressure, much like squeezing toothpaste from a tube (for more detailed descriptions of this technique, see, e.g., [8, 9]) (*see* **Note 1**).

3.2 Preparation of Rat Sciatic Nerves

1. Expose the superficial muscles of the upper leg with a longitudinal incision in the mid-thigh region.
2. Separate the deeper muscles with blunt dissection to avoid bleeding.
3. Expose the sciatic nerve for about 4 cm from the sciatic notch.
4. Crush the sciatic nerve using jeweler's forceps (*see* **Note 2**).
5. A translucent band in the otherwise opaque white nerve indicates that sciatic nerve axons have been cut.

3.3 Preparation of Rat Optic Nerves

1. Make a superficial incision in the sclera surrounding the back of the eye.
2. Rotate the eye forward.
3. Visualize the optic nerve using a dissecting microscope. Identify the retinal artery (*see* **Note 3**).
4. Crush the optic nerve (as described for sciatic nerves), making sure to avoid the retinal artery. A translucent band in the otherwise opaque white nerve indicates that optic axons have been severed.

3.4 Preparation of the Active Subcellular Fractions

Axoplasm:

1. Remove 100 uL of axoplasm from −20 °C storage, and transfer to a 1.2 mL Eppendorf tube.
2. Homogenize samples using a pestle made of dental cement (*see* **Notes 4** and **5**).
3. Wash the Eppendorf tube with 60 uL of 10 mM Tris–HCl, pH 7.4, containing 0.25 M sucrose and 6 mM 2-mercaptoethanol.
4. Pool the wash with the homogenate.
5. Transfer the homogenate to a 200 uL capacity high-speed polypropylene centrifuge tube.
6. Centrifuge at 150 k xg for 60 min at 4 °C in a Beckman Airfuge centrifuge.

Nerve segments:

1. At the designated times after crush injury, anaesthetize the rats and remove nerve segments.
2. Place the nerve segment on a chilled glass slide.
3. Desheath the nerve using fine forceps and mince with a razor blade.
4. Homogenize two to four pooled nerve segments in 0.35 mL of 10 mM Tris–HCl, pH 7.4, containing 0.25 M sucrose and 6 mM 2-mercaptoethanol, in a 10 mL all-glass Ten Broeck homogenizer (*see* **Note 5**).
5. Wash the homogenizing tube with 150 uL of 0.35 mL of 10 mM Tris–HCl, pH 7.4, containing 0.25 M sucrose and 6 mM 2-mercaptoethanol.
6. Transfer the homogenate to a high-speed polypropylene centrifuge tube.
7. Centrifuge at 150,000 g for 60 min at 4 °C.

3.5 Fractionation by Sephacryl S-200 Chromatography

The 150,000 g supernatant is fractionated by column chromatography on a Sephacryl S-200 column (2.5 × 300 mm; Pharmacia). The goal of this procedure is to collect fractions containing high molecular weight protein/nucleic acid fraction while removing molecules of <5 kDa (*see* **Note 6**).

For both axoplasm and vertebrate nerves:

1. Apply the 150 k xg supernatant of tissue homogenates onto a Sephacryl S-200 column (4 °C) (other separation techniques can be used as long as the procedure removes molecules of <5 kDa) (*see* **Note 7**).
2. Collect the effluent of the column to be used as the source of the reaction components.

3.6 Posttranslational Incorporation of Amino Acids into Proteins

The void volume fraction of the S-200 column is used as the source of tRNA, aminoacylating enzymes, putative amino acid transferases, and endogenous acceptor proteins as components of HMW complexes. Keep this fraction on ice until incubation.

1. Add 15 μg protein (approx. 1.0 μg protein/μL) of the gel filtration column effluent to an incubation tube.
2. Add an equal volume of a reaction mixture (60.0 mM Tris–HCl (pH 7.4), 200 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, to the final concentration in the reaction of 30.0 mM Tris–HCl (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP). Typical reactions have a final volume of approx. 150 μL (*see* **Note 3**).
3. Start the reaction by adding 3–5 μL (approx. 5 μCi) of ^{3}H-Arg or other tritium-labeled amino acids.
4. Incubate samples for 20 min at 37 °C.
5. Stop reactions by adding an equal volume of ice-cold 10% TCA to each tube, and mix well (*see* **Note 8**).
6. Place all samples in an ice bucket.

3.7 Determination of Protein Arginylation

The incorporation of ^{3}H-Arg (and other amino acids) into nucleic acid and total protein fractions is measured by precipitation in cold and hot 10% TCA (*see* **Note 9**). The hot and cold precipitatable material is solubilized in tissue solubilizer (0.7 mL Protosol, New England Nuclear Corp.), and radioactivity is counted in a liquid scintillation spectrometer (*see* **Note 10**).

1. Leave samples in ice for 30 min to allow RNA and proteins to form aggregates.
2. Centrifuge at 1100 g for 10 min and remove supernatant.
3. Repeat until the radioactivity in the cold TCA-soluble fraction (free ^{3}H-amino acids) reaches background levels (four to five washes).
4. Add 0.5 mL of 10% TCA to the cold TCA precipitate, and transfer the tubes to a boiling water bath for 20 min (this procedure hydrolyzes the aminoacyl-tRNA bond releasing amino acids bound to tRNA).
5. Return samples to ice for 20 min.
6. Repeat **steps 2** and **3** until the radioactivity in the cold TCA fraction reaches baseline.
7. Dissolve the precipitate in a tissue solubilizer and count in a liquid scintillation counter. This fraction contains the radioactive amino acid that had been covalently bound to proteins (*see* **Note 10**).

Closing Comments When these experiments were performed in the latter part of the twentieth century, the only roles for a 76 nt RNA (the RNA shown to be in abundance in squid axoplasm and axons of regenerating – but not intact – vertebrate nerves) were as an amino acid donor in protein synthesis or, for $tRNA^{Arg}$, as an Arg donor in posttranslational protein modification.

In recent years, a new role for 76 nt RNA has been uncovered. MicroRNAs, 22 nt RNAs that act to regulate translation of mRNAs (see, e.g., [10–14]), have been shown to be derived from 76 nt precursors (pre-miRNAs). The enzyme, Dicer, cleaves the 76 nt precursor into the active 22 nt miRNA [15, 16]. MicroRNAs have been found in axonal growth cones, and interference with specific miRNAs or with Dicer can affect axonal guidance [17, 18].

Based on these recent findings, we speculate that the abundant 76 nt RNA found in growing vertebrate axons is composed of (1) tRNA that functions in classical axonal protein synthesis, (2) tRNAs involved in posttranslational protein modification as described in this volume, and (3) pre-miRNAs that serve as precursors for miRNAs in axonal growth cones that regulate axon guidance.

4 Notes

1. In the preparation of axoplasm, it is critical to keep extruded axoplasm separate from adaxonal glial contamination. To accomplish this, the isolated axon is placed on a slide covered with Parafilm. The end of the axon is cut. As the axoplasm is pushed out, the axon sheath is pulled back. This leaves a "bead" of about 5.0 μL of axoplasm.
2. Nerve crushes are performed by compressing the nerve in curved jeweler's forceps to a count of 10. Nerves are released and crushed again at the same site for an additional 10 s. Following the second crush, the nerve is intact (proximal and distal segment are still connected) but should turn translucent (from the usual white, opaque) indicating that sciatic nerve axons have been transected.
3. It is critical to avoid the retinal artery when crushing the rat optic nerve, since cutting it will lead to the death of retinal ganglion cells and a physiological condition where no response to injury or regenerative growth is possible. If there is bleeding, the preparation should be discarded.
4. Pestles are made by placing dental cement in the bottom of an Eppendorf tube to the point where it just fills the tapered portion of the tube. A metal rod is placed in the center, and the cement is allowed to harden. Once the cement has formed,

the Eppendorf tube can be broken away. The pestles are stable and can be washed and reused in subsequent homogenizations of axoplasm.

5. Homogenization for axoplasm and sciatic nerves is performed by moving the pestle into the homogenate with smooth complete strokes for 3 min, while the sample is in an ice bucket. The degree of homogenization is critical and should be consistent among samples as over-homogenization leads to the disruption of the high molecular weight protein-RNA complexes and a loss of physiological activity.
6. In our experiments in both axoplasm and vertebrate nerves, we were unable to demonstrate posttranslational arginylation of protein in unfractionated 150,000 g supernatants. However, reactions did take place in fractions containing high molecular weight nucleic acid/protein complexes but lacking molecules between 1000 Da and 5000 Da.
7. Gel filtration chromatography of the 150,000 g supernatant is performed in order to remove small molecules from the fraction being assayed for posttranslational aminoacylation. These small molecules inhibit the posttranslational addition of amino acids to protein. The mechanism of this inhibition has been investigated [19, 20] but is not well understood.
8. Controls for non-specific binding can be done in three separate ways: (1) Place the active fraction in a boiling water bath for 20 min (inactivating enzymes in the fraction) prior to incubation at 37 °C. These boiled control samples are incubated along with the experimental samples. (2) Add cold TCA to the samples before incubation. (3) Run parallel samples using ^{3}H taurine as the labeled amino acid. Taurine is a sulfonic amino acid not incorporated into any protein. In our experiments, the levels of taurine associated with protein following incubation and acid extraction were always negligible.
9. Two important keys to demonstrating these reactions in tissue extracts should be noted: (1) The "active fraction" must contain both RNA and protein. We monitored this by measuring absorbance at 260 nm and 280 nm for each fraction as it came off the filtration column. (2) The "active fraction" must be void of molecules of less than 5 kDa. The best method to remove small molecules from the active fraction should be determined empirically for the tissue being studied.
10. It is important to be sure that the label being measured as incorporated into protein is still present as the parent amino acid. Under the incubation and extraction conditions we used, we have not found evidence that significant levels of ^{3}H are transferred to molecules other than the parent compound.

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Chapter 5

Preparation of ATE1 Enzyme from Native Mammalian Tissues

Anna S. Kashina

Abstract

Early studies of protein arginylation preceded the wide availability of recombinant protein expression and relied heavily on the fractionation of proteins from native tissues. This procedure has been developed in 1970 by R. Soffer, in the wake of arginylation discovery in 1963. This chapter follows the detailed procedure originally published by R. Soffer in the 1970, adapted from his article in consultation with R. Soffer, H. Kaji, and A. Kaji.

Key words Arginyltransferase, Native enzyme purification, Arginylation

1 Introduction

The method described below was developed and originally described by R. Soffer [1]. Back in those days, it constituted the "gold standard" of ATE1 purification and was used in the earlier biochemical studies of ATE1 (including, e.g., the studies described in the next chapter). It should be noted however that the method is based on using rabbit liver and isolation of ATE1 from other tissues would require appropriate modifications (e.g., to adjust for potentially limited abundance of starting material, which might require scaling down to milligrams rather than hundreds of grams of tissue and using batch bead purifications instead of columns). The original discovery of ATE1 activity was made in the rat liver [2], and the subsequent chapter of the book describes the use of other tissues and organs, as well as cultured cells, as the source of ATE1.

The method described below takes advantage of the fact that the ATE1 enzyme is not retained by carboxymethyl (Cm) cellulose until after it has been subjected to chromatography on DEAE

Adapted from the original article by R. Soffer in consultation with R. Soffer, H. Kaji, and A. Kaji

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cellulose. In brief, the method consists of two ammonium sulfate cuts, followed by acid precipitation and three consecutive chromatographic steps on Cm cellulose (collecting the flow-through), DEAE cellulose, and another Cm cellulose (collecting the high salt eluate).

In the original paper, three consecutive large-scale fractionations by this procedure yielded preparations which were purified 5000-, 7000-, and 11,000-fold and which had specific activities of at least 110, 114, and 112 units per mg. Disc polyacrylamide gel electrophoresis of these preparations showed a major component and a few minor bands, including the unresolved low molecular weight material, suggesting that the preparation was highly enriched in ATE1 enzyme but not fully purified (our ballpark estimate suggests ~70% purity, assuming that the major polypeptide in the preparation represents ATE1).

2 Materials

1. Freshly excised mammalian tissue. In the original paper, rabbit liver was used. Other researchers also used calf kidney and rat liver and brain. In principle, any tissue containing ATE1 can be used instead.
2. Buffer A: 50 mM Tris–HCl (pH 7.8), 5 mM magnesium acetate, 30 mM KCl, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, and 0.25 M sucrose.
3. SAS: saturated solution of ammonium sulfate in 40 mM Tris–HCl buffer (pH 7.8) containing 10 mM 2-mercaptoethanol.
4. Buffer B: 20 mM Tris–HCI (pH 7.8) containing 5 mM 2-mercaptoethanol.
5. Buffer C: 20 mM Tris–HCl (pH 9.0) containing 0.1 M 2-mercaptoethanol.
6. Buffer D: 1 mM potassium citrate (pH 6.5) containing 0.1 M 2-mercaptoethanol.
7. Two carboxymethyl cellulose (Cm cellulose) columns, 2.2 × 26 cm, one pre-equilibrated with buffer D and one with buffer D containing 80 mM KCl.
8. One DEAE cellulose column, 3 × 28 cm, pre-equilibrated with buffer D.

3 Methods

The steps below represent a continuous procedure, divided into sections so that they would be easier to follow. All these steps must be performed at the temperatures of 0–4 °C.

3.1 Preparation of Tissue Extract

1. Homogenize three freshly excised rabbit livers (300 g wet weight) in 600 mL of buffer A for three 1-min periods in a Waring Blendor pre-chilled with a water-ice mixture for 30 s between each homogenization.
2. Follow-up with one stroke with a Teflon tissue grinder, and pass the homogenate through a double layer of cheese cloth to remove large debris.
3. Centrifuge the homogenate at 20,000 × g for 30 min. Discard the pellet and re-centrifuge under the same conditions. Discard the pellet.
4. Centrifuge the supernatant from **step 4** at 105,000 g for 120 min. Take the upper four-fifths of the supernatant fraction for the next steps, and discard the pellet and the remaining supernatant.
5. Dialyze the supernatant from **step 4** overnight against two changes of 4 liters each of 20 mM Tris–HCl buffer (pH 7.8), containing 5 mM 2-mercaptoethanol (*see* **Notes 1** and **2**).

3.2 Ammonium Sulfate Fractionation

1. Slowly add pre-chilled SAS to the dialyzed high-speed supernatant from the previous step, using 167 mL of SAS per 450 mL of the dialyzed supernatant (approximately 27% of SAS). Stir for 10 min to allow the formation of a visible precipitate.
2. Centrifuge the suspension at 20,000 g for 30 min. Discard the pellet.
3. Slowly add pre-chilled SAS to the supernatant from **step 2**, using 231 mL of SAS per 570 mL of the supernatant solution (approximately 29% or 56% total from **steps 1** to **3**). Stir for 10 min.
4. Centrifuge the suspension at 20,000 g for 30 min. Discard the supernatant.
5. Redissolve the pellet from **step 4** in buffer B, bringing it to the total volume of 75 mL.
6. Dialyze the solution overnight against two changes of 6 liters each of buffer B. After dialysis, dilute the solution to 150 mL with the same buffer (*see* **Note 3**).

3.3 Precipitation at pH 5

1. Add glacial acetic acid to the solution drop-wise with simultaneous stirring until the pH reaches 5.2. Stir for additional 15 min. A visible precipitate should form.
2. Centrifuge the suspension at 20,000 g for 30 min. It is important to maintain the temperature of the rotor between 3 and 6 °C during this centrifugation. Discard the supernatant.
3. Add 40 mL of buffer C to the precipitate. Homogenize the suspension with five strokes of a Teflon tissue grinder.

4. Dialyze the suspension for 2 h against 2 L of buffer D. The precipitate should dissolve during this dialysis.
5. Dialyze the solution overnight against buffer D, using two changes of 6 L each. Dilute the dialyzed solution to 100 mL with buffer D.

3.4 Column Chromatography

1. Load the solution from the previous step onto the Cm cellulose column (2.2 × 26 cm) pre-equilibrated with buffer D. Continue the elution with this buffer, and collect 10 mL fractions at a flow rate of 3 mL per min (*see* **Note 4**).
2. Combine the protein-containing fractions, which should be detectable by their yellow color (or could also be detected by OD^{280} measurement). Approximately 160 mL of the eluate should be collected.
3. Load the eluate from **step 2** immediately onto DEAE cellulose column (3 × 28 cm) pre-equilibrated with buffer D. Wash the column with 400 mL of the same buffer, and discard the effluent.
4. Elute the enzyme with buffer D containing 0.2 M KCI. Collect 6 mL fractions at a flow rate of 3 mL per min. Combine the peak protein-containing fractions (should be yellow). Approximately 72 mL of the eluate should be collected. The eluate can be stored overnight in an ice bucket prior to the final step.
5. Dilute the DEAE cellulose eluate from **step 4** to the final volume of 180 mL using buffer D.
6. Load the diluted eluate onto a new Cm cellulose column pre-equilibrated with buffer D containing 80 mM KCI.
7. Wash the column with 200 mL of 0.1 M KC1 in buffer D. Discard the effluent.
8. Elute the enzyme with the buffer D containing 0.2 M KCl. Protein-containing fractions can be combined and used for ATE1 activity measurements. (Note: in a typical preparation, after 75 mL of the elution buffer has passed into the resin, 25 3 mL fractions are collected at a flow rate of 3 mL/mL) (*see* **Note 5**).

4 Notes

1. Preparations made from 50 rabbit livers by this procedure were found to have specific activities ranging from 0.007 to 0.049 unit per mg with a mean of 0.019 unit per mg. The average protein concentration was 26 mg per ml. The dialyzed supernatant fractions could be stored at −20 °C for 2 months without loss of activity.

2. In modern purification procedures, buffer A would have been supplemented with protease inhibitor cocktail prior to homogenization. However, according to our pilot data, some of the standard protease inhibitor cocktails can inhibit ATE1 activity.
3. In some of the subsequent studies, including those described in the next chapter, 70% ammonium sulfate cut was used at **step 3**.
4. At this step, ATE1 is not retained by the column and is recovered in the flow-through.
5. This purification procedure resulted in 10,000-fold enrichment with the yield of about 10%. According to the originally published protocol, protein concentration of the second Cm cellulose eluate was usually between 5 and 10 pg per mL, and if kept under these conditions, the preparations were found to lose as much as 85% of their activity within 24 h. They were therefore used at once or concentrated at least 30-fold by dialysis against dry Sephadex G-200 or against 30% polyethylene glycol in 20 mM Tris–HCl (pH 9.0) containing 0.1 M 2-mercaptoethanol. Between 20% and 60% of the activity was lost during concentration, depending on the time required for the operation. For storage, concentrated solution fractions were dialyzed for 180 min against 20% glycerol in 20 mM Tris–HCI (pH 9.0) containing 0.1 M 2-mercaptoethanol and were stored at 0 °C. They were found to lose about 50% of their activity within 1 week.

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Chapter 6

Correlated Measurement of Endogenous ATE1 Activity on Native Acceptor Proteins in Tissues and Cultured Cells to Detect Cellular Aging

Hideko Kaji and Akira Kaji

Abstract

Following our early discovery of arginylation in 1963, we have performed several studies to correlate its activity with essential biological processes. We employed cell- and tissue-based assays to detect both the level of acceptor proteins and the level of ATE1 activity under different conditions. Remarkably, in these assays, we found a close correlation between arginylation and aging, a discovery that we believe has longer-term implications in uncovering the importance of ATE1 in normal biology and disease therapies. Here, we describe the original methods we used to measure ATE1 activity in tissues and correlate it with key biological events.

Key words Arginylation, Aging, Cell proliferation, SV40 transformation, ATE1, Arg acceptor proteins

1 Introduction

Following up on our discovery of protein modification by aminoacyl tRNA and aminoacyl tRNA protein transfer enzymes [1–5], we investigated several biological processes regulated by this modification. We consider the relationship between arginylation and cellular aging as one the most exciting and promising subjects that emerged from these studies. Our finding uncovered a close relationship between the cellular aging and arginylation, which, as we hope, will eventually lead to uncovering a novel mechanism of aging and possible relationship between age-related cognitive impairment and arginylation. In this chapter, we describe the method used to study this question at the time of our discovery and three representative fundamental findings using those methods to show how the simple approach can lead to basic fundamental facts.

The very first finding related to aging and the level of ATE1 enzyme began with the simple question of comparison of the level

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of ATE1 enzyme between normal and regenerating rat liver. This was based on a naive speculation that cellular transformation or proliferation may cause different level of arginylation which may have a controlling role in cellular activity related to cell division. It was found that the ribosome-free incorporation of ^{14}C arginine into hot trichloroacetic acid-insoluble fraction expressed per unit weight of cellular protein was higher with regenerating liver about three times, suggesting that higher cellular proliferation is reflected as higher activity of the arginylation. At this time, we did not know whether it was due to the increased level of the acceptor proteins or higher ATE1 enzyme level. At least, we got the basic notion that increased modification is related to higher cell division [6–8]. We then examined the relationship between aging and the level of ATE1 enzyme in a more direct way, by extracting the enzyme from various rat organs at various ages. It appears that the brain is the exceptional organ where ATE1 enzyme activity remains constant from 2 months old up to 30 months old. Assuming that the average lifespan of experimental rats in cages is about 36 months, we can conclude that the level of ATE1 enzyme in the brain is kept constant throughout the adult life (Fig. 1). This is a striking finding, because in the two other organs tested, kidney and liver, ATE1 enzyme steadily decreased as the animals aged. In these experiments, we added an Arg acceptor protein (α-lactalbumin) to ensure that the level of ATE1-mediated Arg incorporation does not depend on the availability of the natural acceptors in the organs.

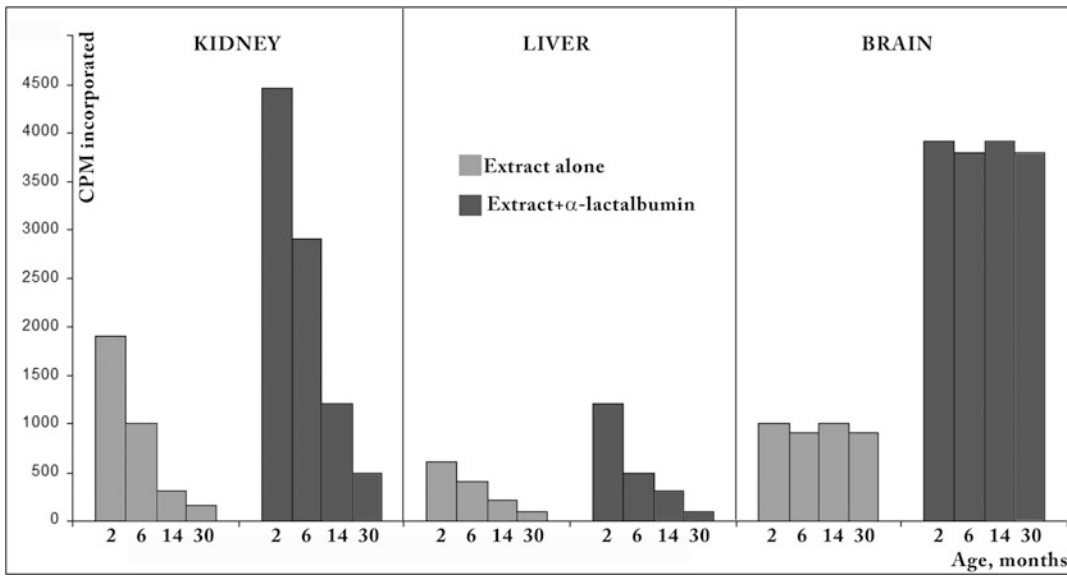

Fig. 1 Arginyl-tRNA transferase (ATE1) activity in aging rat tissues up to 30 months. Transferase activity is presented as hot (95 °C) trichloroacetic acid (TCA)-insoluble radioactivity (CMP) (see [20] for the originally published result)

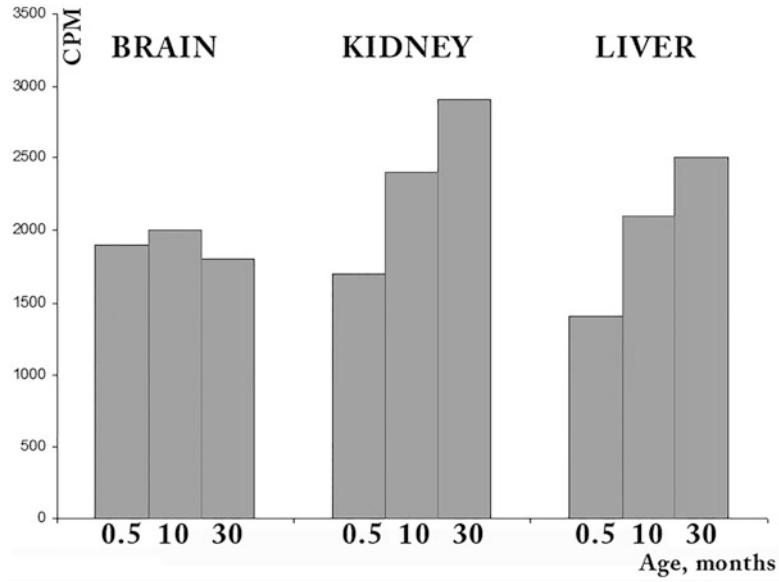

Fig. 2 Modification of endogenous acceptor proteins by arginyl-tRNA transferase. The amount of the arginine acceptor proteins in the brain remains constant, while those in the kidney and liver increase with age. The assay was carried out as described in the text using 45/μg of soluble proteins from aging rat brain, kidney, or liver. Endogenous arginyl-tRNA transferase was inactivated before assaying by heating the preparations at 100 °C for 2 min (see [20] for the originally published result)

We also examined the level of arginine acceptor protein in each organ by inactivating the ATE1 enzyme in each organ and then adding exogenous purified ATE1 to measure the incorporation of arginine into protein. To our surprise, the acceptor protein levels increased in the kidney and liver as the animals aged. In clear contrast, the acceptor protein levels in the brain remained constant up to 30 months (Fig. 2). It appears that in the kidney and liver, the acceptor protein levels increases despite the fact that the ATE1 enzyme activity is decreased. We assume that this will leave more and more non-arginylated acceptors in these organs as the animals age. These concepts, derived from our early studies, were later confirmed with *C. elegans* [9], where ATE1 enzyme increased as *Caenorhabditis elegans* matured followed by rapid decline during the senescence. In contrast, in the brain, the acceptor proteins were kept constant throughout the lifespan of the organism. Thus, it must be important to keep the acceptor molecule constantly arginylated in the brain. As discussed later, this would lead to a speculation that arginylation must be kept constant in the brain to maintain healthy mental behavior.

At the time of these findings, we interpreted them as an indication of the replication activity. Indeed, unlike the other organs, the majority of the fully formed brain does not have a lot of active cell

division, and neither the Arg acceptor proteins nor the ATE1 enzyme levels change. This primitive concept was clearly wrong because later studies by another laboratory showed that during the recovery after heat shock, about 46% increase of arginyl-tRNA protein transferase was observed in the brain [10]; however, we believe that our original discovery has merit and is likely related to key brain-dependent processes, including, possibly, age-related dementia.

To further support the concept that ATE1 enzyme is closely related to cell division and cellular aging, we turned our attention to the cellular senescence which is accompanied by slowing down or cessation of cell division. Very fortunately, an ideal system for studying the cellular aging was just developed by Hayflick at the Wistar Institute which is right across the street from the University of Pennsylvania. One of us was a personal acquaintance of Dr. Hayflick. It has been established that populations of normal diploid human fibroblasts (called WI38) can proliferate in culture for only finite periods of time [11]. The strain has three phases: phase I for the initial ex-plantation period, phase II for a period of rapid proliferation with the cells dividing logarithmically, and phase III for a period of declining division. These cells have population doubling potentials of 50 ± 10 divisions. Comparison of the population doubling potential of cell strains derived from older donors with those derived from younger donors indicated that the relationship is inversely proportional to the age of the host. Changes associated with phase III are regarded as representing cellular aging. The system is even more attractive because it has been shown a few years earlier [12] that transformation of human fibroblasts by SV40 virus is possible. This makes it possible to study the relationship between senescence and cellular transformation [13].

In the paper entitled "Fixation of cellular aging processes by SV40 virus transformation" [14], we reported a finding that ATE1 activity in human fibroblasts decreases together with the aging of the WI38 cells. This is consistent with the notion we formed during our studies of kidneys and liver of aging rats, as indicated in the preceding section. Arginyl-tRNA protein transferase, derived from the soluble fraction of eukaryotic cells, has been implicated in cell regulatory and proliferative functions via posttranslational modifications of specific acceptor proteins of various cellular components (e.g., membranes [15]). Using WI38 and related cell strains, the activity of arginyl-tRNA protein transferase (ATE1) was examined in relation to cellular aging and SV40 viral transformation of normal fibroblasts (Fig. 3). In both transformed and non-transformed strains, the level of ATE1 enzyme was linearly related to the age of the human fibroblasts, expressed as the number of divisions they went through. The relationship was surprisingly linear [14]. As a matter of fact, one could tell the age of the cells (how many times they have doubled) by measuring the level of the ATE1 enzyme. This is surprising, because the total amount of protein per cell and

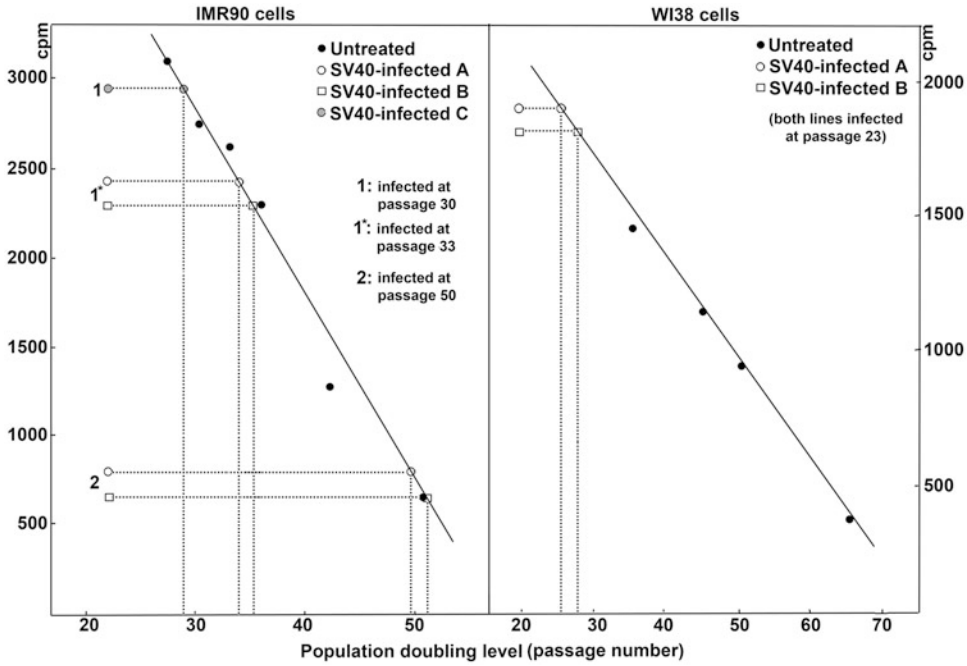

Fig. 3 ATE1 activity decreases with the cellular aging, but oncogenic transformation stops this decrease. ATE1 activity in normal and SV40 viral transformed fibroblasts (IMR90 and WI38 strains) was assayed using 20 pg of soluble protein derived from each cell type and 30 μg of α-lactalbumin (a receptor protein). Comparison of the transformed cell lines (SV40-infected A, B, C) with normal uninfected cell lines of the same strains (untreated) indicates a remarkable correlation between the observed level of transferase activity (y-axis, shown as counts per minute (cpm) of ATE1-dependent ^{3}H Arg incorporation into α-lactalbumin) and the population doubling level (passage number) at which the cells were initially exposed to SV40 virus (see [14] for the originally published result)

the size of the cell increase together with the aging of WI38 cells, which reach senescence after approximately 50 divisions.

After we accepted the invitation to contribute a chapter on aging and ATE1 enzyme in this book, we wanted to update our knowledge on this matter because we are not currently working on this subject. Surprisingly, very small number of contributions turned up about the relationship between aging and arginylation. Our attention and great interest were aroused by the original finding by Bongiovanni et al. [16] entitled "The post-translational incorporation of arginine into a β-amyloid peptide increases the probability of α-helix formation." It is well-known that β-amyloid peptide accumulation is a hallmark of Alzheimer dementia. It is either the cause or results of the disease. A notable symptom of this disease is the loss of short-term memory which is controlled by the hippocampus. Excitingly, of the various regions of the brain studied, the hippocampus had the highest specific activity of ATE1 enzyme followed by the striatum, medulla oblongata, cerebellum, and cerebral cortex [17]. It is quite possible as speculated by

Bongiovanni et al. [16] that arginylation of β-amyloid peptide may facilitate the breakdown of this protein and this is an active ongoing process in the brain. This original finding of Bongiovanni et al. was recently confirmed and extended to tau protein, another hall mark of Alzheimer disease by Brower et al. [18]. As the human average lifespan increases, cognitive diseases such as Alzheimer disease become a very important issue which needs immediate attention of biomedical research. Based on the review of our classic papers and the recent literature regarding arginylation and aging, we like to describe briefly our intention to make our classic discovery more applicable to human health. In healthy aging, ATE1 enzyme maintains its level in neurons and the central nervous system. There must be a special mechanism in the brain, especially in the hippocampus, which is responsible for short memory, to keep the ATE1 enzyme level from deteriorating with aging. We like to propose very naively that the cure and prevention of the dementia may reside in the boosting of the ATE1 enzyme of aging brain. With modern technology of biomedical sciences, this may not be a distant dream.

The methods described here are represented in [6, 14, 19–21]. Examples of related more recent work are in [10, 18, 22–24], but the real possible role(s) of arginylation in aging and possible cause of cognitive impairment or dementia are not known. This makes the extensive research in this area of arginylation very exciting.

Below we describe the method used at the time these experiments were done. Many of the procedures can be simplified with the current advanced technology.

2 Materials

1. Male Fischer inbred rats ranging in age from 2 weeks to 30 months (for rat aging studies).
2. Cell cultures (for cell aging studies): human fibroblast strains IMR90, AG-2804, and WI38 sublines 9WI and 8A8, maintained in either McCoy's or Dulbecco's modified Eagle's culture medium supplemented with 10% fetal calf serum containing streptomycin 50 μg/mL and penicillin G 275 units/mL. The cells are grown at 37 °C in a humidified 5% CO_2 atmosphere on 10 cm culture dishes, and sub-cultivation is carried out at an 8:1 split ratio using Puck's EDTA-trypsin solution.
3. PBS (phosphate buffered saline containing 137 mM NaCI/8.1 mM Na_2HPO_4/2.6 mM KCI/1.4 mM $KH_2.PO_4$).
4. Buffer A (0.3 M sucrose containing 50 mM Tris–HC1, pH 7.8; 4.0 mM Mg acetate; 10 mM β-mercaptoethanol; 12.5 mM KC1).

5. Buffer B (50 mM Tris–HCl, pH 7.4; 25 mM KCI; 5 mM $MgCI_2$).
6. Buffer C (50 mM Tris–HCl, pH 7.4/25 mM KCI/5 mM $MgCI_2$/10 mM β-mercaptoethanol).
7. 10 mM Tris–HCl, pH 7.8
8. Dounce B glass homogenizer.
9. Wild-type and tsA30 of SV40 viruses (for virus infection as needed – *see* Subheading 3.5).

3 Methods

3.1 Preparation of Chromatin (Arg Acceptor) and Post-microsomal Supernatant (Source of the ATE1 Enzyme) from Rat Tissues for Rat Aging Studies

1. Kill the rats by decapitation, and remove the tissues to be tested for ATE1, aiming for approximately 1 g of tissue for each sample. All the subsequent steps must be performed with cold solutions at 4 °C unless otherwise indicated.
2. Wash the excised tissues two times with PBS, mince using a blade or scissors, and homogenize in 4.0 mL buffer A using a Dounce B glass homogenizer.
3. Centrifuge at 3000 g for 15 min to obtain the nuclear pellet.
4. Re-suspend the nuclear pellet in 1.0 mL buffer B, and centrifuge over a 0.2 M sucrose cushion in buffer B for 5 min. at 2000 g.
5. Repeat **step 4** two more times using buffer B containing 0.5% triton X-100 for re-suspending the pellet.
6. Homogenize the purified nuclei in 2.0 mL 10 mM Tris–HCl, pH 7.8, and layer onto 2.5 mL 10 mM Tris–HCl, pH 7.8, containing 1.7 M sucrose. Gently mix the resulting interface, and centrifuge the samples in a swinging bucket rotor (e.g., Beckman SW50.1) at 130,000 g for 3 h to obtain the chromatin pellet and the post-nuclear supernatant.
7. Wash the chromatin pellet twice with 10 mM Tris–HCl, pH 7.8, by suspending and centrifuging at 12,000 g for 10 min, and freeze at −80 °C. This fraction is used as the source of ATE1 acceptor proteins below.
8. Simultaneously, centrifuge the post-nuclear supernatant at 12,000 g for 10 min to pellet the mitochondria. Discard the pellet, and centrifuge the resulting supernatant at 130,000 g for 3 h to obtain the post-microsomal supernatant.
9. Dialyze the post-microsomal supernatant from **step 8** overnight against 3 L of buffer C with three changes of buffer.
10. Centrifuge the dialyzed solution for 10 min at 12,000 g, separate into 0.2 mL aliquots, and freeze at −80 °C until needed. This fraction is used as the source of ATE1 enzyme.

3.2 Preparation of Chromatin and Post-microsomal Supernatant from Cultured Cells for Cell Aging Studies

1. Grow the cultured cells to confluence. To harvest, aspirate the medium, wash the cell monolayer two times with PBS, and harvest with a rubber policeman.
2. Homogenize the cells, and follow the steps described above for rat tissues to obtain chromatin and post-microsomal supernatant.

3.3 Preparation of Arginyl-tRNA Transferase (ATE1), tRNA, and Arginyl-tRNA

Transfer RNA (tRNA) and arginyl-tRNA were prepared as described elsewhere [15] except that tRNA was extracted from cultured mouse plasmacytoma NRK-RSV ts cultured cells grown at 35 °C.

ATE1 enzyme for these experiments can be prepared from the post-microsomal supernatant described in Subheading 3.1 (for measurements of endogenous ATE1 activity) or from calf kidneys, rabbit liver, or other freshly excised mammalian tissue when used as an external ATE1 standard for measurements of acceptor protein levels. We used the method originally published by Soffer [25], which is described in the preceding chapter in this book, except that as the second ammonium sulfate precipitation step 70% ammonium sulfate cut was used.

3.4 Assay of Arginyl-tRNA Synthetase and Arginyl-tRNA Transferase Activity Using an ATP Regenerating System

1. Mix a 0.075 mL reaction containing:
   - 9.0 μmoles of Tris–HCl, pH 7.8.
   - 0.72 μmoles of magnesium acetate.
   - 0.35 μmoles of β-mercaptoethanol.
   - 4.5 μmoles of KCI.
   - 0.36 μmoles of ATP (potassium salt).
   - 0.72 μmoles of phosphoenolpyruvate (sodium salt).
   - 0.006 mg of pyruvate kinase.
   - μCi H^3-arginine (specific activity: 22–27curie/mM).
   - 30 μg tRNA.
   - 30 μg of acceptor protein (chromatin fraction or α-lactalbumin).
2. Incubate the mixture at 37 °C.
3. To obtain a time course of Arg incorporation, take aliquots (20 μL) from the incubation mixture at various time intervals, and measure for incorporated radioactivity using scintillation counter by the filter disc method [26]. The radioactive products insoluble in a mixture of cold (5 °C) 5% trichloroacetic acid, ether/alcohol (1:1, v:v), and ether represent arginyl-tRNA synthetase activity, while insolubility in the same mixture at 90 °C represents arginyl-tRNA transferase activity (*see* **Note 1**).

3.5 Determination of Cell Population Doubling Time

1. Plate cells in a Linbro 24-well dish and count the number of cells after trypsinization at various times.
2. Calculate population doubling levels according to the formula: 2^x = number of cells harvested/no. cells plated, where x is the population doubling increase at each subcultivation. Average generation times can be calculated by dividing the time between sub- cultivations by the population doubling increase during that period.
3. ATE1 activity in post-microsomal supernatant can be determined as a function of cell population doubling time as a marker of aging.

3.6 Transformation of Cells with SV40 Virus

This procedure was used during our studies to determine the effect of cell transformation on ATE1-dependent arginylation [14].

1. Infect IMR90 cells at various population doubling levels with wild-type and tsA30 of SV40 viruses [27] at the ratio of ten virus particles per cell for 2 h at 34 °C.
2. Passage cells by trypsinization once a week, examining morphological alterations during cell growth. Detection of cellular antigens in situ can be performed using immunofluorescence procedures as described in [28]. Parallel examination of uninfected cells should also be carried out.

4 Notes

1. This reaction mixture can also be employed to evaluate arginyl-tRNA transferase activity with different protein substrates. Amounts of added acceptor protein can be varied depending on the purpose of the assay. In some cases, endogenous ATE1 can be inactivated by heating for 2 min at 100 °C.

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Chapter 7

Assaying the Posttranslational Arginylation of Proteins in Cultured Cells

Mauricio R. Galiano and Marta E. Hallak

Abstract

To evaluate the posttranslational arginylation of proteins in vivo, we describe a protocol for studying the ^{14}C-Arg incorporation into proteins of cells in culture. The conditions determined for this particular modification contemplate both the biochemical requirements of the enzyme ATE1 and the adjustments that allowed the discrimination between posttranslational arginylation of proteins and de novo synthesis. These conditions are applicable for different cell lines or primary cultures, representing an optimal procedure for the identification and the validation of putative ATE1 substrates.

Key words Arg-tRNA-protein transferase, Posttranslational arginylation, Metabolic labeling, N-terminal modification, ATE1

1 Introduction

A vast majority of proteins synthesized in eukaryotic cells is modified at the time of synthesis (co-translational modification) or after its translation (posttranslational modification) [1–3], probably also involving translocation of the protein substrate [4, 5]. These modifications usually promote structural changes that modulate the protein properties and functions in vivo. Posttranslational arginylation of proteins is a modification related to many physiological processes and is gaining special consideration during the last years. The enzyme responsible of this modification is Arg-tRNA-protein transferase (ATE1) [6]. In a process that also requires the presence of Arg-tRNA synthetase (also implicated in protein translation), ATE1 mediates the covalent binding of an Arg (Arg) residue from Arg-tRNA to the amino acidic sequence of different proteins or peptides exposing Asp or Glu as N-terminal amino acid [7–14]. More recently, it was found that ATE1 also transfers Arg onto a mid-chain Glu residue or a different internal-chain

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residue probably using an alternative mechanism or cofactor(s) [15–17].

Originally, the enzyme ATE1 was characterized from in vitro experiments using a soluble fraction of protein samples derived from rat liver [7]. Its characterization together with few substrates raised the hypothesis that posttranslational arginylation is a preliminary modification of proteins that will be degraded by the ubiquitin-proteasome system [11, 18–20]. However, only a small number of identified ATE1 substrates are degraded by the proteasome after arginylation [6]. In addition, identification of several arginylated proteins [16, 21–24], whose functions are modulated by ATE1, has expanded the roles that can be assigned to this particular modification. Thus, it is important to corroborate that the arginylation of any substrate take place in vivo.

A few years ago, our laboratory adjusted the conditions for the study of posttranslational arginylation of proteins in living cells [25]. Traditionally, the radiolabeled amino acid incorporation into proteins has been used to assess both de novo synthesis of proteins and posttranslational aminoacyl modification of proteins in living cells [26]. To study posttranslational arginylation of proteins in vivo, the procedure detailed below takes advantage of the metabolic labeling of proteins, particularly, by the addition of a set of protein synthesis inhibitors that do not affect ATE1 activity, thus avoiding the artifacts that could be derived from de novo synthesis of proteins [25]. To confirm the N-terminal modification of proteins by arginylation, the protocol is complemented with the method of Sanger [27] and the Edman degradation method [28, 29]. Both methods determine, by different detection methods, the identity of the amino acid at the N-termini of protein sequences, after chemical derivation with specific reagents (*see* details on Subheading 3). As ATE1 modifies proteins by transferring Arg at the N-terminal or mid-chain amino acid [30], the latter would be underestimated by the mentioned confirmation methods. A more precise determination for mid-chain arginylated proteins includes mass spectrometry analysis of modified proteins. Representing the first physiological evidence of this modification in vivo [25], the protocol described below to study posttranslational arginylation of proteins using primary cell cultures or distinct cells lines has shown a successful replicable performance. As an in vivo system, this method has been extensively used on high-throughput screenings using fibroblast derived from ATE1 full knockout, allowing the identification of new ATE1 substrates [16]. Defining which particular protein is arginylated in vivo is essential in order to elucidate how this modification regulates protein function and also whether it has any relevance on pathological processes.

2 Materials

2.1 Cell Culture

1. Cultured mammalian cells

   In our original experiments, we used two cell lines (PC12 and COS-7) as well as primary brain cells derived from chicken embryos [25]. However, other cell lines or primary cultures can also be used. Cells are cultured until confluence in order to have abundant starting material for higher efficiency of the method. The confluence of cells in a 100 mm culture dish will contain 0.5–2 $\times$ 10^7 cells, depending on cell type.

2. Cell culture media

   For PC12 cells: RPMI 1640 media supplemented with 5% fetal bovine serum (FBS) and 10% heat-inactivated horse serum.

   For COS-7 cells: DMEM media supplemented with 10% FBS.

   For primary brain cells derived from chicken embryos: DMEM media supplemented with 10% FBS.

3. Arg-free media. Different types of Arg-free media are available from different suppliers. It is recommended to search the proper type of media required for the selected cell line.

2.2 Buffers and Solutions

1. 0.6 M $NaHCO_3$: dissolve 10.08 g of $NaHCO_3$ in 150 mL of distilled water. Adjust the volume to 200 mL to get a final concentration of 0.6 M. The solution is sterilized by filtration and stored at room temperature.
2. 5% 1-fluoro-2,4-dinitrobenzene (FDNB): dissolve 250 mg of FDNB with ethanol absolute to get a 5% final concentration.
3. Trichloroacetic acid (TCA) 10% solution: 100% (w/v) TCA stock solution is prepared by dissolving 500 g TCA in approximately 227 g of distilled water, which yield a final volume of 500 mL. By dilution, a 10% (w/v) TCA working solution is employed to get a 5% final concentration. Caution: protect eyes and avoid skin contact with TCA solutions.
4. Hydrochloric acid (HCl) 6 N: 400 mL of 6 N HCl solution is prepared by the addition of 200 mL concentrated HCl to 200 mL distilled water. A 0.02 N HCl solution is prepared by dilution with distilled water.

   Caution: handling and preparing dilutions with concentrated HCl with water produce an exothermic reaction. Preparation of solutions must be done under extraction hood with extreme caution.

2.3 Reagents

1. [^{14}C] Arg is required for metabolic labeling of proteins by addition to culture media at a final concentration 16 μM (1 mL of culture media will have 16 nmols =5 μCi) [25]. In a

similar way, other Arg isotopes can be employed as L-[2,3,4-^{3}H] Arg [30].

2. Poly-D-lysine (10 μg/mL) in PBS is required for preliminary coating of the dishes employed for culturing PC12 cells. As this method is suitable for different kinds of cells, the selected cell line or primary culture may require a different coating condition.
3. Mixture of protein synthesis inhibitors: stock solutions of camptothecin (10 mg/mL solubilized in DMSO), anisomycin (10 mg/mL solubilized in DMSO), and cycloheximide (10 mg/mL solubilized in ethanol) were employed as mixture in culture media at a final concentration of 20 μg/mL camptothecin, 20 μg/mL anisomycin, 5 μg/mL actinomycin, and 10 μg/mL cycloheximide [25]. Other procedures have employed a mixture of cycloheximide (100 μg/mL) and chloramphenicol (40 μg/mL), showing apparently less efficiency on protein synthesis inhibition [30].
4. Solution containing 5% triethylamine (dilution from stock reagent ≥99.5% concentration), 10% SDS (20% stock solution is prepared by dilution of corresponding amount of sodium dodecyl sulfate with distilled water, warming at ≤60 °C, and stirring till complete drug dissolution), and 10 μL of phenyl isothiocyanate (PITC, from stock reagent).

   Caution: Preparation of solutions must be done under extraction hood with protection for the eyes, skin, and lungs.
5. Chromatography mobile solution: phenol-water (75:25, w/v).

   Caution: Mixture and chromatography development must be performed under extraction hood with protection for the eyes, skin, and lungs. Biohazard handling of waste is also required.

2.4 Equipment

1. Cell culture incubator: Cells are cultured in a humidified, 37 °C, 5% CO_2 incubator.
2. Ultrasonic processor: A 200 watt ultrasonic device was employed in our experiments [13]; however, other devices suitable for controlled sonication of cells can be used.

   Caution: It is recommended to use devices with acoustic cabinet and ears protection during operation.
3. Refrigerated centrifuge.
4. Vacuum concentrator.
5. Liquid scintillation counter.

2.5 Chemicals

1. Trifluoroacetic acid (TFA): 99% stock reagent. Caution: Handling must be performed under extraction hood with protection for the eyes, skin, and lungs.

2. Butyl acetate: ≥98% stock reagent. Caution: Handling must be performed under extraction hood with protection for the eyes, skin, and lungs.
3. Plaques of silica gel G (20 × 20 × 0.025 cm) are employed according to the manufacturer's instruction for the identification of DNP-derivatized amino acids by thin layer chromatography, using DNP-Arg as standard.

3 Methods

The method detailed in this section has been mainly used in cultures of PC-12 cells and COS-7 cells [25]. Nonetheless, the condition set for in vivo arginylation of proteins can be adjusted for distinct cell lines or primary cell cultures [16, 25]. This method describing the in vivo arginylation assay using cells in culture consists of two procedures, the metabolic labeling by [^{14}C]Arg incorporation into proteins from cultured cells, followed by the evaluation of [^{14}C]Arg incorporated to the N-terminal position of proteins, which can be performed by two distinct procedures (*see* Subheading 3.2) [25]. Extended use of this method has made it useful for the identification of arginylated proteins in vivo ([16]; *see* **Note 1**).

3.1 Incorporation of [^{14}C]Arg into Proteins from Cultured Cells

1. Once the cells in culture reach confluence, the system is ready to perform the metabolic labeling by the incorporation of [^{14}C]Arg (*see* **Note 2**). Aspirate culture media, and rinse cells three times by gently swirling with Arg-free pre-warmed media.
2. Incubate the cell cultures for 1 h in the same Arg-free media to set the growing condition of the cells for appropriate metabolic labeling.
3. Supplement the media in the dish with 1 mL of fresh Arg-free media without serum (*see* **Note 3**) containing the mixture of protein synthesis inhibitors (see Subheading 2.3), and incubate for 2 more hours. This incubation will transiently arrest de novo synthesis of proteins (*see* **Note 4**); hence, [^{14}C]Arg incorporation will correspond to posttranslational modification of the proteins.
4. Add 16 nmol of [^{14}C]Arg (5 μCi) to culture media for additional 3 h of incubation. At this step, [^{14}C]Arg will incorporate posttranslationally into proteins.
5. Stop the reaction by setting the cells on ice. Replace the incubation media with fresh Arg-free media (1 mL per dish), keeping the dishes on ice.
6. Scrape off the cells with the added Arg-free media using rubber policeman, and transfer the material to a small tube with lid.

7. Break the cells by sonication. To minimize thermal degradation of the samples, four pulses (10 s each at 40% output) should be applied per sample, keeping the tubes on ice during the procedure.
8. Centrifuge the lysed cells at 100,000 g for 1 h at 4 °C. Collect the supernatant containing cytosolic proteins, and discard the pellet. Typically, at this stage, ~3 mg of proteins containing 0.5–1 × 10^5 cpm of [^{14}C]Arg can be collected from a confluent 10 cm dish.

3.2 Evaluation of [^{14}C]Arg Incorporation to the N-Terminal Position of Proteins

Two traditional procedures can be applied to evaluate the [^{14}C]Arg bound to the N-terminal position of proteins, the method of Sanger modified by Levy [27] and the Edman method [28, 29] (*see* **Note 5**).

The Method of Sanger

To determine the N-terminal sequence of peptides and proteins, this technique employs the compound 1-fluoro-2,4-dinitrobenzene (FDNB) which, under basic conditions, reacts with the free N-amino group of peptides (FDNB also reacts with the phenolic group of tyrosine, the imidazole group of histidine, the ε-amino group on lysine, and the hydroxy group on tyrosine of all free amino acids or peptides) generating colored dinitrophenyl (DNP) derivatives. By acidic hydrolysis and ether extractions, the N-terminal DNP-derivatized amino acids can be separated from those non-N-terminal amino acids that have side chains that react with FDNB (as listed above) and identified by chromatographic methods. The main limitation of this method is that it only allows the analysis of one N-terminal amino acid in the sample.

The whole method must be performed under extraction hood to avoid toxic exposition.

1. Lyophilize the solution of arginylated cytosolic proteins obtained in the previous step, and resuspend them in 0.6 M $NaHCO_3$ (1 mL per 3 mg protein sample).
2. Mix the protein suspension with an equal volume (1 mL) of 5% FDNB solution to induce derivatization of the protein N-termini. This mixture is incubated for 18 h with agitation in the dark.
3. Stop the reaction by acidification (to pH 2.0, approximately) by the addition of concentrated HCl. Remove non-reacted FDNB by extraction with ethylic ether (5 mL – three times), and discard the organic phase.

4. Precipitate the proteins contained in aqueous phase by the addition of TCA to 5% final concentration. Centrifuge the mixture at 1000 g for 15 min. Discard the supernatant. Wash the precipitate once with 2 mL absolute ethanol absolute, and centrifuge again under the same conditions. Discard the supernatant.
5. Incubate the pellet containing TCA-insoluble material with 5 mL of 6 N HCl at 105 °C (refluxed on a hot plate) for 18 h for acidic hydrolysis. This reaction hydrolyses the derivatized N-terminal amino acids.
6. Extraction of derivatized amino acids with sulfuric ether. After acidic hydrolysis, cool the solution containing derivatized amino acids to room temperature, and dilute with 10 mL of distilled water. Extract this suspension three times with 5 mL of sulfuric ether, and discard the organic phase. Under this conditions, derivatized DNP-[^{14}C]Arg amino acids remain in the aqueous phase, which is then concentrated by vacuum centrifugation.
7. Identify the DNP-[^{14}C]Arg by thin layer chromatography (TLC). Run the concentrated aqueous phase containing the DNP-[^{14}C]Arg on a thin layer of silica gel G using DNP-Arg as a standard. The solvent system required is phenol-water (75: 25, w/v). After saturation of the plaque inside the chromatographic cube during 2 h, the chromatogram is developed around 15 cm (front migration). The band that co-migrates with the standard is scrapped and quantified by liquid scintillation counting to determine the level of radioactive Arg incorporation.

The Edman Method

In this method, the α-amino group of the N-terminal amino acid residue reacts with phenyl isothiocyanate (PITC) under basic conditions to generate phenylthiocarbamyl (PTC)-derivatized peptides. Mild acidic hydrolysis of the protein or peptide releases N-terminal-derivatized residues that are converted to phenylthiohydantoin (PTH)-derivatized amino acids, which are extracted from the reaction mixture. The stabilized (PTH)-derivatized residues are identified by chromatography (or analyzed through different methods; see below). The main advantage of this method over Sanger method is that the remainder of the same peptide or protein sample can be subjected repeatedly to the same treatment, allowing the determination of the sequence beyond the N-terminal residue.

1. Lyophilize the cytosolic proteins, and resuspend them in 250 μL of solution containing 5% triethylamine, 10% SDS,

and 10 μL of PITC. The mixture is incubated for 45 min at 50 °C, producing PTC-peptides/proteins.

2. Extraction of the unbound PITC and the secondary reaction products. Add 1 mL water and 0.8 mL of cold acetone to the mixture, and discard the organic phase. Repeat the procedure for a total of three times.
3. Dry the aqueous phase containing PTC-peptides/proteins using a SpeedVac concentrator.
4. Hydrolyze the samples by the addition of 150 μL trifluoroacetic acid (TFA) and incubation at 50 °C for 10 min. This reaction cleaves the weakened N-terminal peptide bond of PTC-peptides producing shorter peptides and thiazolinone-derivatized amino acids.
5. Dry the sample again. Add 1 mL 0.02 N HCl to convert thiazolinone-derivatized amino acids on (PTH)-derivatized amino acids.
6. Extract the sample three times with 1 mL butyl acetate at 0 °C. Collect and pool the organic phase containing PTH-derivatized amino acids, and quantify the level of radioactive Arg by liquid scintillation counting. This [^{14}C]Arg detected after a first round of Edman degradation method corresponds to Arg N-terminally incorporated into proteins.

4 Notes

1. The described method for studying posttranslational arginylation of proteins in cell cultures has been subsequently optimized for the identification of arginylated proteins in vivo, given the current availability of fibroblasts derived from ATE1$^{-/-}$ mice [16, 30].
2. To confirm optimal conditions for the assay, the cell viability can be checked by the trypan blue exclusion.
3. The protocol requires the incubation of the cells in media without Arg for at least 6 h. Even if Arg is an essential amino acid for cellular physiology, the absence of Arg during the procedure should not be detrimental for the cells. Moreover, the Arg-free media employed for metabolic labeling does not have serum, to avoid dilution of added [^{14}C]Arg by the non-labeled Arg from the serum. Nevertheless, at least a visual inspection of the cells in culture by phase contrast microscopy is recommended. Further studies may be required if morphological changes or dish attachment impairments are observed during Arg-free incubation of the cells.

4. The confirmation of arrested de novo protein synthesis is checked by metabolic labeling with [^{35}S]-methionine. Using this procedure, the efficiency of each antibiotic on protein synthesis inhibition was assayed in the different cell cultures employed. When different protein synthesis inhibitors were applied in combination, it was determined a 98% of protein synthesis inhibition [25]. In addition, the effect of each antibiotic on posttranslational incorporation of [^{14}C]-Arg into proteins was evaluated by in vitro assays using soluble proteins derived from rat brain. Any protein synthesis inhibitor evaluated affects the global posttranslational arginylation of proteins in vitro [25].
5. Using the Edman method, a robust increase in the proportion of N-terminally arginylated proteins in the presence of protein synthesis inhibitors was described in our studies [25]. For example, a proportional change of N-terminally arginylated proteins from 1.4% to 20% was observed in PC12 cells when protein synthesis inhibitors were added. In view of these changes, it is important to consider that the analysis by Edman method is evaluating the [^{14}C]Arg incorporated at the N-terminal residue of proteins. Taking into account that ATE1 seems to also modify mid-chain residues of proteins [15, 16, 30], the values derived from this method are likely underestimating the in vivo activity of ATE1. A global evaluation of ATE1 activity would involve a more precise method to characterize which residue of a protein is modified by this enzyme, as well as the identity of modified proteins, mainly derived from mass spectrometry studies [16, 31, 32].

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Chapter 8

Assaying ATE1 Activity in Yeast by β-Gal Degradation

Anna S. Kashina

Abstract

In the 1980s, it was found that addition of N-terminal Arg to proteins induces their ubiquitination and degradation by the N-end rule pathway. While this mechanism applies only to the proteins which also have other features of the N-degron (including a closely adjacent Lys that is accessible for ubiquitination), several test substrates have been found to follow this mechanism very efficiently after ATE1-dependent arginylation. Such property enabled researchers to test ATE1 activity in cells indirectly by assaying for the degradation of such arginylation-dependent substrates. The most commonly used substrate for this assay is *E. coli* beta-galactosidase (beta-Gal) because its level can be easily measured using standardized colorimetric assays. Here, we describe this method, which has served as a quick and easy way to characterize ATE1 activity during identification of arginyltransferases in different species.

Key words Arginyltransferase, Yeast complementation, Beta-galactosidase, N-end rule, Ubiquitin, Proteasome

1 Introduction

The method described below utilizes the fact that ATE1-mediated arginylation can render certain proteins metabolically unstable via the ubiquitin-dependent N-end rule pathway [1]. In the original study, the authors discovered that synthetic constructs of beta-galactosidase (beta-Gal) expressed in yeast with different amino acid residues engineered into the N-terminal position have different metabolic stability (N-end rule pathway), measured by the beta-Gal activity in yeast lysates using color substrates. Met-beta-Gal, containing the naturally occurring Met at the N-terminus, was stable, resulting in high beta-Gal activity, while Arg-beta-Gal was so unstable it became virtually undetectable in color reactions.

The same tests showed that Asp-beta-Gal and Glu-beta-Gal were greatly destabilized compared to Met-beta-Gal in the presence of ATE1, which favors these N-terminal residues for arginylation and renders the resulting proteins metabolically unstable. These

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assays have been used in many studies characterizing arginylation as a branch of the N-end rule pathway of protein degradation [2–10].

In a typical assay for testing ATE1 activity through beta-Gal arginylation and degradation, ATE1 is co-transfected into yeast cells together with Asp- or Glu-beta-Gal, and the beta-Gal activity (reflecting its intracellular levels) is measured in the transfected yeast extracts in comparison to yeast extracts transfected with Met-beta-Gal (positive control) and Arg-beta-Gal (negative control).

All the procedures described below are standard procedures used for yeast transformation and beta-Gal assays and can be found described in detail in multiple yeast manuals, with the exception of the cloning strategy utilized for the design of protein substrates containing different N-terminal residues (Subheading 3.1).

This method enables the expression of proteins encoding any amino acid residue in the N-terminal position, and it has been originally used in the studies of the N-end rule pathway of protein degradation to demonstrate that in vivo half-life of some proteins can depend on the identity of their N-terminal residue [1, 11]. To achieve this, ubiquitin is cloned in a position directly adjacent to the protein's N-terminus, and this ubiquitin moiety is then cleaved off co-translationally to expose the adjacent amino acid residue that then becomes the protein's N-terminus (Fig. 1).

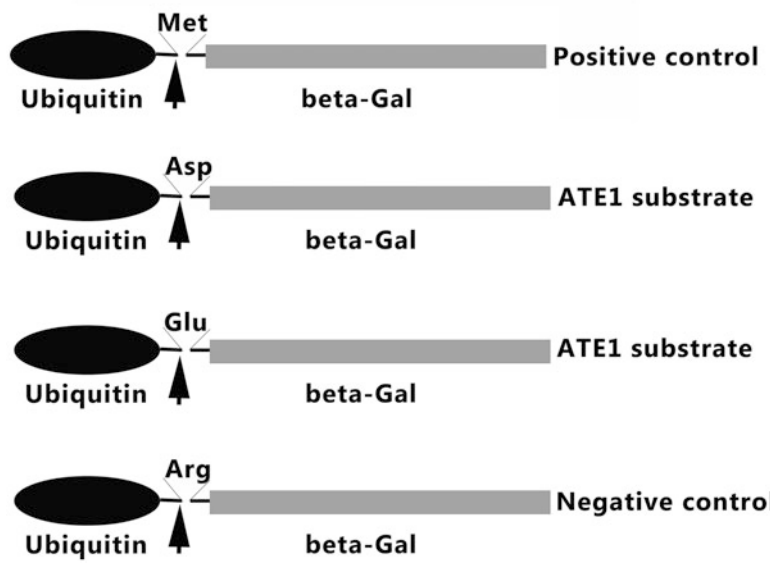

Fig. 1 Ubiquitin fusion constructs for testing the activity of ATE1. The arrow denotes the site of co-translational cleavage by de-ubiquitinating enzymes, exposing the N-terminal residue of the beta-Gal-derived test substrate

For this cloning, the end of ubiquitin is modified to insert silent mutations that transform this site into the site for SacII restrictase, and subsequent SacII cleavage can then be used for the direct in-frame fusion of ubiquitin to the protein of interest. In the method described below, this strategy is applied to preparing the construct of beta-Gal, but the same strategy can be used for any other protein to modify it into a favorable substrate for arginylation by introducing Asp or Glu into the N-terminal position.

2 Materials

1. Yeast strains lacking ATE1 (*S. cerevisiae ate1Δ*) (*see* **Note 1**).
2. Yeast extract-peptone-dextrose (YPD) media (Cold Spring Harbor Protocols http://cshprotocols.cshlp.org/content/2010/9/pdb.rec12315.full?text_only=true).
3. Single-strand carrier DNA from salmon sperm or calf thymus (or an equivalent source), 10 mg/mL solution.
4. 10× TE buffer: 100 mM Tris-HCl, pH 7.5, 10 mM EDTA.
5. 1 M lithium acetate (LiAc) solution, pH 7.5 (pre-adjusted using dilute acetic acid).
6. TEL buffer: TE buffer containing 0.1 M LiAc, pH 7.5 (mixed from the stocks of 10× TE buffer, 1 M LiAc, and distilled water).
7. Plate solution: 8 mL of 50% w:v PEG 4000, 1 mL of 10× TE, 1 mL of 10× LiAc.
8. Sterile distilled water.
9. DMSO (dimethyl sulfoxide).
10. Plasmids encoding Arg-beta-Gal (negative control), Met-beta-Gal (positive control), Asp-beta-Gal, Glu-beta-Gal, and ATE1 constructs to be tested (*see* **Note 1**), containing appropriate selection markers, e.g., URA for beta-Gal plasmids and TRP for ATE1 plasmids to enable the double selection of transformants.
11. Complete minimal (CM) dropout plates: 17% yeast nitrogen base minus amino acids, 0.5% $(NH_4)_2SO_4$, 0.2% dextrose, -Ura/-Trp dropout supplement (for the example in pp. 9 above; other supplements should be used for the plasmids with other selection markers).
12. Galactose media for recombinant protein expression: liquid media (for ONPG assay) and plates (for X-gal overlays) prepared the same as above but with 2% galactose in place of dextrose.
13. 1 M Na_2PO_4, pH 7.0.

14. 10% SDS.
15. X-gal solution in dimethylformamide (DMF): ~10 mg or more in 2 mL of DMF.
16. Agarose (any grade) for X-gal overlays.
17. Z buffer: 60 mM Na_2HPO_4:$7H_2O$, 40 mM NaH_2PO_4: H_2O, 10 mM KCl, 1 mM $MgSO_4$:$7H_2O$, 50 mM 2-mercaptoethanol, pH 7.0.
18. Chloroform.
19. 4 mg/mL ortho-nitrophenyl-beta-galactoside (ONPG) in 0.1 M KPO_4, pH 7.0 (sterile-filter and store frozen).
20. 1 M Na_2CO_3.

3 Methods

3.1 Preparation of Yeast Competent Cells

1. One day before the experiment, inoculate 3 × 5 mL yeast cultures for each transformation, and grow overnight at 30 °C.
2. The next morning, combine all 3 cultures (15 mL total) into 300 of fresh YPD medium. Grow at 30 °C to OD_{600} ~0.7 (approximately 6.5 h).
3. Centrifuge the yeast cultures at 3000 rpm for 5 min at room temperature to collect the yeast cells (6 × 50 mL conical tubes).
4. Discard the supernatant and resuspend the cells in 5 mL sterile distilled water. Combine all cells into two tubes to make up the volume to 50 mL in each tube.
5. Centrifuge at 3000 rpm for 5 min at room temperature, and discard the supernatant.
6. Resuspend the pellets in 5 mL sterile distilled water each, combine both into one tube, and add another 40 mL of sterile distilled water to make up the volume to the total of 50 mL.
7. Centrifuge at 3000 rpm for 5 min at room temperature, and discard the supernatant.
8. Resuspend the pellet in 4 mL TEL buffer. Store at 4 °C (this suspension can be stored up to 1 week).

3.2 Yeast Transformation

1. Prepare 1 Eppendorf tube for each transformation, and label them.
2. To each tube, add 2 μL of each plasmid to be transformed plus 2 μL carrier DNA. For testing ATE1 activity, a mixture of Asp- or Glu-beta-Gal and ATE1 plasmids, cloned into the vectors optimized for yeast expression, should be used. For control transfections, Met-beta-Gal and Arg-beta-Gal (with or without ATE1) should be used.

3. Add 100 μL of yeast competent cells to each tube and mix.
4. Add 600 μL of plate solution to each tube and vortex vigorously.
5. Incubate for 30 min in a 30 °C shaker.
6. Add 70 μL of DMSO to each tube, and mix gently by inverting.
7. Heat shock the cells for 15 min in a 42 °C water bath.
8. Place the cells on ice for 2 min.
9. Centrifuge in a benchtop centrifuge for 5 min at 14,000 rpm, and discard the supernatant.
10. Resuspend the pellet in 100 μL of TE and plate onto CM dropout plates.

3.3 Color Overlay Assay for Beta-Gal Activity Using X-Gal

This method can be used for quick estimation of beta-Gal degradation in the presence of ATE1.

1. Two days before the experiment, streak the colonies of yeast transformants in segments onto a fresh CM dropout plate with galactose (for Gal promoter induction) or otherwise optimized for the expression of recombinant proteins. Grow at 30 °C to obtain visible colonies or a lawn of cells.
2. Melt 0.5 g of agarose (any grade) in 50 mL of distilled water. Add 50 mL of 1 M Na_2PO_4, pH 7.0, mix, and keep at 70 °C to prevent the agarose from solidifying.
3. Add 1 mL of 10% SDS to the solution.
4. Add 10+ mg of X-gal in 2 mL DMF (*see* **Note 2**).
5. Mix the solution by gentle swirling to avoid bubbles.
6. Using a pipette, gently overlay 10 mL of the solution per yeast plate. Pour to the side of the plate to avoid dislodging the yeast colonies, and tilt the plate to ensure that the agar evenly covers the entire surface. The agar should solidify shortly after the overlay.
7. Incubate the plate at 37 °C until the development of intense blue color in the yeast colonies transfected with Met-beta-Gal positive control. For a strong Met-beta-Gal signal, this should happen within an hour. At this point, the intensity of the blue color directly correlates with the levels of beta-Gal, reflecting the extent of its ATE1-mediated degradation.

3.4 Liquid Assay for Beta-Gal Activity Using ONPG

This method enables quantitative estimation of beta-Gal levels and activity in liquid cultures and can be used as an indirect measurement of ATE1 activity. Due to the relatively poor sensitivity of the method, multiple parallel measurements should be performed for each sample to ensure reproducibility.

1. Pick individual yeast colonies transformed with appropriate plasmids, and grow overnight at 30 °C in 5 mL of selective media with galactose (for Gal promoter). Testing five to ten colonies from each condition is recommended (*see* **Note 3**).
2. Inoculate 20–50 μL of each overnight culture into fresh media. Grow to ~OD_{600} 0.5–1.0.
3. Centrifuge the cultures in individual tubes for 5 min at 2500 rpm in a tabletop centrifuge. Discard the supernatants.
4. Resuspend the cell pellets in an equal volume of Z buffer and place on ice.
5. Determine the OD_{600} for each sample and write down the numbers.
6. For each tube, set up the following 1 mL reactions: (i) 100 μL of cell suspension plus 900 μL of Z buffer and (ii) 50 μL of cell suspension plus 950 μL of Z buffer.
7. Add one drop of 0.1% SDS and two drops of chloroform to each tube using a Pasteur pipette. Vortex for 10–15 s and incubate for 15 min in a 30 °C water bath.
8. Prepare a timer. Add 0.2 mL ONPG solution to each tube. Vortex for 5 s, place in a 30 °C water bath, and start the timer.
9. When a medium-yellow color has developed (this should take, on average, 10–15′ in the arginylation assay), stop the reaction by adding 0.4 mL of 1 M Na_2CO_3, and write down the time. The color will become deeper after 1 M Na_2CO_3 addition (*see* **Note 4**).
10. Centrifuge the reaction mixture for 5 min at 2500 rpm in a tabletop centrifuge to remove the debris. Determine the OD_{550} (should be close to zero for a well-clarified sample) and OD_{420} (reflecting the beta-Gal-mediated conversion of ONPG).
11. Calculate beta-Gal activity in each sample using the following formula:

$$\text{Activity (U)} = 1000 \times (OD_{420} - OD_{550} \times 1.75)/(t) \times x(v) \times (OD_{600}),$$

where (t) is the time of the reaction (min) and (v) is the volume of the culture used in the assay (mL).

This activity serves as a reflection of beta-Gal level in the yeast extract, which is dependent on the extent of its ATE1-mediated degradation. A typical result is shown in Fig. 2.

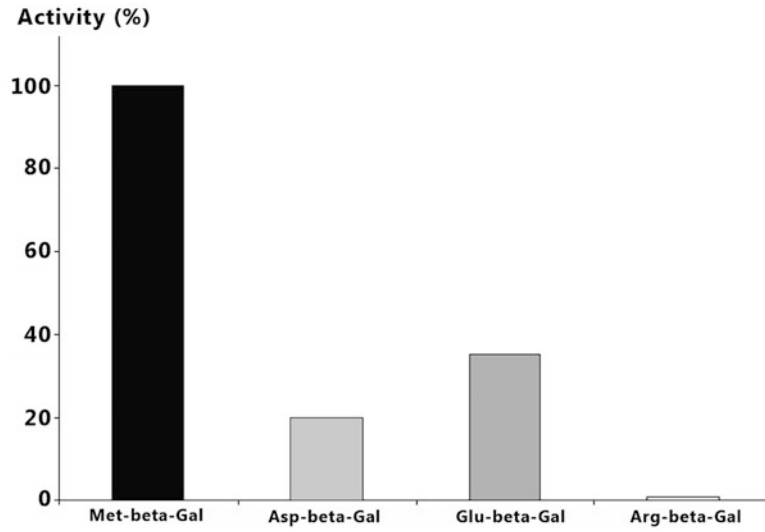

Fig. 2 Typical results of arginylation-dependent beta-Gal degradation assays. Activities of beta-Gal bearing different N-terminal residues are denoted as percentages of the Met-beta-Gal positive control, which remains metabolically stable and does not undergo detectable arginylation in this assay. Glu-beta-Gal is typically more stable in these assays compared to Asp-beta-Gal, suggesting that this substrate may be a less efficient target for arginylation during yeast complementation. *Note:* Asp and Glu do not appear to differ significantly in in vitro arginylation assays

4 Notes

1. Yeast strain with the deletion of Ate1 gene is needed only if exogenously introduced ATE1 is involved in the assay. Use this strain in combination with ATE1-expressing plasmids to be co-transfected with the beta-Gal substrates. Otherwise, for testing endogenous ATE1 activity, wild-type yeast should be used, and no ATE1 plasmid is required.
2. X-gal amount can be adjusted slightly to achieve the desired color. DMF solution is essential for cell permeabilization, and its volume should not be altered.
3. For multiple parallels, the assay should be done in batches to avoid delays at critical points in the experiment.
4. It is recommended to do a pilot experiment with Met-beta-Gal control to determine the approximate incubation time prior to the experiment.

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Chapter 9

Assaying Arginylation Activity in Cell Lysates Using a Fluorescent Reporter

Akhilesh Kumar and Fangliang Zhang

Abstract

Here, we describe an antibody-based method to evaluate the enzymatic activity of arginyltransferase1 (Ate1). The assay is based on the arginylation of a reporter protein, which contains the N-terminal peptide of beta-actin, a known endogenous substrate of Ate1, and a C-terminal GFP. The arginylation level of the reporter protein is determined on an immunoblot with an antibody specific for the arginylated N-terminus, while the total amount of substrate is evaluated with anti-GFP antibody. This method can be used to conveniently and accurately examine the Ate1 activity in yeast and mammalian cell lysates. Moreover, the effect of mutation on Ate1 critical residues and effect of stress and other factors on Ate1 activity can also be successfully determined with this method.

Key words Arginylation, Arginyltransferase1, Ate1, Reporter, Antibody, Lysate, Western blot, Immunoblot

1 Introduction

Posttranslational arginylation is a process that transfers an arginine residue from Arg-tRNA to proteins or peptides in a ribosome-independent manner [1, 2]. The new arginine predominately links to the free amine group of the N-terminus, although side-chain modification has also been reported [3, 4]. The N-terminal arginylation may lead to the degradation of its target proteins or changes the function of the proteins by altering their surface charge [5]. Arginylation regulates several biological pathways including stress response [6–8]. Therefore, the measurement of arginylation activity in the cell or tissue is important to understand the various biological processes regulated by arginylation.

Arginyltransferase1 (Ate1) enzyme catalyzes arginylation reaction, which is conserved in all eukaryotes [9–11]. In the past, several methods have been developed to measure the activity of Ate1. Traditionally, the incorporation of radio-labeled arginine into

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an exogenously added substrate protein such as α-lactalbumin or bovine serum albumin has been used to assess posttranslational arginylation. Since arginine is also naturally used for protein synthesis, translation inhibitors were added in the arginylation reaction mixture to minimize artifacts arising from de novo synthesis of proteins [12, 13]. Another colorimetric method described to assay the Ate1 activity took the advantage of arginylation-mediated degradation of beta-galactosidase enzyme. In this method, the *ATE1* gene was co-transfected into *ate*1-deleted yeast cells together with beta-galactosidase enzyme exposing destabilizing residues (Asp- or Glu) at its N-terminus, which is found to be preferentially arginylated. To express the Asp-beta-gal or Glu-beta-gal, in-frame N-terminal fusion of ubiquitin was used, which would be co-translationally removed and expose the penultimate residue as the N-terminus. In this assay, the protein level of the beta-galactosidase carrying the Asp or Glu residue at its N-terminus is expected to be inversely correlated with the Ate1 activity. Thus, by measuring the beta-Gal activity in the transfected yeast extracts, Ate1 activity was indirectly measured [14, 15]. More recently, an ELISA-based method has been developed to assay the activity of purified Ate1. In this method, a 16-amino acid-long peptide (DDIAALVVDNGSGMCK) was used as a substrate for Ate1. This peptide was derived from beta-actin, which is a known substrate for arginylation in vivo. An antibody specific to arginylated form of this peptide was then applied to measure the level of arginylation in a reconstituted system with purified proteins [16]. These methods have played important roles in the studies of arginylation; however, they suffer from one or other demerits. For example, radioactivity-based method involves radiation hazards, and also translation-mediated incorporation of labeled arginine into proteins (due to the leaky nature of most translation inhibitors) compromises its accuracy. The beta-Gal-based method requires co-transfection of *ATE1* gene in yeast along with the beta-Gal reporter. Moreover, this method has poor sensitivity and requires multiple parallel measurements and controls to ensure the reproducibility. The ELISA-based method is poorly compatible for measuring arginylation activity in the cell or in cell lysates, which contains many endogenous arginylated proteins that cross-react with the antibody in the ELISA assay. Furthermore, the short peptides used in this method cannot be reliably quantified without a complex method and high-precision instrument.

This chapter describes an Ate1 activity assay that is based on the arginylation of N-terminal beta-actin peptide and its detection on an immunoblot originally described by Kumar et al. [6]. In this method, a stretch of 15 amino acids (DDIAALVVDNGSGMC) derived from the N-terminus of mammalian beta-actin, a known substrate of Ate1 [17], was in-frame fused with ubiquitin at its N-terminus and GFP at its C-terminus. When expressed in

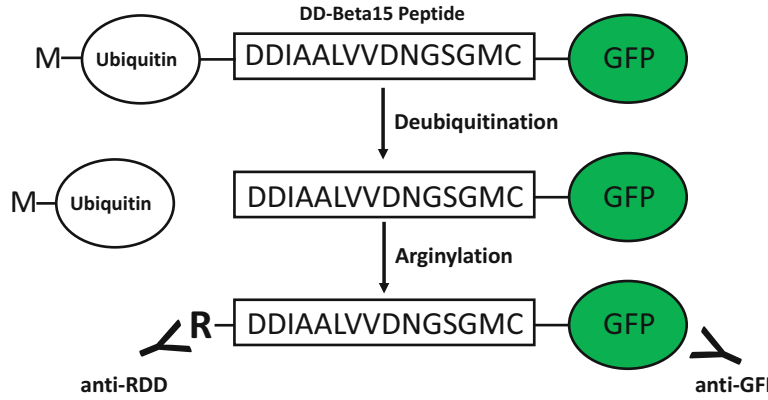

Fig. 1 A scheme illustrating how the DD-beta15-GFP arginylation reporter is used to evaluate the Ate1 activity. A 15-amino acid-long peptide starting with two aspartic acid residues (D) derived from the N-terminus of mammalian beta-actin was in-frame fused with ubiquitin at its N-terminus, which is cleaved co-translationally by endogenous deubiquitination enzymes in eukaryote and leaves the aspartic acid residues as the new N-terminus. The Ate1 activity is measured by determining the arginylation level of this reporter protein with an anti-RDD antibody, which only recognizes the arginylated form of this reporter protein. The C-terminal GFP is used to determine the steady-state level of the reporter protein by immunoblotting it with anti-GFP antibody

eukaryotic system, the co-translational removal of ubiquitin by endogenous deubiquitination enzymes resulted in a GFP protein with a new N-terminus of 15 amino acids derived from beta-actin, which is termed "DD-beta15-GFP." This reporter protein was expressed in *ate1*-deleted yeast to avoid inadvertent arginylation and then harvested using anti-GFP magnetic beads. The purified protein was used as a substrate to measure the Ate1 activity in the lysate of cells or tissues. The level of arginylation of this protein was determined using Western blot with a custom synthesized antibody (anti-RDD) which specifically recognizes the arginylated form of the beta-actin peptide. The total amount of the substrate protein (arginylated or non-arginylated) was determined with an anti-GFP antibody (Fig. 1).

Notably, a similar method can be employed in mammalian cells by using mammalian expression reporter plasmids.

2 Materials

1. Yeast strain lacking *ATE1* gene (*Saccharomyces cerevisiae*, *ate1Δ*) for the production of DD-beta15-GFP arginylation reporter and wild-type yeast strain with functional *ATE1* gene to test arginylation activity.

2. Wild-type mouse embryonic fibroblast (MEF) cells and *ATE1*-KO MEF cells.
3. Yeast expression plasmids pGPD2 containing DNA construct to express Ub-DD-beta15-GFP arginylation reporter and with URA selection markers for the selection of transformants.
4. Yeast extract-peptone-dextrose (YPD) media.
5. Complete minimal (CM) dropout media and plates: 17% yeast nitrogen base minus amino acids, 0.5% ammonium sulfate, 0.2% dextrose, −Ura, and dropout supplements.
6. 10× TE buffer: 100 mM Tris–HCl, pH 7.5, 10 mM EDTA.
7. 1 M lithium acetate (LiAc) solution, pH 7.5 (pH adjusted using dilute acetic acid).
8. TE-LiAc buffer: 1× TE buffer containing 0.1 M LiAc, pH 7.5 (mixed from the stocks of 10× TE buffer, 1 M LiAc, and distilled water).
9. Single-strand carrier DNA from salmon sperm, 10 mg/mL solution.
10. PEG solution: 8 mL of 50% w/v PEG 4000, 1 mL of 10× TE, 1 mL of 10× LiAc.
11. Yeast cell lysis buffer: 10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, PMSF 0.1 M, and protease inhibitor 0.1 M.
12. Magnetic bead wash buffer: 10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA.
13. Acidic elution buffer: 200 mM glycine, pH 2.5.
14. Neutralization buffer: 1 M Tris–HCl, pH 10.4.
15. Arginylation reaction buffer: 50 mm HEPES buffer pH 7.5, 25 mM KCl, 15 mM $MgCl_2$, 2.5 mM ATP, and 0.2 mM arginine. Reducing reagents should be avoided.
16. 4× Laemmli buffer: 8% SDS, 40% glycerol, 20% 2-mercaptoethanol, 0.008% bromophenol blue, and 0.250 M Tris–HCl, pH 6.8.
17. Nitrocellulose membrane and precast SDS-PAGE gel (10%).
18. SDS-PAGE running buffer: 3.0 g Tris base, 14.4 g glycine, 1 g SDS (final volume 1 L).
19. Transfer buffer: 3.0 g Tris base, 14.4 g glycine, 200 mL methanol (final volume 1 L).
20. PBS buffer: Phosphate buffered saline.
21. PBST buffer: Phosphate buffered saline containing 0.1% Tween20 (final volume 1 L).

22. Blocking buffer: 0.5% casein in 1× PBS buffer.
23. Antibodies: Anti-RDD antibody (custom produced or purchased from a commercial source; currently, such antibody is commercially available from Millipore Sigma, catalog number ABT264), anti-GFP antibody, anti-yeast-phosphoglycerate kinase (anti-PGK) antibody, anti-tubulin antibody, anti-beta-actin antibody, anti-mouse/rabbit-HRP conjugated antibodies.
24. GFP-TRAPS nanobody conjugated with magnetic beads (Bulldog Bio) (in the case of the protocol described in Subheading 3.2).
25. HRP chemiluminescence kit (Thermo Fisher Scientific Super-Signal Femto) or similar products for Western blot detection.
26. Restore Stripping Buffer (Thermo Fisher Scientific).

3 Methods

3.1 Cloning of DD-beta15-GFP Arginylation Reporter

1. Clone the chimeric gene downstream of constitutive GAP promoter in pGPD2 yeast expression plasmid vector between EcoR1 and XhoI restriction sites (Fig. 2) (*see* **Note 1**). The DNA sequence encoding a stretch of 15 amino acids (DDIAALVVDNGSGMC) derived from the N-terminus of mammalian beta-actin should be cloned directly adjacent to ubiquitin using SacII and BamH1 restriction sites to generate an N-terminal in-frame fusion of ubiquitin.
2. Clone the GFP encoding gene in-frame after the peptide sequence using BamH1 and XhoI restriction sites, to generate a C-terminal in-frame GFP fusion.

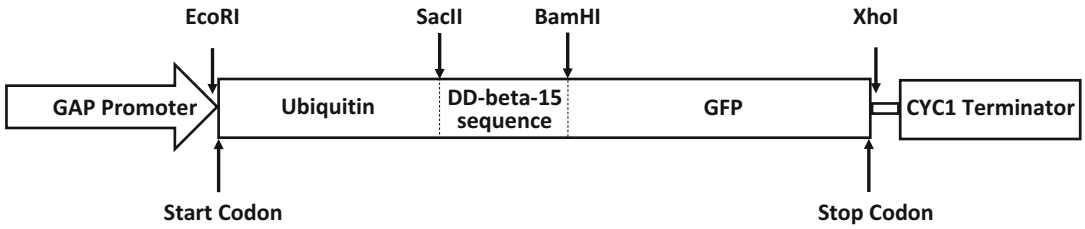

Fig. 2 Cloning strategy of DD-beta15-GFP arginylation reporter. The construct is cloned in pGPD2 yeast expression vector between a constitutively active GAP promoter and a CYC1 terminator. The arrows indicate the restriction sites, start and stop codon positions. The DD-beta15 sequence, DDIAALVVDNGSGMC, was derived from the N-terminal region of beta-actin with the first methionine and the second Asp (D) removed

3.2 Production of DD-beta15-GFP Arginylation Reporter in Yeast and Its Purification Using GFP-TRAPS Nanobody Conjugated with Magnetic Beads

1. Transform yeast cells with the Ub-DD-beta15-GFP arginylation reporter construct using conventional LiAc-based yeast transformation method in a BY4741 yeast strain lacking *ATE1* gene [6]. Plate the transformed yeast culture on CM media lacking uracil (-Ura) (*see* **Note 2**). Select the transformed yeast cells by picking outgrown colonies on Ura-minus plate.
2. Inoculate yeast transformants expressing DD-beta15-GFP arginylation reporter from constitutive GAP promoter in 100 mL selective media, and grow them overnight.
3. Centrifuge the overnight grown culture for 5 min at ~4000 × g in a tabletop centrifuge in 50 mL conical tubes to harvest the cells.
4. Resuspend cell pellets in 5 × volume of yeast cell lysis buffer, and transfer the mixture into six 1.5 mL tubes.
5. Add glass beads equal to 1 ~ 2× volume of cell pellet into each tube.
6. Lyse the cells by vortexing the cell suspension 15 times, in cycles of 1 min on and 1 min off, at 4 °C.
7. Centrifuge the lysed cell suspension at 12000× g, and transfer the supernatant into fresh tubes.
8. Add 1/10 of the volume of GFP-TRAPS nanobody conjugated with magnetic beads, pre-washed in the lysis buffer, to the lysate. Purify GFP-conjugated protein as described in the manual of GFP-TRAPS nanobody manufacturer (Bulldog Bio) by mixing 500 μl of cell lysate with 500 μL solution containing the GFP-TRAPS magnetic beads, followed by incubation for 1 h at 4 °C on a rotator and bead collection using a magnet. Wash the beads three times using 500 μL wash buffer for 5 min on a rotator, and collect the beads using a magnet. Elute the proteins bound to the beads using 100 μL acidic elution buffer added in beads and mixed with pipette. Collect the beads using a magnet. Transfer the supernatant into a new tube, and immediately add 10 μL neutralization buffer to neutralize the eluate fraction.
9. Analyze the purified protein by SDS-PAGE and conventional Coomassie blue staining. The N-terminal ubiquitin should be completely removed at this stage, as seen by the molecular weight of the recombinant protein.
10. The purified DD-beta15-GFP reporter protein can be stored at 4 °C for up to a week or at −20 °C with 50% added glycerol for a few months.

3.3 In-Lysate Arginylation Assay in Yeast

1. One day before the experiment, inoculate wild-type and *ate1*-deleted yeast cells into 20 mL complete minimal (CM) medium, and grow overnight at 30 °C or at the desired test

conditions. The wild-type and *ate1*-deleted yeast cells grown in CM medium and at 30 °C are meant to serve as positive and negative controls, respectively, for the assay.

2. The next morning, collect yeast cells by centrifuging yeast cultures (OD 600 ~ 1.0 for the two controls) at 4000 × g for 5 min at room temperature.
3. Discard the supernatant and wash the cell pellet with sterile distilled water.
4. After wash, weigh the cell pellet, and resuspend it in 2× volume of arginylation reaction buffer supplemented with 0.1 M PMSF and yeast protease inhibitor cocktail (final dilution 100×; Sigma-Aldrich).
5. Add glass beads equal to cell weight to the cell suspension.
6. Lyse the cells by vortexing the cell suspension 15 times using 1 min on and 1 min off cycles at 4 °C.
7. Centrifuge the lysed cell suspension at 12000 g at 4 °C for 10 min. Collect the supernatant into fresh tubes and place it on ice.
8. For arginylation activity assay, transfer 20 μL of cell lysates of wild-type and *ate1*-deleted yeast into fresh tubes. Add up to 10 μL of purified DD-beta15-GFP arginylation reporter protein solution to the yeast lysates, and mix it using a pipette. Add arginylation buffer to adjust the reaction volume in each tube to 50 μL.
9. Carry out the arginylation reaction for 30 min at 37 °C. Time points can be collected instead, to observe the increase of Arg incorporation into the reporter over time.
10. Stop the reaction by addition of 1/3 volume of 4 × SDS sample buffer and incubating in boiling water bath for 5 min.

3.4 In-Lysate Arginylation Assay in Mammalian Cells

1. One day before the experiment, seed the desired test cells into 100 cm culture plates. Also seed similar cultures of wild-type and *ATE1* knockout MEFs cells to be used as positive and negative controls for the assay.
2. The next morning, collect the control cells and the desired test cells by scraping or by a brief digestion with trypsin. Resuspend the cells in a small volume of culture media, and transfer the suspension into pre-weighted 1.5 mL microcentrifuge tubes. Pellet the cells by centrifugation at 2000× g for 1 min. Gently discard the supernatant without disturbing the cell pellet.
3. Wash the cells by gently pipetting up and down in 1 mL dPBS, and then centrifuge at 2000× g for 1 min. Remove the supernatant as described in **step 2**. Centrifuge again at 2000× g for

30 s to remove residual liquid. Weigh the tubes to calculate the wet weight of the cell pellet by subtracting the weight of the empty tubes.

4. Lyse the cell pellet by pipetting in a 2 × volume of a modified arginylation reaction buffer (50 mM TRIS/HCl, 32 mM Na_3PO_4, pH 7.4, 5 mM MgCl2, 1 mM EDTA, 2.5 mM ATP, 0.2 mM arginine, and 0.2% NP-40). To avoid bubbles formation, always keep the pipette tip inside the cell suspension. Reducing reagents should be avoided at this step.
5. Centrifuge the lysed cell suspension at 12000× g at 4 °C, and collect the supernatant into fresh tubes. Place the tubes on ice.
6. For arginylation activity assay, use 40 μL of cell lysates from the test cell culture, as well as wild-type or *ATE1* knockout MEF cells for the positive and negative controls, respectively. Add 10 μL of purified DD-beta15-GFP arginylation reporter. Incubate at 37 °C for 60 min, or a series of time points, to carry out the reporter arginylation.
7. Terminate the reaction by addition of equal volume of 4 × SDS sample buffer, and incubate in boiling water bath for 5 min.

3.5 Western Blot Analysis

1. For the Western blot analysis, 25 μL of terminated reaction mixtures obtained as described in the sections 3.2, 3.3, and 3.4 were separated on SDS-PAGE (yeast and mammalian cells on separate gels).
2. Transfer the proteins onto the nitrocellulose membrane at 120 volts for 2 h at 4 °C or at 30 volts for overnight.
3. Incubate the nitrocellulose membrane in blocking buffer for 1 h on a rocker at room temperature.
4. To evaluate the arginylation level of DD-beta15-GFP, incubate the membrane in anti-RDD antibody for 2 h on a rocker at room temperature or at 4 °C overnight. The concentration of the antibody should be predetermined by using the positive and negative controls to reach a clear distinction of the two controls.
5. Wash the membrane with PBST buffer four times, 5 min each time.
6. Incubate the membrane in anti-rabbit HRP-conjugated secondary antibody for 1 h on a rocker.
7. Wash the membrane with PBST buffer four times, 5 min each time.
8. Visualize the HRP signal by chemiluminescence kit.
9. Strip the anti-RDD antibody from the membrane using 5 mL Restore Stripping Buffer. After the stripping, wash the membrane with 5 mL PBS buffer.

10. To determine the total amount of substrate protein (DD-beta15-GFP), incubate the membrane in anti-GFP antibody, preferably originated from a different host organism compared to the anti-RDD antibody (e.g., mouse if anti-RDD comes from rabbit). Perform the washes and detection as described in **steps 5–8**.
11. The GFP level is the loading control that is needed for determining the arginylation level (*see* **Note 3**). For additional controls, the protein concentration of the cell lysates should also be assessed. This can be quantified by a conventional method (such as the Bradford assay) or reflected by the level of housekeeping genes whose degradation is not significantly affected by arginylation (e.g., yeast phosphoglycerate kinase (PGK) or mammalian tubulin or beta-actin [6, 17, 18]).

4 Notes

1. For arginylation detection in mammalian cells, clone the reporter into a mammalian expression plasmid, and follow similar steps for mammalian, rather than yeast, transfection. The described method was first used to assay the activity of Ate1 in cell or tissue lysates by adding purified reporter protein. However, this method can also be adapted to determine the arginylation activity of Ate1 enzyme in intact cells by expressing the DD-beta15-GFP reporter in the target cells.
2. Absence of Ura in CM media would prevent the growth of non-transformed cells, so that only the transformed yeast cells would form colonies on the -Ura CM plates, as Ura marker in these cells will be expressed from the transformed plasmid.
3. In the Western blot analysis, the levels of the arginylated and the total reporter proteins were measured by chemiluminescence assays on the same membrane, where the signals of two different antibodies were separated by using stripping buffer. This measurement can also be done by using secondary antibodies with different fluorescent dyes to simultaneously detect the two signals on the same membrane in an Odyssey imager (or other fluorescence-based imagers). It should be noted that the GFP band on the membrane may emit fluorescence if activated. For this reason, the choices of the fluorescence dyes should avoid the absorbance spectrum of GFP. The typical far-red 680 nm and 800 nm dyes used for Odyssey imager should fulfill this requirement. However, it is strongly recommended that the background fluorescence of the membrane with the GFP band should be pre-determined on the imager, before the fluorescent secondary antibody was applied.

Acknowledgments

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Chapter 10

Assaying Intracellular Arginylation Activity Using a Fluorescent Reporter

Brittany MacTaggart and Anna S. Kashina

Abstract

In this chapter, we present a simplified version of the method described in Chapter 9 of this book, adapted for fast and convenient evaluation of intracellular arginylation activity in live cells. As in the previous chapter, this method utilizes a GFP-tagged N-terminal β-actin peptide transfected into cells as a reporter construct. Arginylation activity can then be evaluated by harvesting the reporter-expressing cells and analyzing them directly by Western blot using an arginylated β-actin antibody and a GFP antibody as an internal reference. While absolute arginylation activity cannot be measured in this assay, different types of reporter-expressing cells can be directly compared, and the effect of genetic background or treatment can be evaluated. For its simplicity and broad biological application, we felt this method merited presentation here as a separate protocol.

Key words Arginylation, Fluorescent reporter, Assay

1 Introduction

Intracellular arginylation measurements are a broad challenge in the field. Since the majority of Arg incorporates into proteins during translation, posttranslational arginylation activity tends to be drowned out as noise. While accurate comparisons of translation versus arginylation have not been made, it is likely that arginylation accounts for only a very small fraction of Arg utilization in cells. Thus, measurement of arginylation in vivo requires specialized tools that are generally challenging to develop.

In many past studies, intracellular arginylation of target proteins has been measured using mass spectrometry or incorporation of radioactively labeled arginine under the suppression of protein synthesis. However, both methods are labor-intensive and are challenging to perform quantitatively. The development of the arginylation sensor based on the N-terminal β-actin peptide, described in the previous chapter, provides a relatively simple and reliable

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method accessible in nearly every lab. This method requires the use of an arginylation sensor plasmid, DD-β15-GFP, first described in an in vitro assay [1], to measure the relative arginylation levels in cultured cells. The sensor contains 15 amino acids from the processed N-terminus of β-actin, flanked by an N-terminal ubiquitin moiety and a C-terminal GFP [1]. Once transfected into cells, this construct undergoes rapid N-terminal deubiquitination by the native intracellular machinery, revealing the N-terminal D (Asp) of the processed N-terminus of β-actin, which is a well-known target of arginylation [2–6]. Notably, while some N-terminally arginylated proteins have been previously shown to undergo ubiquitin-dependent degradation, this sensor lacks the Lys residues that can serve as sites for ubiquitin conjugation and is thus metabolically stable.

Similar to the method described in the previous chapter, the total level of the construct in cells can be detected by a GFP antibody, and the level of arginylation of the construct can be detected by an arginylated-actin (R-actin) antibody [4]. However, in this case, such detection is performed by Western blot on whole cell lysates, rather than on in-lysate arginylation assays.

Control experiments show that when the DD-β15-GFP is expressed in cells, its arginylated form is only detected in wildtype cells, not in *Ate1* knockout cells. At the same time, expression of a pre-arginylated construct with an Arg (R) residue encoded in the peptide sequence (RDD-β15-GFP) can be detected using the R-actin antibody in both wildtype and *Ate1* knockout cells. Finally, a construct resembling the full-length, unprocessed N-terminus of β-actin (MDDD-β15-GFP) can be weakly detected by the R-actin antibody in both wildtype and *Ate1* knockout cells (Fig. 1).

The ratio of the R-actin signal to the GFP signal can be used to compare the relative arginylation levels in cells. As an example, *Ate1* knockout cells co-transfected with DD-β15-GFP and Ate1.1-GFP show a similar relative arginylation level to wildtype cells (Fig. 2). Thus, this method can potentially be used to compare the relative arginylation levels in cells under different conditions (e.g., cell density, cell stress, etc.) or in different genetic backgrounds.

2 Materials

1. Cell transfection kit or reagent (e.g., Lipofectamine 2000).
2. Sensor-expressing plasmid: DD-β15-GFP cloned in frame into a mammalian expression vector (*see* **Note 1**).
3. Arginylated control plasmid: RDD-β15-GFP (*see* **Note 1**).
4. Unprocessed control plasmid MDDD-β15-GFP (*see* **Note 1**).
5. Negative control plasmid: pEGFP-N2 (*see* **Note 1**).

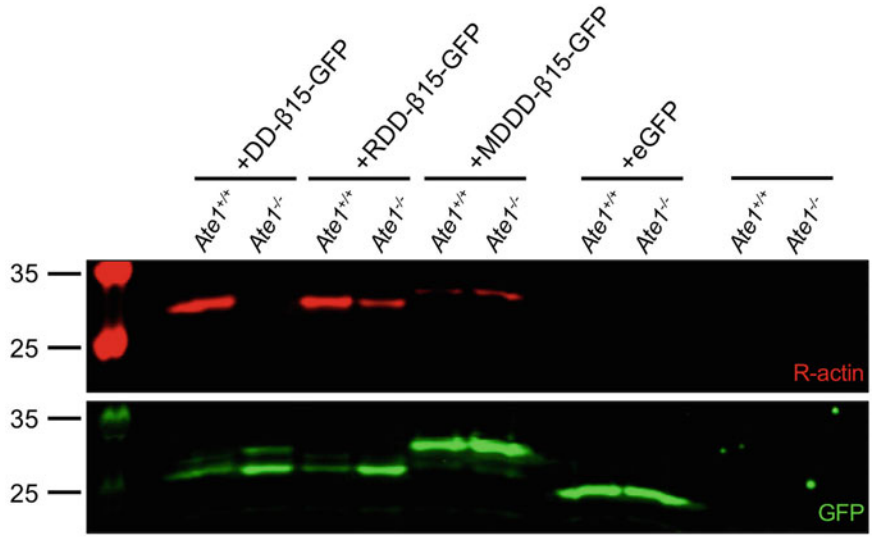

Fig. 1 Sensor arginylation detection in cultured cells. Western blots of total cell extracts from wild type (Ate1+/+) and Ate1 knockout (Ate1-/-) mouse embryonic fibroblasts transiently transfected with arginylation sensor plasmid as well as its pre-arginylated and N-terminally unprocessed versions, as well as untransfected control. Sensor plasmids used for each transfection are listed on top. Top panel, R-actin antibody signal. Bottom panel, GFP antibody signal

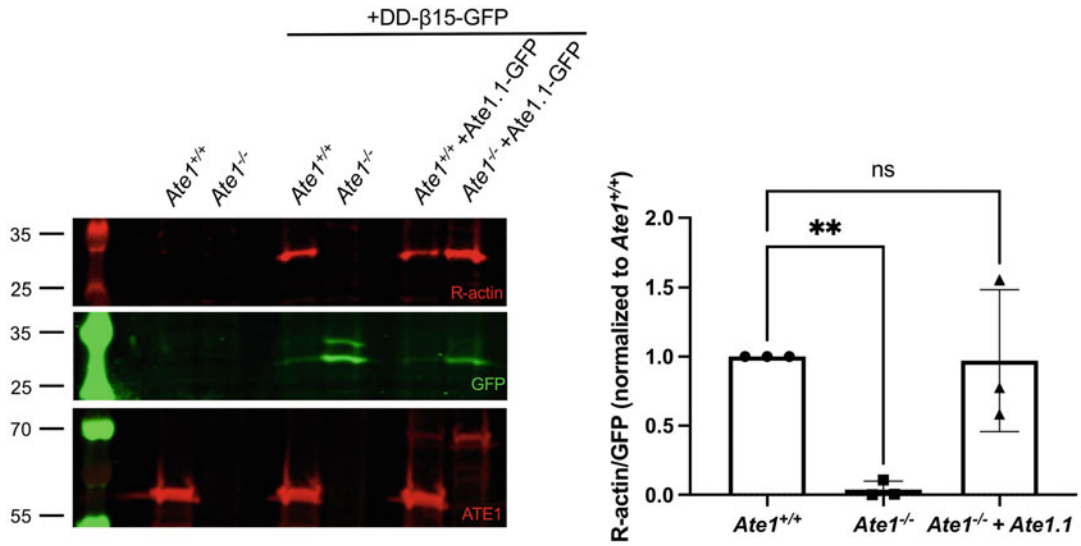

Fig. 2 Full output of an arginylation detection experiment using inracellular arginylation sensor. Left, Western blots of whole cell lysates from sensor-transfected wild type and Ate1 knockout cells (annotated similarly to Fig. 1), as well as Ate1 knockout cells re-transfected with Ate1.1-GFP to restore arginylation activity. Top, middle, and bottom panel show signal from R-actin, GFP, and ATE1 antibody, respectively. The higher molecular weight ATE1 band in the last lane corresponds to ATE1-GFP fusion. GFP blot in the middle panel is cropped to exclude this band. Right, quantification of arginylation in the cells extracts shown on the left, derived as R-actin/GFP signal in the band corresponding to the arginylation sensor

6. Optional: Ate1 rescue plasmid, expressing enzymatically active ATE1 (e.g., Ate1-1-GFP) (*see* **Note 2**).
7. Arginylated β-actin antibody. Currently, we use Anti-R-actin (EMD Millipore, ABT264) at 1:2000 dilution for Western blot.
8. Anti-GFP antibody (e.g., Abcam, ab1218 at 1:3000 dilution for Western blot).
9. Anti-ATE1 antibody. The current best antibody is a rat monoclonal antibody, clone 6F11, commercially available from Sigma-Aldrich, mabs436, used at 1:1000 dilution for Western blot.
10. Cultured cells and inhibitors for the desired experimental tests.
11. Phosphate buffered saline (PBS).
12. Tissue culture media (formulated for the specific cell types used).
13. SDS sample buffer and reagents for SDS electrophoresis and Western blot.

3 Methods

1. Plate cells into six-well plates and grow to ~80% confluency.
2. Transfect cells with 2.5 μg total plasmid using Lipofectamine 2000 (1:3 DNA-to-Lipofectamine ratio) (*see* **Note 3**). Grow for 24–48 h to reach maximum sensor expression (*see* **Note 4**).
3. Wash the cells once with PBS. Harvest the cells by scraping and centrifugation (1000 rpm, 5 min in a tabletop centrifuge). Resuspend the cell pellet in 2× SDS sample buffer at a 1:20 w/v ratio. Boil the sample for 10 min.
4. Run 10 μL of each sample on a 12% SDS-PAGE gel (*see* **Note 5**). Transfer to a 0.45 μm nitrocellulose membrane at 100 V for 60 min. Block the membrane in 3% BSA in PBS for 1 h at room temperature, and incubate in primary antibody overnight at 4 °C, followed by washes and secondary antibody incubation and detection (*see* **Note 6**).

4 Notes

1. The expression plasmid should be chosen based on the desired expression system. While this method describes mammalian cells, the same approach could be used for other eukaryotic cell types, such as yeast, and in this case, a system-appropriate expression plasmid should be chosen. In our case, we use the sensor plasmid cloned into pEGFP-N2 vector.

2. This plasmid should be used to reintroduce ATE1 into cells showing no detectable arginylation activity, such as *Ate1* knockout cells, to prove that the lack of sensor arginylation is due to ATE1 depletion and not some secondary effect. Co-expression of ATE1 and the sensor should result in high arginylation activity. As in **Note 1**, a system-appropriate expression plasmid should be chosen for different cell types.
3. For different experimental treatments, cells should be transfected with the sensor and control plasmids described in **steps 2–5** of Subheading 2. At least three technical repeats for each data point should be used to allow for statistical analysis.
4. Transfection efficiency and sensor expression can be evaluated under a microscope by observing GFP fluorescence. Low transfection efficiency and sensor expression should not preclude accurate arginylation measurements.
5. The arginylation sensor construct should run in the 30 kDa range, and higher percentage gels should result in a more central position and thus improve its detection. In principle, gradient gels could also be used.
6. The detection system should be sufficient to enable direct quantification of the R-actin and GFP antibody signals, e.g., LiCor Odyssey gel scanner. Secondary antibodies should be chosen based on compatibility with the detection system.

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Chapter 11

Bacterial Expression and Purification of Recombinant Arginyltransferase (ATE1) and Arg-tRNA Synthetase (RRS) for Arginylation Assays

Junling Wang and Anna S. Kashina

Abstract

Here, we describe the procedure for the expression and purification of recombinant ATE1 from *E. coli*. This method is easy and convenient and can result in one-step isolation of milligram amounts of soluble enzymatically active ATE1 at nearly 99% purity. We also describe a procedure for the expression and purification of *E. coli* Arg-tRNA synthetase essential for the arginylation assays described in the next two chapters.

Key words Arginyltransferase, Arg-tRNA synthetase, Recombinant protein, Arginylation

1 Introduction

All the methods and assays described up to this point in the book have been employed in early days of arginylation studies, before the development of standardized methods for protein expression and purification. The development of such methods enabled a new era of protein arginylation studies, starting in the early 2000s and actively ongoing today.

The present chapter describes a method for the expression and purification of recombinant ATE1 using 6xHis-tagged construct and Ni-NTA affinity chromatography. This method can yield milligram amounts of protein at nearly 99% purity, possessing high enzymatic activity [1–3].

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2 Materials

1. ATE1 construct cloned into a standard vector optimized for *E. coli* expression, e.g., pET29 (Novagen), containing C-terminal 6xHis tag (*see* **Note 1**).
2. *E. coli* arginyl-tRNA synthetase (RRS) cloned into pTYB1 vector with Intein tag (New England Biolabs).
3. *E. coli* competent cells, BL21-CodonPlus® (DE3)-RIL (for ATE1) and BL21(DE3) (for RRS) (*see* **Note 2**).
4. LB media supplemented with 100 mg/mL kanamycin and 50 mg/mL chloramphenicol (for ATE1) or 100 mg/mL ampicillin (for RRS).
5. IPTG (isopropyl-1-thio-D-galactopyranoside).
6. Lysis buffer A: 0.5 M NaCl, 1 mM $MgCl_2$, 50 mm Tris–HCl, 10 mM β-mercaptoethanol, 5 mM imidazole, 1 mM PMSF, pH 7.5.
7. Lysis buffer B: 0.5 M NaCl, 20 mM Tris–HCl, 1 mM EDTA, 0.1% Triton X −100, 1 mM PMSF, pH 8.5.
8. Cleavage buffer: Freshly dissolve 30 mM DTT in lysis buffer B.
9. Wash buffer: 1 M NaCl, 1 mM mgCl, 50 mm Tris–HCl, 10 mM β-mercaptoethanol, 25 mM imidazole, pH 7.5.
10. Elution buffer: 0.5 M NaCl, 1 mM $MgCl_2$, 50 mM Tris–HCl, 10 mM β-mercaptoethanol, 0.5 M imidazole, pH 7.5.
11. Dialysis buffer: 25 mM HEPES, 1 mM DTT, 0.5 M NaCl, 0.1 mM EDTA, 1 mM $MgCl_2$, 50% glycerol, pH 7.5.
12. Ni-NTA affinity column (for ATE1).
13. Chitin column (for RRS).

3 Methods

3.1 Expression and Purification of ATE1

1. Transform the *E. coli* BL21-CodonPlus® (DE3)-RIL competent cells with ATE1 constructs following the manufacturer's protocol.
2. Grow an overnight 5–10 mL culture of *E. coli* cells harboring a plasmid of pET29a-ATE1, and inoculate the culture into 1 L LB supplemented with 100 mg/mL kanamycin and 50 mg/mL chloramphenicol.
3. Grow on a shaker at 37 °C until OD_{600} reaches 0.4–0.5.
4. Cold-shock the culture by placing the entire flask on ice for 30 min.

5. Add 0.8 mM IPTG to induce ATE1 expression. Incubate for approximately 18 h at 16 °C on a shaker at 220 rpm.
6. Collect the cells by centrifuging at 6000 rpm for 30 min. Discard the supernatant.
7. Resuspend the cell pellet in 10 mL lysis buffer A, and lyse the cells using a French press (alternatively, cells could be sonicated at level 5, 6 × 10 s with 1 min intervals, on ice).
8. Centrifuge the cell lysate at 20,000 rpm for 30 min at 4 °C. Discard the pellet.
9. Load the supernatant onto the Ni-NTA agarose column pre-equilibrated with 10 column volumes of lysis buffer A.
10. Wash the column with 10 column volumes of wash buffer.
11. Elute the protein with elution buffer, collecting 1 mL fractions.
12. Check the purity of the eluted fractions by 10% SDS-PAGE. The fractions should contain one major band running at ~60 kDa, corresponding to the ATE1 protein (Fig. 1).
13. Combine the peak fractions and dialyze against with dialysis buffer overnight at 4 °C.
14. Determine the final protein concentrations using Bradford assay with the bovine serum albumin (BSA) standard curve.
15. Aliquot the dialyzed protein into 1.5 mL Eppendorf tubes, and keep them at −80 °C. The working fraction can be kept at −20 °C for at least 2 weeks (*see* **Note 3**).

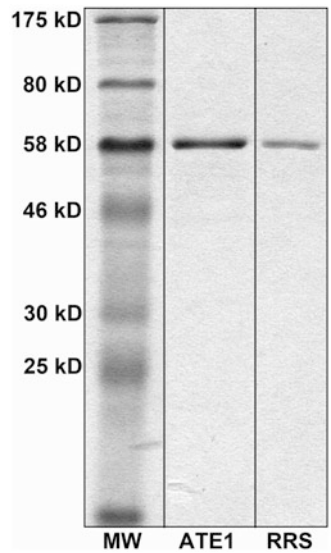

Fig. 1 Gel of a typical preparation of ATE1 and RRS using the method described in this chapter

3.2 Expression and Purification of RRS

1. Transform the *E. coli* BL21DE3 competent cells with RRS construct following the manufacturer's protocol.
2. Grow an overnight 5–10 mL culture of *E. coli* cells harboring a plasmid of pTYB1-RRS, and inoculate the culture into 1 L LB supplemented with 100 mg/mL ampicillin.
3. Grow on a shaker at 37 °C until OD_{600} reaches 0.4–0.5.
4. Cold-shock the culture by placing the entire flask on ice for 30 min.
5. Add 0.5 mM IPTG to induce *E. coli* RRS expression. Incubate for approximately 16 h at 37 °C on a shaker at 220 rpm.
6. Collect the cells by centrifuging at 6000 rpm for 30 min. Discard the supernatant.
7. Resuspend the pellet in 10 mL lysis buffer B, and lyse the cells using a French press (alternatively, cells could be sonicated at level 5, 6 × 10 s with 1 min intervals, on ice).
8. Centrifuge the cell lysate at 20,000 rpm for 30 min at 4 °C. Discard the pellet.
9. Load the supernatant onto a chitin column (New England Biolabs) pre-equilibrated with the lysis buffer B.
10. Wash the column with >10 column volumes of lysis buffer B.
11. Flush the column quickly with cleavage buffer, and leave it at 4 °C overnight for on-column cleavage.
12. Elute the protein by adding 3 column volumes of lysis buffer B to the column. Collect 1 mL fractions.
13. Check the purity of the eluted fractions by 10% SDS-PAGE. The fractions should contain one major band running at ~60 kDa, corresponding to the RRS protein (Fig. 1).
14. Combine the peak fractions and dialyze against with dialysis buffer overnight at 4 °C.
15. Determine the final protein concentrations using Bradford assay with the bovine serum albumin (BSA) standard curve.
16. Aliquot the dialyzed protein into 1.5 mL Eppendorf tubes, and keep them at −80 °C. The working fraction can be kept at −20 °C for at least 2 weeks.

4 Notes

1. Tagging the ATE1's N-terminus should be avoided, since the N-terminal portion of ATE1 has been found to be essential for its enzymatic activity [4, 5].

2. CodonPlus cells are necessary for the expression of mammalian ATE1 isoforms in *E. coli* and are not used for *E. coli* RRS, which is expressed in a native system. For bacterial expression of RRS from other species, CodonPlus cells may be necessary.
3. 50% glycerol introduced into this fraction during dialysis would prevent it from freezing solid at −20 °C.

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Chapter 12

Preparation of tRNA^{Arg} for Arginylation Assay by In Vitro Transcription

Irem Avcilar-Kucukgoze and Anna S. Kashina

Abstract

This chapter describes the preparation of tRNA^{Arg} by in vitro transcription. tRNA produced by this method can be efficiently utilized for in vitro arginylation assays, following aminoacylation with Arg-tRNA synthetase, either directly during the arginylation reaction or separately to produce the purified preparation of Arg-tRNA^{Arg}. tRNA charging is described in other chapters of this book.

Key words tRNA, In vitro transcription, Arginylation

1 Introduction

In vitro transcription is widely used to efficiently produce any RNA of interest. This method provides micrograms of an individual tRNA in a short period of time. In this chapter, we base the primers and procedure on specific type of mouse tRNA, tRNA^{Arg}ACG; however, a similar method could in principle be used for the preparation of any other tRNA^{Arg} from any species. It should be noted that tRNA generated by this method does not contain any natural posttranscriptional modifications; however, our prior studies suggest that this lack of modifications does not affect the activity of this tRNA in arginylation assays.

2 Materials

1. 200 mM Tris-HCl, pH 7.5.
2. dNTP mix.
3. DNA polymerase I, large (Klenow) fragment (5 U/μL).
4. 10 × NEB buffer 2.

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5. Crush and soak buffer: 50 mM potassium acetate (KOAc), 200 mM KCl, pH 7.5 (should be prepared by DEPC-treated water).
6. Phenol/chloroform/isoamyl alcohol (25:24:1).
7. 100% ethanol.
8. Agarose.
9. MEGAshortscript Kit (Invitrogen) for in vitro transcription; alternatively, HiScribe™ T7 High Yield RNA Synthesis Kit (NEB) or RevertAid H Minus Reverse Transcriptase (Thermo Fisher) can be used.
10. RNaseZap.
11. UreaGel System 19:1, which includes UreaGel concentrate, UreaGel diluent, and UreaGel buffer (National Diagnostics).
12. 10% ammonium persulfate (APS), freshly prepared.
13. TEMED.
14. RNA ladder should cover around 80 bases. Low-range ssRNA ladder (NEB) can be used.
15. SYBR Gold.
16. 0.22 μm syringe filter.
17. 3–5 mL sterile syringe.
18. 100% isopropanol.
19. DEPC-treated water, which can be purchased or prepared. % 0.1 DEPC is added to ultrapure water and stirred overnight at dark, followed by autoclaving.
20. Nuclease-free water.
21. tDNA oligonucleotides for mouse $tRNA^{Arg}ACG$.

Example sequences:

Full-length $tRNA^{Arg}ACG$: 5′- GGCCAGTGGCGCAATGGATAACGCGTCTGACTACGGATCAGAAGATTCCAGGTTCGACTCCTGGCTGGCTCG cca-3′ (where the anticodon ACG is underlined and CCA tail is lowercased).

Full-length sequence with the addition of T7 promoter: 5′-**TAATACGACTCACTATAG**GGCCAGTGGCGCAATGGATAACGCGTCTGACTACGGATCAGAAGATTCCAGGTTCGACTCCTGGCTGGCTCGcca-3′ (where the T7 promoter is bold and the rest are marked as above).

Forward tDNA oligonucleotide: 5′-TAATACGACTCACTATAGGGCCAGTGGCGCAATGGATAACGCGTCTGACTACGGATCA-3′.

Reverse tDNA oligonucleotide: 5′-TGGCGAGCCAGCCAG GAGTCGAACCTGGAATCTTCTGATCCGTAGTCAGAC GCGTTATCC-3′.

3 Methods

3.1 Transcription Template Design

1. Select the tRNA sequence of interest from the database (*see* **Note 1**).
2. Design two single-stranded tDNA oligonucleotides: forward oligonucleotide, encoding the 5′-end of sequence of the sense strand, and reverse oligonucleotide, encoding the 3′-end of the sequences of antisense strand. These two oligonucleotides should overlap by 20–25 nt in the middle of the tRNA sequence. The forward oligonucleotide should contain an upstream T7 promoter (5' TAATACGACTCACTATAG 3′, where the underlined G is the first nucleotide to be included in the newly transcribed RNA) (*see* **Note 2**). The reverse oligonucleotide should contain CCA sequence at the 3′ end (*see* **Note 3**).

3.2 Preparation of the Transcription Template from tDNA Oligonucleotides

1. Mix the tDNA oligonucleotides as follows: 10 μL of the forward oligonucleotide (100 μM), 10 μL of the reverse oligonucleotide (100 μM), 4 μL of 200 mM Tris-HCl (pH 7.5), 16 μL of nuclease-free water.
2. Heat the mixture in a heat block for 2 min at 95 °C to denature any secondary structures and anneal the tDNA oligonucleotides at room temperature for 3 min.
3. To fill in the 5′ overhangs and form blunt ends, mix the following in the given order: 20 μL of 10× buffer (NEB buffer 2); 129.4 μL of nuclease-free water; 6.6 μL of 1 mM dNTP mix; 4 μL of DNA polymerase I, large (Klenow) fragment (5 U/μL); and 40 μL of the tDNA oligonucleotide mix from **step 1** (*see* **Note 4**).
4. Incubate the reaction for 15 min at 25 °C.
5. Add one volume (200 μL) of the crush and soak buffer and two volumes (400 μL) of phenol/chloroform/isoamyl alcohol. Vortex 30 s and centrifuge at 21,000 × *g* for 5 min at 4 °C.
6. Take the aqueous (top) phase carefully with a pipette tip, transfer to a new tube, and precipitate with 2.7 volumes of ethanol (100%). Incubate for 30 min at −80 °C, followed by centrifugation at 21,000 × *g* for 30 min at 4 °C.
7. Air-dry the pellet for 5–10 min at room temperature. Dissolve into 50 μL of nuclease-free water. Determine the concentration by NanoDrop and the integrity by 2% agarose gel.

Alternatively, 10% TBE polyacrylamide gel electrophoresis can be used. Store the synthesized double-stranded DNA template at −20 °C.

3.3 In Vitro Transcription

1. Use MEGAshortscript Kit with 200 ng of DNA template according to the manufacturer's protocol. Incubate for 16 h at 37 °C (*see* **Notes 5** and **6**).
2. After the incubation, add 1 μg of TURBO DNase provided by MEGAshortscript Kit to the reaction, and mix gently. Incubate at 37 °C for further 15 min (*see* **Note 7**).
3. Load the mixture onto 12% PAGE/7.5 M urea gel. The dimension of the gel should be 14 cm × 14 cm × 0.1 cm.
4. Visualize the transcribed tRNA by SYBR Gold under the UV light, and excise the tRNA band that is around 80 nt (*see* **Note 8**).
5. Place the gel slice into a 0.2 mL tube with holes at the bottom. This tube is made by punching five to six times by sterile 21-gauge needle. Place the 0.2 mL tube into 1.5 mL tube, and centrifuge at 16,000 × *g* for 1 min at room temperature to crush the gel slice through the holes into the bigger tube (Fig. 1). Discard the 0.2 mL tube.
6. Add two volumes of crush and soak buffer to the gel pieces, and elute tRNA transcript by shaking the tube on a rotator for 16 h at 4 °C (*see* **Notes 9** and **10**).
7. To remove the gel pieces, filter the sample through 0.22 μm syringe filter. Add one volume of isopropanol (100%) to the resulting tRNA eluate, and incubate for 30 min at −80 °C to precipitate the tRNA.
8. Centrifuge the mixture from the previous step at 21,000 × *g* for 30 min at 4 °C. Carefully remove the supernatant, making sure that the tRNA pellet remains in the tube (*see* **Note 11**).
9. Wash the tRNA pellet by adding 70% ethanol, and vortex briefly, followed by centrifugation at 21,000 × *g* for 10 min at 4 °C and removal of the supernatant similar to the previous step (*see* **Note 11**). Repeat the procedure two to three times.
10. Remove the supernatant as completely as possible without disturbing the pellet, and air-dry the pellet in an open tube (*see* **Note 12**).
11. Dissolve the pellet into 50 μL of DEPC-treated water. Determine the tRNA concentration by NanoDrop. The ratio of A_{260}/A_{280} should be ≥ 2.
12. To ensure correct folding of tRNA, heat the solution from **step 11** containing the tRNA transcript at 95 °C for 2 min, followed by 3 min incubation at room temperature and then 5 min at 37 °C to obtain the final tRNA preparation (*see* **Notes 13–15**).

4 Notes

1. The sequence of tRNA of interest can be found at Genomic tRNA Database (http://gtrnadb.ucsc.edu/) [1]. In our experience, any $tRNA^{Arg}$ from any species can be used for the arginylation reaction, provided that it can be charged by the selected Arg-tRNA synthetase, which also seems to have low species specificity.
2. T7 promoter sequence at the beginning of the forward primer is required for T7 RNA polymerase (T7 RNAP) binding. Addition of two more G's provides efficient transcription [2]. Since T7 RNAP is best to initiate with a G nucleotide (G + 1), transcription of RNA transcripts starting with a nucleotide other than G may result in lower efficiency [3].
3. 3' CCA at the end of tRNA is genetically encoded in prokaryotes and posttranscriptionally added in eukaryotes; therefore, it should be added manually to the 3′ of tRNA sequence if the origin of tRNA is eukaryotic.
4. T7 RNAP requires a double-stranded promoter to initiate transcription [4, 5]. This method provides a fully double-stranded transcription template, which is a good substrate for in vitro transcription. It is also possible to form a partially double-stranded transcription template by annealing of a short oligonucleotide which is complementary to the T7 promoter (Fig. 2).
5. Higher amount of DNA template can be used for in vitro transcription reaction.
6. After this step, the samples should be handled in RNase-free conditions. It is necessary to use sterile and disposable plasticware. All buffers should be prepared using DEPC-treated water. Reused glassware should be washed with RNaseZap followed by ultrapure water.
7. This step removes the DNA template used for the in vitro transcription reaction.
8. This step should be done rapidly because excessive UV exposure can cause several types of damage to the RNA molecule, e.g., photochemical modification and oxidative damage [6].
9. Alternatively, the gel slice can be crushed using the disposable pipette tip; however, this method results in relatively larger gel pieces which would affect the diffusion of RNA transcript to the buffer.
10. RNA transcript can also be cleaned by Monarch RNA Cleanup Kit according to the manufacturer's instruction. It should be noted, however, that T7 RNAP generally produces one or a

Fig. 1 The procedure to crush the gel slice into small pieces

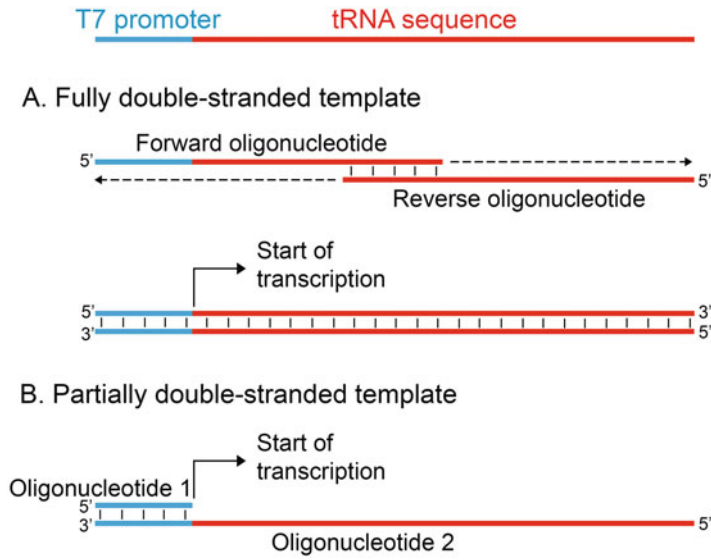

Fig. 2 Two different types of transcription templates can be used for in vitro transcription reaction. A. Fully double-stranded template is constructed by two single-stranded oligonucleotides that overlap in the middle of the sequence. Dashed lines show the fill-in of 5′-protruding ends. This method allows the rapid production of a variety of tRNA transcripts. B. Partially double-stranded template is formed by annealing of a short oligonucleotide which is complementary to the T7 promoter

few nucleotides longer products [7]. Gel extraction method is much more efficient in getting rid of these contaminants, although it is time-consuming.

11. tRNA pellet at this step appears whitish and opaque and may be very small. Care should be taken not to disturb it during supernatant removal.
12. Care should be taken not to overdry the RNA pellet, as over-drying might make the pellets difficult to dissolve. Overdried pellet looks transparent.

13. tRNA preparation at this step can be used directly for arginylation or stored at −80 °C. For repeated use, the tRNA transcript should be aliquoted to prevent multiple freeze/thaw cycles.
14. This denaturing and annealing step should be done only once.
15. Quality of tRNA folding can be determined during the Arg-tRNA-synthetase charging, described in a later chapter. If problem arise, tRNA can be denatured and refolded in the presence of Mg^{+2}, which may promote the correct conformation of tRNA transcript. It has been previously found that divalent and monovalent cations, especially Mg^{+2}, are effective at folding and stabilizing RNA molecules [8–10].

Acknowledgments

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Chapter 13

Preparation of an Enriched tRNA^{Arg} Fraction for Arginylation by Expression in *E. coli*

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Abstract

The method described here provides a fast and efficient way to obtain an enriched preparation of tRNA of interest, which is also posttranscriptionally modified by the intracellular machinery of the host cells, *E. coli*. While this preparation also contains a mixture of total *E. coli* tRNA, the enriched tRNA of interest is obtained in high yields (milligram) and is highly efficient for biochemical assays in vitro. It is routinely used in our lab for arginylation.

Key words tRNA, In vitro transcription, Arginylation

1 Introduction

This method involves the construction of an overexpression plasmid for the tRNA gene of interest, introduction of the plasmid into an *E. coli* host, and isolation of total tRNA from the expression host cells. We routinely introduce the tRNA gene of interest using the *Eco*R1 and *Pst*I restriction sites of the pKK223-3 plasmid for expression under the control of the IPTG-inducible Tac promoter. The resulting total tRNA mixture is greatly enriched for the expressed tRNA, which contains an estimated 40–50% of tRNA^{Arg} according to our experience. This preparation is suitable for routine arginylation reaction, despite the presence of other tRNA species. This method provides an overexpressed tRNA in the mixture of all bacterial native tRNA species containing the full complement of natural posttranscriptional modifications. The overexpressed tRNA in the mixture can be directly added into an in vitro arginylation reaction.

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2 Materials

Prepare all buffers for use with RNA with DEPC-treated water free of RNases. Other buffers can be prepared with ultrapure water. The buffers for RNA can be aliquoted to avoid RNase contamination.

2.1 Materials for Bacterial Cell Transformation and Growth

1. The tRNA expression plasmid. In our lab, we typically use *E. coli* $tRNA^{Arg}ACG$ cloned into the pKK223-3 vector. In principle, any other bacterial tRNA gene cloned into this, or a similar expression vector, could be used.
2. *E. coli* JM109 competent cells.
3. Luria broth (LB) agar plate with 100 μg/mL of ampicillin.
4. Luria broth (LB).
5. Ampicillin stock at 100 mg/mL in water, stored at −20 °C.
6. 1 M IPTG stock in water, stored at −20 °C.

2.2 Materials for tRNA Isolation

1. 0.9% NaCl.
2. 50 mM sodium acetate (NaOAc), pH 5.0, 10 mM magnesium acetate ($Mg(OAc)_2$).
3. Acidic phenol, pH 4.5.
4. 5 M NaCl.
5. 100% isopropanol.
6. 100% ethanol.
7. 70% ethanol.
8. 1 M NaCl.
9. 0.3 M NaOAc, pH 5.0.
10. 1.5 M Tris-HCl, pH 9.0.
11. 3 M NaOAc, pH 5.0.

3 Methods

3.1 tRNA Expression in Bacterial Cells

1. Use the tRNA expression plasmid to transform *E. coli* JM109 competent cells according to the manufacturer's protocol.
2. Plate the transformed cells on a Luria broth (LB) agar plate containing 100 μg/mL of ampicillin, and incubate the plate at 37 °C overnight.
3. Pick a single colony from the plate, and resuspend it into 20 mL of LB broth supplemented with 100 μg/mL of ampicillin. Grow overnight on a shaker at 37 °C to obtain the pre-culture.
4. Add 10 mL of pre-culture into 1 L LB broth supplemented with 100 μg/mL of ampicillin, and continually grow with

shaking at 37 °C for approximately 3–4 h, until OD_{600} reaches 0.4–0.6 (*see* **Note 1**).

5. To induce the expression of $tRNA^{Arg}ACG$, add 300 μL of 1 M IPTG to a final concentration of 0.3 mM. Incubate on a shaker for approximately 10 h at 37 °C (*see* **Note 2**).
6. Spin down the culture at 5000 × *g* for 25 min at 4 °C to pellet the cells. Discard the supernatant.
7. Wash the cell pellet with 30 mL of 0.9% NaCl.
8. Centrifuge the cell suspension at 5000 × *g* for 15 min at 4 °C. Discard the supernatant (*see* **Notes 3** and **4**).

3.2 tRNA Isolation

1. Resuspend the cell pellet from **step 8** in 18 mL of 50 mM sodium acetate (NaOAc), pH 5.0, 10 mM magnesium acetate ($Mg(OAc)_2$).
2. Add 17.2 mL of commercial acidic phenol, pH 4.5. Shake the emulsion in a 125 mL flask at 215 rpm for 30 min at 37 °C.
3. Centrifuge the mixture at 10,000 × *g* for 15 min at 4 °C. At this step, the mixture should visibly separate into three phases. Upper aqueous phase contains RNA and DNA, bottom organic phase includes phenol, and the interphase has protein. Use a pipette to carefully collect the upper aqueous phase, and transfer it to a new tube without disturbing the interphase (*see* **Note 5**).
4. Add 14 mL of 50 mM NaOAc, pH 5.0, 10 mM $Mg(OAc)_2$ to the original tube containing the phenol phase and the interphase for a second extraction. Shake at 215 rpm for 15 min at 37 °C.
5. Centrifuge the mixture, and recover the aqueous phase as described in **step 3**. Discard the phenol phase and the interphase.
6. Combine aqueous phases from **steps 3** and **5** (should be approximately 36 mL).
7. To precipitate total nucleic acid, add 1.5 mL of 5 M NaCl and 1 volume (37.5 mL) of isopropanol to the mixture of aqueous phases from **step 6**. Store briefly at room temperature, and centrifuge at 14,500 × *g* for 15 min at room temperature. Carefully remove the supernatant and discard it (*see* **Note 5**).
8. Wash the nucleic acid-containing pellet by adding cold 70% ethanol, followed by centrifugation at 14,500 × *g* for 15 min at room temperature. Discard the supernatant (*see* **Note 5**).
9. Remove the supernatant after the last wash as carefully as possible, and air-dry the pellet at room temperature for 5–10 min.

10. Resuspend the pellet in 10 mL of cold 1 M NaCl by pipetting and/or vortexing. The pellet can be hydrated with the 1 M NaCl solution prior to resuspension.
11. Centrifuge at 9500 × *g* for 20 min at 4 °C, and collect the supernatant (*see* **Note 6**).
12. Optional: repeat **steps 10** and **11** with 5 mL of cold 1 M NaCl, and combine the supernatants (total 15 mL).
13. To precipitate the remaining nucleic acids (including DNA and tRNA), add 30 mL (2 volumes) of cold ethanol to the supernatant from **steps 11** and **12**, and incubate for 30 min at −20 °C. Centrifuge at 14,500 × *g* for 5 min at 4 °C.
14. Wash the pellet with 70% ethanol by adding cold 70% ethanol, followed by centrifugation at 14,500 × *g* for 5 min at room temperature. Discard the supernatant (*see* **Note 5**).
15. Air-dry the pellet for 5–10 min.
16. To remove DNA from the pellet, dissolve the pellet into 6 mL of 0.3 M NaOAc, pH 5.0. This step may require heating to 60 °C and pipetting. Add 3.4 mL of isopropanol (0.56 volume) to the nucleic acid solution, and incubate for 10 min at room temperature. Centrifuge at 14,500 × *g* for 5 min at room temperature. Collect the supernatant (*see* **Notes 7–8**).
17. For the final precipitation of tRNA, add 2.3 mL of isopropanol to the supernatant (volume ratio of water/isopropanol should be 1.00:0.95), and incubate for 30 min at −20 °C.
18. Centrifuge the suspension 14,500 × *g* for 15 min at 4 °C. Remove the supernatant (*see* **Note 5**).
19. Wash the pellet by adding cold 70% ethanol, followed by centrifugation at 14,500 × *g* for 15 min at room temperature. Discard the supernatant (*see* **Note 5**). Repeat this wash two to three times.
20. Air-dry the pellet for 5–10 min (*see* **Note 10**). Dissolve the pellet into 500 μL of DEPC-treated water (*see* **Note 11**).
21. To deacylated tRNA, add 35 μL of 1.5 M Tris–HCl pH 9.0 to the preparation from **step 20**, and incubate for 45 min at 37 °C.
22. Add 53.5 μL of (0.1 volume) of 3 M NaOAc, pH 5.0, and add 1.6 mL (2.7 volumes) of ethanol. Incubate for 30 min at −80 °C (*see* **Note 9**).
23. Centrifuge at 16,000 × *g* for 30 min at 4 °C. Wash the pellet by adding cold 70% ethanol, followed by centrifugation at 16,000 × *g* for 10 min at 4 °C. Discard the supernatant (*see* **Note 5**). Repeat this wash two to three times.
24. Air-dry the pellet for 5–10 min (*see* **Note 10**). Dissolve the pellet into 500 μL of DEPC-treated water.

25. Determine the concentration by NanoDrop. The A_{260}/A_{280} ratio should be ~2. Typical yield of total tRNA is 5–20 mg per L of culture.
26. Store at −80 °C. Aliquot the sample to prevent damage during multiple freeze-and-thaw cycles.

4 Notes

1. Cells at this stage need to be actively growing to maintain the highest expression level of the plasmid. It is recommended to induce the culture at the OD_{600} 0.4–0.6. If the culture overgrows to above OD_{600} 0.6, a new culture should be started.
2. This method provides the tRNA with the full complement of all natural posttranscriptional modifications synthesized by enzymes in *E. coli* JM109 cells. If tRNA of interest is eukaryotic, the method may not reproduce those provided in a eukaryotic host. For $tRNA^{Arg}ACG$, the wobble A is typically modified to inosine (I) [1, 2], which can be produced in both *E. coli* and eukaryotes.
3. Typical yield at this step is 3.5–5 g of wet cells from 1 L of culture.
4. After this step, the sample should be handled in RNase-free conditions. It is necessary to use sterile, RNase-free, disposable plasticware. All buffers should be prepared with DEPC-treated water. Reused glassware should be washed with RNaseZap followed by ultrapure water.
5. Nucleic acid pellet at this stage is white and opaque. It should not be disturbed or dislodged when the supernatant is removed.
6. This step removes ribosomal RNA and other large RNA contaminants, which are pelleted at this step while tRNA remain in the supernatant.
7. The pellet can be hydrated with 0.3 M NaOAc, pH 5.0, up to overnight.
8. The pellets from multiple 1 L cultures can be combined into 6 mL of 0.3 M NaOAc pH 5.0 if the yield is low.
9. The sample may freeze. The frozen sample can be directly centrifuged.
10. Over-dried pellet looks transparent and is hard to dissolve, so it is important for the pellet not to be over-dried.
11. This solution contains the final tRNA yield. Since it is purified from native cells, some of it might still be charged to amino acids, so it is necessary to perform deacylation steps to ensure that this tRNA is arginylation-competent.

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Chapter 14

Enzymatic Aminoacylation of $tRNA^{Arg}$ Using Recombinant Arg-tRNA Synthetase

Irem Avcilar-Kucukgoze and Anna S. Kashina

Abstract

This chapter describes the preparation of pre-charged Arg-tRNA that can be used in arginylation reaction. While in a typical arginylation reaction arginyl-tRNA synthetase (RARS) is normally included as a component of the reaction and continually charges tRNA during arginylation, it is sometimes necessary to separate the charging and the arginylation step, in order to perform each reaction under controlled conditions, e.g., for measuring the kinetics or determining the effect of different compounds and chemicals on the reaction. In such cases, $tRNA^{Arg}$ can be pre-charged with Arg and purified away from the RARS enzyme prior to arginylation.

Key words tRNA, Aminoacylation, Arginylation

1 Introduction

The use of pre-charged Arg-tRNA enabled a number of arginylation studies that elucidated key properties of arginyltransferase. Using pre-charged tRNA, it was found that ATE1 acts independently of ATP, presumably utilizing the energy of the Arg-tRNA bond [1]. Pre-charged tRNA also enabled studies of the kinetic parameters of the arginylation reaction, as well as comparative efficiency of different tRNA isoacceptors and isodecoders [Irem]. Finally, it was discovered that Arg-tRNA-derived fragments, lacking a large portion of the tRNA molecule but still containing the conjugated Arg, can act in arginylation reaction with comparable efficiency to full-length Arg-tRNA [2]. This discovery expanded the scope of ATE1's biological effects and created a potential functional link between arginylation and the global role of tRNA-derived fragments in vivo. This chapter describes the procedure for preparing Arg-charged tRNA. The preparation of recombinant bacterially expressed RARS used in this protocol is described in another chapter in this book.

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2 Materials

Prepare all solutions using DEPC-treated water in RNase-free conditions. To avoid RNase contamination, the buffers can be aliquoted and stored at −20 °C.

1. 100 mM ATP, stored at −20 °C.
2. Free arginine. Depending on the purpose of the experiment, this arginine could be unlabeled, L-Arg, L-[^{13}C, ^{15}N]-Arg labeled with heavy isotopes, or radioactively labeled L-[2,3,4-^{3}H]-Arg for scintillation counting, or L-[^{14}C(U)]-Arg for autoradiography. While stock solutions may vary, this protocol is written for L-[2,3,4-^{3}H]-Arg typical stock solution at 18.3 μM.
3. $tRNA^{Arg}$ stock solution (typical: 5200 ng/μL or 211.6 μM).
4. Purified recombinant arginyl-tRNA synthetase (RARS) (typical: 1.8 mg/mL or 24 μM) (see Chapter 11 of this book for RARS preparation).
5. 10× assay buffer: 500 mM HEPES, 250 mM KCl, 150 mM $MgCl_2$, 1 mM DTT, pH 7.5. DTT should be freshly added.
6. 100 mM NaOAc, 1 mM EDTA, pH 4.8.
7. Acidic phenol, pH 4.5.
8. Chloroform.
9. 3 M NaOAc, pH 5.0.
10. 100% isopropanol.
11. 70% ethanol.
12. 10 mM NaOAc, 1 mM EDTA, pH 4.8.

3 Methods

1. Prepare a 150 μL reaction containing 8 μM $tRNA^{Arg}$, 3.3 mM ATP, 14 μM free Arg, and 1.5 μM RARS in the 1× assay buffer; for stock solutions listed above, add 5.67 μL $tRNA^{Arg}$, 4.95 μL ATP, 115 μL L-[2,3,4-^{3}H]-Arg, 9.38 μL of RARS, and 15 μL of 10× assay buffer. Meanwhile, set up the control reaction containing the above components, but no RARS (*see* **Notes 1–3**).
2. Incubate the reaction at 37 °C for 2 h.
3. Add 250 μL of the 100 mM NaOAc, 1 mM EDTA, pH 4.8 solution (*see* **Note 4**).
4. Add 400 μL of freshly prepared acidic phenol/chloroform (5:1). Vortex for 30 s.

5. Centrifuge at 16,000 × *g* for 10 min at 4 °C. The solution in the tube should separate into the upper aqueous phase and the lower organic phase, with a clear boundary in between, interphase.
6. Use a pipette to carefully remove the aqueous phase without disturbing the interphase, and transfer it to a new tube.
7. Precipitate the tRNA by adding 0.1 volume of 3 M NaOAc, pH 5.0, and 1 volume of isopropanol. Incubate for 30 min at −80 °C, followed by centrifugation at 16,000 × *g* for 30 min at 4 °C.
8. To remove the unbound Arg, wash the pellet by adding cold 70% ethanol followed by centrifugation at 16,000 × *g* for 10 min at 4 °C. Repeat the wash two to three times (*see* **Note 5**).
9. Air-dry the pellet and dissolve it into 23.5 μL of 10 mM NaOAc, 1 mM EDTA, pH 4.8 (*see* **Note 6**).
10. The final concentration of aa-tRNA after this step should be ~1 μg/μL, assuming 80% recovery after phenol/chloroform extraction. For radioactive samples, to determine the level of aminoacylation, use 1 μL of sample directly in a scintillation counter (*see* **Notes 7** and **8**).
11. Calculate the charging level of tRNA as follows:
    - Measure the cpm value of 1 μL stock L-[2,3,4-^{3}H]-Arg.
    - Measure the cpm value of 1 μL of Arg-tRNA (1 μg/μL) at **step 10**.
    - Calculate the moles of L-[2,3,4-^{3}H]-Arg for 1 μg (40.70 pmole) of Arg-tRNA.
    - Calculate the molar ratio of L-[2,3,4-^{3}H]-Arg:tRNA (*see* **Note 9**).

4 Notes

1. As the protocol requires the usage of radioactive material, the sample should be handled in a hot lab according to the institutional rules and regulations. The researcher should handle the sample with a proper personal protective equipment, e.g., disposable gloves, laboratory coat and goggles, etc.
2. If cognate aminoacyl-tRNA synthetase is not available, a crude cell extract of the organism of interest or a commercial aminoacyl-tRNA synthetase mix from *E. coli* (A3646, Sigma) can be used. Despite considerations related to organism specificity, *E. coli* and human RARSs work well for both mouse and *E. coli* tRNA^{Arg} according to our experience [3].

3. In order to detect the arginylation by autoradiography or mass spectrometry, L-[^{14}C(U)-Arg or L-[^{13}C,^{15}N] should be used, respectively. Note that the U in L-[^{14}C(U)]-Arg indicates that all carbons are uniformly labeled.
4. It is critical to keep aminoacyl-tRNAs (aa-tRNA) in acidic conditions, because the acyl linkage in aa-tRNAs, formed through an ester bond that connects the amino acid carboxyl group with the tRNA terminal 3'-OH group, is sensitive to the physiological pH [2].
5. If needed, washing efficiency at this step for radioactive Arg could be monitored by scintillation counting of the wash solution (supernatant) after centrifugation, and the number of washes could be increased or reduced based on these counts. After the washing is complete, the cpm of the wash solution should be similar to background.
6. The acidic buffer is critical to preserve the aminoacyl moiety.
7. aa-tRNA at this stage can in principle be stored at −80 °C, but due to the highly labile bond between Arg and tRNA, it may lose charge over time. It is recommended to prepare freshly charged Arg-tRNA for each experiment. aa-tRNA should never be stored at temperatures less than −80 °C, even for a short time.
8. Our tests indicate that these conditions result in 20–30% of aminoacylation of $tRNA^{Arg}$.
9. An example of calculating the aminoacylation level of $tRNA^{Arg}$:

   Stock L-[2,3,4-^{3}H]-Arg: 54.5 Ci/mmol, 1 mCi/mL, or 1 μCi/μL.

   1 μL of stock L-[2,3,4-^{3}H]-Arg gives 825,008 cpm.

   1 μCi: 825,008 cpm

   Measured cpm of 1 μg of Arg-tRNA after the charging reaction: 527,503 cpm

   527,503/825,008 = 0.63 μCi

   54.5 μCi = 1 nmol

   0.63 μCi/54.5 μCi = 0.012 nmol = 12 pmol μCi

Thus, 1 μg of aminoacylated Arg-tRNA (40.70 pmol) in this reaction contains 12 pmol of L-[2,3,4-^{3}H]-Arg, and the aminoacylation level is 29.5% (=12/40.70).

Acknowledgments

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Chapter 15

Assaying ATE1 Activity In Vitro

Junling Wang and Anna S. Kashina

Abstract

Here, we describe a standard arginyltransferase assay in vitro using bacterially expressed purified ATE1 in a system with a minimal number of components (Arg, tRNA, Arg-tRNA synthetase, and arginylation substrate). Assays of this type have first been developed in the 1980s using crude ATE1 preparations from cells and tissues and then perfected recently for the use with bacterially expressed recombinant protein. This assay represents a simple and efficient way to measure ATE1 activity.

Key words Arginylation, ATE1, Arginylation assay

1 Introduction

A version of the in vitro assay of ATE1 activity was originally developed in the late 1980s [1]. At that time, only partial purification of ATE1 was possible, similar to that described in Chapter 5 of the current book, and the bacterial expression and purification of ATE1 have not yet been developed. However, the principles of the assay remained largely the same. The standard reaction mixture contained an ATE1 preparation (usually a crude fraction obtained with different fractionation methods), a protein or peptide substrate, Arg-tRNA synthetase, Arg, and tRNA. Arg incorporation was usually detected by radioactive label on Arg, which transferred from the low molecular weight fraction containing Arg and tRNA into the protein fraction and could subsequently be analyzed by scintillation counting or autoradiography.

The development of the bacterial expression and purification method described in Chapter 11 of this book enabled the characterization and optimization of this assay. It was found that pre-charged Arg-tRNA obtained in a separate RRS-mediated reaction can be utilized by ATE1 directly in an ATP-independent manner [2]. However, such pre-charging and subsequent Arg-tRNA purification are not only more labor-intensive but can also

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result in significant losses of charged Arg-tRNA through subsequent purification steps. For this reason, a typical working assay for ATE1 described here includes RRS, tRNA, and free Arg plus all the components essential for tRNA charging, in addition to ATE1 and its substrate.

This chapter describes the individual format assay using protein and peptide substrates, originally described by Wang et al. [2, 3].

2 Materials

1. ATE1 and RRS obtained as described in Chapter 11 of this book.
2. 4× assay buffer: 200 mM HEPES, 100 mM KCl, 60 mM MgCl2, 0.4 mM DTT, pH 7.5.
3. Arginylation substrates. In our assays, we used bovine serum albumin (BSA or α-lactalbumin) as protein Arg acceptor (Subheading 3.1) and angiotensin II (DRVYIHPF, Subheading 3.2) as peptide Arg acceptors (*see* **Note 1**). Other Arg acceptors, including substrates to be tested, can also be used in this assay.
4. ATP (100 mM stock).
5. Peptide wash buffer: 0.1% trifluoroacetic acid (TFA) in water (Subheading 3.2).
6. Peptide elution buffer: 60% acetonitrile, 0.1%TFA in water (Subheading 3.2).
7. Labeled Arg: L-[2,3,4-^{3}H]-Arginine (PerkinElmer; other sources of radiolabeled Arg could be used). For detection of arginylation by autoradiography, ^{14}C-labeled Arg should be used in place of ^{3}H-labeled one. For detection by mass spectrometry, unlabeled L-Arginine (Sigma) or L-[^{13}C,^{15}N]-Arginine (Pierce) could be used. These non-radioactive Arg variants usually come dry and should be dissolved in water to 100 mM prior to the experiment.
8. tRNA^{Arg} (Arg-specific tRNA) obtained by expression in *E. coli* or in vitro transcription as described in other chapters in this book. If unavailable, bulk *E. coli* tRNA from Sigma, Roche, or other vendors could be used; however, this tRNA has significantly lower efficiency in arginylation assays (*see* **Note 2**).
9. Trichloroacetic acid (TCA; for protein substrate precipitation, Subheading 3.1).
10. C18 spin columns (The Nest Group, Inc.) (for peptide substrate purification, Subheading 3.2).
11. Acetonitrile (for peptide substrate purification, Subheading 3.2).

3 Methods

3.1 Individual Format Assay Using a Protein ATE1 Substrate

1. On ice, mix a 50 μL reaction containing 1× assay buffer, 2.5 mM ATP, 12.5 μM [^{3}H]-Arginine (or other Arg type as described in the "Materials" section), 40 μM $tRNA^{Arg}$, 2 μM RRS,1 μM ATE1, and 8.3 μM BSA substrate. Keep the reaction on ice until ready.
2. On ice, mix control reactions: two excluding ATE1, one excluding RRS, and one excluding BSA.
3. Prepare quenching tubes, one for each time point (see below): 40 μL of 20% trichloroacetic acid (TCA) containing 1 mM of unlabeled Arg.
4. When all the reactions are mixed, take a 10 μL aliquot from each tube (0 time point), and immediately quench it into the quenching tube to stop the reaction. Place all the tubes with the remaining mixtures simultaneously into a heat block pre-equilibrated to 37 °C, and start the timer.
5. Take out a 10 μL aliquot at each time point (usually 10, 20, 30, and 40 min), and immediately quench it into the quenching tube. Keep the quenched aliquots at room temperature for at least 10 min.
6. Set aside one of the control sets containing no ATE1 to be used as RRS charging control. Heat the rest of the quenched aliquots at 95 °C for 15 min to destroy the excess of the labeled Arg-tRNA.
7. Put all the tubes on ice and keep them for 20 min, and then spin at 13,000 rpm for 30 min at room temperature to collect the pellets containing precipitated proteins.
8. Wash the pellets three times by adding 5% cold TCA without disturbing the pellet and re-centrifuging at 13,000 rpm for 10 min at room temperature. Repeat the wash one more time using cold acetone in place of TCA. Air-dry the pellets.
9. Put the air-dried tubes into vials filled with liquid scintillation solution, and count with a scintillation counter. ATE1 activity (or RRS charging for the RRS control) will be measured as counts per minute (cpm) of [3H]-Arg at each time point.

For Alternative Detection Methods

1. For detection of arginylation by autoradiography, use [^{14}C]-Arg instead of [^{3}H]-Arg in the reaction. In this case, instead of TCA quenching, samples at each time point should be mixed with an equal volume of 2× SDS sample buffer and boiled, then separated on the SDS page, dried, and exposed to X-ray film at −80 °C (usually for several days or longer). Since ^{14}C is very stable, gels could be stored and re-exposed indefinitely.

2. For detection of arginylation by mass spectrometry (described in a later chapter), unlabeled or [^{13}C,^{15}N]-labeled Arg should be used in the reaction. The use of heavy isotope-labeled Arg in parallel with unlabeled one increases the accuracy of the assay. Instead of time points, samples should be arginylated for 40 min, precipitated with 20% TCA, and processed as described elsewhere [4, 5] and later in this book.

3.2 Individual Format Assay Using a Peptide ATE1 Substrate

1. On ice, mix a 100 μL reaction containing 1× assay buffer, 3 mM ATP, 100 μM tRNA^{Arg}, 6 μM RRS, 3 μM ATE1, 100 μM peptide substrate, and 1 mM Arg (*see* **Note 3**). Simultaneously, set up the control reactions as described in Subheading 3.1.
2. Place the reactions into a pre-equilibrated 37 °C heat block and incubate for 1 h.
3. Terminate the reactions by heating at 95 °C for 15 min.
4. Keep the tubes on ice for 20 min, and then spin at 13,000 rpm for 15 min at room temperature.
5. Load the supernatants onto C18 spin columns prewashed with 100% acetonitrile and water by centrifugation at 110*g* for 1 min.
6. Wash the columns with 150 μL peptide wash buffer by centrifugation at 110*g* for 1 min.
7. Elute the columns with 150 μL peptide elution buffer by centrifugation at 110*g* for 1 min. For radioactive Arg, eluted peptides at this stage should be transferred into scintillation vials and analyzed on a liquid scintillation counter. For other analysis methods, **steps 8** and **9** should be performed.
8. Dry the eluted peptides using speed vacuum until the disappearance of any visible liquid from the tube. The peptide should form a thin film-like pellet that may not be clearly visible.
9. Re-dissolve the arginylated peptide in peptide wash buffer for further analysis by mass spectrometry or Edman sequencing.

4 Notes

1. Any soluble peptide containing N-terminal Asp or Glu could in principle be used as an efficient Arg acceptor in the peptide-based arginylation reaction. In our work, we tried several other peptides in addition to angiotensin II [2, 3].
2. Since tRNA charging with Arg depends on the activity of RRS, tRNA and RRS should ideally be a match by species – e.g., *E. coli* tRNA and *E. coli* RRS.

3. For mass spectrometry, use L-[^{13}C,^{15}N]-Arginine; for subtractive Edman sequencing described later in the book, use L-Arg; for radioactive detection by scintillation counting, use 12.5 μM L-[2,3,4-^{3}H]-Arg, and adjust the concentrations of $tRNA^{Arg}$ and peptides to 50 μM.

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Chapter 16

High-Throughput Arginylation Assay in Microplate Format

Sougata Saha, Junling Wang, and Anna S. Kashina

Abstract

Here, we describe the biochemical assay for ATE1-mediated arginylation in microplate format, which can be applied to high-throughput screens for the identification of small molecule inhibitors and activators of ATE1, high-volume analysis of AE1 substrates, and other similar applications. Originally, we have applied this screen to a library of 3280 compounds and identified 2 compounds which specifically affect ATE1-regulated processes in vitro and in vivo. The assay is based on in vitro ATE1-mediated arginylation of beta-actin's N-terminal peptide, but it can also be applied using other ATE1 substrates.

Key words ATE1 inhibitors, Arginylation, Tannic acid, Cell motility, Angiogenesis

1 Introduction

This chapter describes in vitro arginylation assay in microplate format originally developed by S. Saha [1] based on the methods by Wang et al. described in the previous chapter [2, 3]. In the original assays, N-terminal beta-actin peptide DDIAALVVDNGSGMCK was used as a substrate for arginylation, and antibodies against the N-terminally arginylated beta-actin were then applied as the detection reagent. It has been found that such an assay is highly sensitive and has very low background, due likely to the high efficiency of peptide substrates in the arginylation reaction [1]. Since then, antibodies to the arginylated beta-actin N-terminus have been commercially developed and licensed by EMD Millipore (ABT264), making this a good method for fast and reliable high-throughput studies.

In principle, a similar approach could be used with another substrate in place of beta-actin peptide, requiring another detection antibody. It should also be possible to develop the same assay for radioactive detection, using radioactively labeled Arg in the reaction, if an appropriate detection system for microplate formats is available.

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The assay is described here for 384-well format, but other plate and non-plate formats could be used.

2 Materials

1. Beta-actin's N-terminal peptide DDIAALVVDNGSGMCK.
2. Microplate coating buffer: 28.6 mM Na_2CO_3, 71.4 mM $NaHCO_3$, pH 9.6.
3. Microplate blocking solution: 5% milk in PBS.
4. Microplate coating solution: 25 μM of DDIAALVVDNGSGMCK peptide in microplate coating buffer.
5. PBST: 0.05% Tween20 in phosphate buffered saline (PBS).
6. Chemiluminescence substrate (Thermo SuperSignal ELISA Femto, Thermo Fisher).
7. ATE1 and RRS obtained as described in Chapter 9 of this book.
8. 4× assay buffer: 200 mM HEPES, 100 mM KCl, 60 mM $MgCl_2$, 0.4 mM DTT, pH 7.5.
9. ATP (100 mM stock).
10. L-Arg (Sigma).
11. $tRNA^{Arg}$ (Arg-specific tRNA) from *E. coli* (Chemical Block, Russia). If unavailable, bulk *E. coli* tRNA from Sigma, Roche, or other vendors could be used; however, this tRNA has significantly lower efficiency in arginylation assays. Note: Since tRNA charging with Arg depends on the activity of RRS, tRNA and RRS should be a match by species – e.g., *E. coli* tRNA and *E. coli* RRS.
12. 384-well high binding white plates (Corning) (other plates could be used instead; high binding plates are recommended).
13. Anti-R-β peptide antibody recognizing the N-terminally arginylated beta-actin (EMD Millipore ABT264).

3 Methods

1. Coat the 384-well plates with 25 μL per well of microplate coating solution. Incubate at room temperature for 90 min. Longer incubation times could be used at this stage.
2. Remove the coating solution, and fill up the wells with microplate blocking solution (110 μL for a standard 384-well plate). Incubate at 37 °C for 1 h.

3. Remove the blocking solution, and wash the plates three times with PBS at room temperature.
4. Set up 25 μL per well of the reaction mixture containing 1× assay buffer, 6 mM ATP, 0.5 mM arginine, 100 nM $tRNA^{Arg}$, 66 nM RRS, and 250 nM ATE1. In addition, set up individual 25 μL negative control reactions lacking ATE1, RRS, or tRNA (*see* **Note 1**).
5. Add 25 μL reaction mixture into each well and incubate at 37 °C for 30 min.
6. Aspirate the reaction mixture from the wells, and wash the plates three times with PBST at room temperature (*see* **Note 2**).
7. Incubate the plates with anti-R-β peptide antibody at room temperature for 1 h (*see* **Note 3**).
8. Wash plates three times with PBST.
9. Incubate plates with HRP-conjugated anti-rabbit IgG (secondary antibody) at room temperature for 1 h.
10. Wash plates three times with PBST.
11. Add 25 μL of chemiluminescence substrate to each well, and analyze the plates in a microplate reader. To ensure the completion of the chemiluminescence reaction and avoid the decay of the chemiluminescence signal, readings should be collected no earlier than 5 min and no later than 15 min after the substrate addition.

4 Notes

1. This assay can be used for screening of small molecule libraries to identify activators and inhibitors of ATE1. To do that, aliquots of the compounds to be tested should be added directly into the arginylation reaction mixture at **step 4**. For this purpose, the reaction mixture can be divided in two parts. Part I, which will be added first, will contain all the components except Arg. After the addition of this mixture to the wells, the compound library can be applied, followed by the addition of part II of the reaction mixture which will contain arginine in 1× reaction buffer. Controls using pre-charged Arg-tRNA in place of RRS, tRNA, and Arg in the reaction mixture should be performed on the positives to exclude the effect of the inhibitors on RRS and tRNA.
2. Arginylated beta-actin peptide remains bound to the microwell plate and can be detected with the anti-R-β peptide antibody. From this step forward, the procedure resembles an ELISA assay using antibodies for the detection of arginylated beta-actin peptide in the wells. The strength of the antibody signal

detected by this method should directly reflect the activity of ATE1 in each well.

3. The antibody should be pre-titered using control arginylated peptide to determine the optimal dilution in the assay.

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Chapter 17

Assaying for Arginyltransferase Activity and Specificity by Peptide Arrays

Junling Wang and Anna S. Kashina

Abstract

Here, we describe arginylation assays performed on peptide arrays immobilized on cellulose membranes via chemical synthesis. In this assay, it is possible to simultaneously compare arginylation activity on hundreds of peptide substrates to analyze the specificity of arginyltransferase ATE1 toward its target site(s) and the amino acid sequence context. This assay was successfully employed in prior studies to dissect the arginylation consensus site and enable predictions of arginylated proteins encoded in eukaryotic genomes.

Key words Arginylation, ATE1, Peptide arrays, Site specificity

1 Introduction

This chapter describes arginylation assays on peptide arrays, originally described in [1]. Other studies also employed a similar method [2]. This method enables high-throughput analysis of target site specificity of arginyltransferases and side-by-side comparisons of the arginylation efficiency of hundreds of peptide substrates by ATE1 enzymes from different sources.

In principle, this assay resembles other in vitro arginylation assays described in this volume, in terms of the reaction components and general conditions of the reaction. It requires pre-synthesized peptide arrays, which can be performed by qualified facilities by employing peptide synthesis directly on the membrane. During this synthesis, membranes can be created that contain dozens of peptides of the same length but different sequences, bound to the membranes in an oriented way to ensure the identity and positions of the N-terminal and mid-chain residues. These membranes can then be treated with a mixture of ATE1, RARS, tRNA, and labeled Arg similar to the way it is done for in-solution and microplate assays described in other chapters of this volume. In the case of radioactive label described here,

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posttranslationally incorporated Arg can then be detected by autoradiography. This detection can be semi-quantitative, as the level of Arg incorporation can be quantified as gray level on film, which can be directly compared with the level of Arg incorporation into other peptide spots on the same membrane, and also normalized between membranes in relation to identically spotted peptide standards.

In our prior work, we used peptide arrays to dissect the target site specificity of different ATE1 isoforms and to develop algorithms that could predict the identity of arginylated proteins in vivo [1].

2 Materials

1. Peptide array(s), designed and pre-synthesized on amino-PEG derivatized cellulose membrane using SPOT synthesis technology [3]. 10 × 15 cm membranes are typically used (*see* **Notes 1–5**). For negative control, one to two positions on the array should be left empty, without peptides (see Fig. 1).

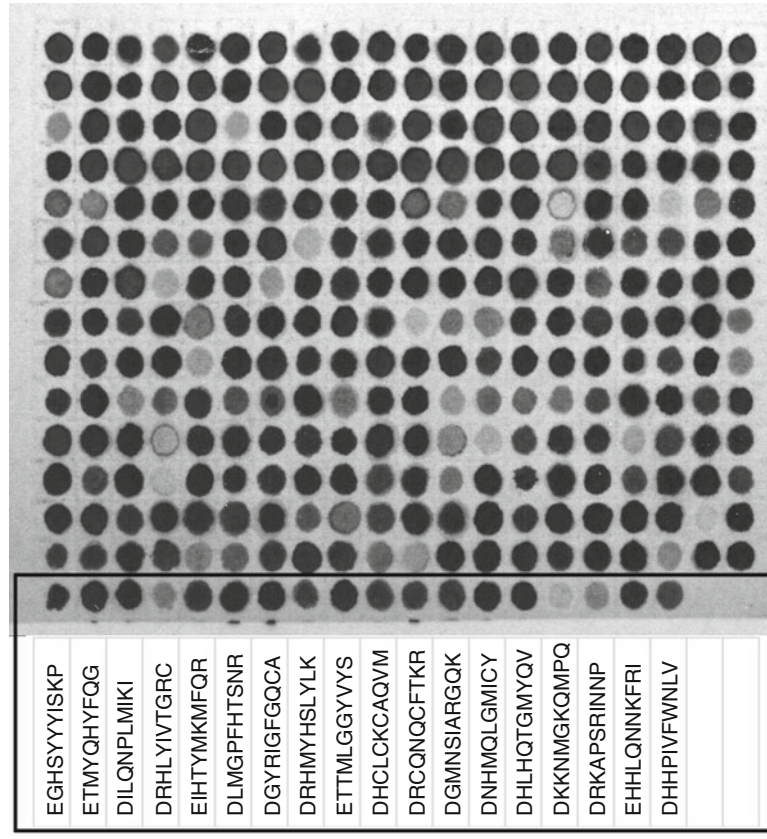

Fig. 1 An example of a developed peptide array. Sequences of the peptides loaded into the bottom-most row of the array are shown underneath, boxed with the corresponding spots on the array. The last two positions on the array are left empty for negative control

2. ATE1 and RRS obtained as described in Chapter 11 of this book.
3. $tRNA^{Arg}$ obtained as described in Chapters 12 and 13 of this book.
4. Total tRNA (commercially available).
5. Acetylated BSA.
6. 4× assay buffer: 200 mM HEPES,100 mM KCl, 60 mM MgCl2, 0.4 mM DTT, pH 7.5.
7. ATP (100 mM stock).
8. Labeled Arg. For detection of arginylation by autoradiography, ^{14}C-labeled Arg is recommended.
9. Ribonuclease A.
10. 3 M Whatman filter paper.

3 Methods

1. Ensure that the membrane with immobilized peptides is legibly gridded with a pencil to detect the position and order of the peptide and the upper side containing the peptide array. The membrane at this point can be cut into smaller pieces, if needed for multiple assays, and cut or marked at the corners to ensure the position of the upper side. During the reaction and film exposure, the upper side needs to face the reaction solution and the film, respectively.
2. Pre-wet the membrane in 1× assay buffer to ensure all peptide spots are uniformly wet. If needed, the membrane can be incubated on a shaker for 30 min–1 h until the membrane looks homogenously translucent.
3. Pre-block the wet membrane (peptide array) in 1× assay buffer supplemented with 0.5% acetylated BSA and 1 mg/mL total tRNA at 37 °C for 1 h (*see* **Note 6**).
4. Add arginylation reaction mixture containing 1.35 μM ATE1, 1.35 μM RARS, 25 μM tRNA, 50 μM ^{14}C-labeled Arg, and 3.3 mM ATP in 1× assay buffer to the pre-blocked membrane (*see* **Note 7**). Incubate at 37 °C for 1 h.
5. Transfer the membrane into 0.5 mg/mL of ribonuclease A in 1× assay buffer. Incubate at 37 °C for 30 min (*see* **Notes 8** and **9**).
6. Remove and discard ribonuclease A solution (*see* **Note 8**). Wash the membrane with PBS on a shaker for 5 min. Repeat the wash for the total of three times.
7. Place the membrane array-up on a piece of dry 3 M Whatman filter paper, and air-dry until no visible moisture remains.

8. Place the dry membrane into an X-ray cassette, and cover it with a piece of X-ray film in the dark. Place the closed cassette at −80 °C, and incubate until signal develops (*see* **Note 10**). See Fig. 1 for an example of a fully developed array (*see* **Note 11**).

4 Notes

1. Amino-PEG derivatization of the cellulose membrane allows the deposition on membranes of individual activated amino acids resulting in peptide formation using standard FMOC HOBT/DCI chemistry.
2. Peptide synthesizer should be modified to increase the number of coupling cycles and FMOC deprotection steps per coupling cycle.
3. XYZ liquid handling robot is typically used, as it enables precise spotting of the peptides on the array.
4. If not performed in the synthesis facility, membrane after synthesis should be treated with trifluoroacetic acid/triisopropylsilane/H_2O 92.5%/5%/2.5% cocktail for 2 h to remove amino acid side chain protection groups followed by 3 × 2 min washes of dichloromethane, 3 × 2 min washes of ethanol, 3 × 2 min washes of H_2O, and 3 × 2 min washes of ethanol. The membrane is then air-dried and gridded with pencil.
5. Test peptides on the membrane can be synthesized using a cleavable linker and assayed by MALDI mass spectrometry as a quality control measure.
6. This step prevents arginylation reaction components from non-specific binding to the array membrane.
7. Reaction volume should be calculated as the minimum volume that enables total submergence of the entire membrane and thus varies depending on the membrane size, which is in turn determined by the number of peptides on the array. One way to minimize the solution volume is to incubate the membrane in a moist chamber while placed face-down on a layer of Parafilm.
8. Since the solution at this point contains ^{14}C-labeled Arg, the reaction mixture and the subsequent washes should be discarded as radioactive waste. The Parafilm, plastic bag, or any other items that came into direct contact with the reaction solution should also be discarded as radioactive waste.
9. This step destroys residual tRNA that may at this point be Arg-conjugated and bound to the membrane non-specifically.
10. Low-temperature incubation limits the flight of the radioactive particles and reduces the signal background. Since ^{14}C signal is very stable over time, multiple exposures can be performed at

this stage to ensure the optimal signal above the background. For strong peptide substrates under optimal reaction conditions, 12–24 h exposures are recommended.

11. Array signal, such as shown, e.g., in Fig. 1, can be quantified using gray level of signal inside the peptide spot against the negative control background. Empty spots with no peptides deposited make for a good negative control.

Acknowledgments

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Chapter 18

Assay of Arginyltransferase Activity by a Fluorescent HPLC Method

Koichi Takao

Abstract

Syntheses of fluorescent substrate and product for arginyltransferase, *N*-aspartyl-4-dansylamidobutylamine (Asp4DNS) and *N*-arginylaspartyl-4-dansylamidobutylamine (ArgAsp4DNS), respectively, including their precursor 4-dansylamidobutylamine (4DNS), are described. Then, HPLC conditions are summarized for a baseline separation of the three compounds in 10 min. The present method, which permits the simultaneous determination of Asp4DNS, 4DNS, and ArgAsp4DNS (in eluting order), is advantageous in measuring arginyltransferase activity and detecting the unfavorable enzyme(s) in 105,000 × *g* supernatant of tissues to ensure accurate determination.

Key words Arginyltransferase assay, Fluorescent HPLC, *N*-Aspartyl-4-dansylamidobutylamine, *N*-Arginylaspartyl-4-dansylamidobutylamine, 4-Dansylamidobutylamine

1 Introduction

Widely used method for measuring arginyltransferase activities is the classic but still convenient method which measures the incorporation of ^{14}C- or ^{3}H-labeled arginine to an acceptor protein such as α-lactalbumin, bovine serum albumin, or other proteins [1–6]. Recently, a non-radioisotopic ELISA method was reported [7]. These methods have contributed to elucidating the role of arginyltransferase, but the problem of whether the arginylated protein substrate can be measured correctly in crude samples containing various proteases remains to be solved. The fluorescent HPLC method [8, 9] described here will assure a reliable measurement of the enzyme activity in 105,000 × *g* supernatant of tissues. This chapter deals with chemical syntheses of fluorescent substrate and product, HPLC analysis, and assay of arginyltransferase activity.

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2 Materials

2.1 Materials for Chemical Syntheses of Fluorescent Substrate and Product

All the organic solvents used for the syntheses are dehydrated grade.

1. Ammonia solution (28%).
2. Acetic acid (AcOH).
3. Trifluoroacetic acid (TFA).
4. 1,4-Diaminobutane.
5. Dansyl chloride (DNS-Cl).
6. *tert*-Butyloxycarbonyl-L-aspartic acid-β-benzylester.
7. *N*-Hydroxysuccinimide (*N*-hydroxySu), *N*,*N*′-dicyclohexylcarbodiimide (DCC).
8. α-Carbobenzyloxy-Arg(di-carbobenzoxy)-α-succinimidyl ester (Z-Arg(di-Z)-OSu).
9. Pd/C (palladium on carbon).
10. Ninhydrin spray.
11. TLC plate (MERCK Silica gel 60 F_{254}).
12. Silica gel (Wakogel C-300).
13. Filter paper (ADVANTEC, No. 2).

2.2 Materials for HPLC Analysis

1. Methanol (liquid chromatography grade).
2. Sodium octanesulfonate (ion-pair reagent grade).
3. Citric acid (amino acid analysis grade).
4. Disodium hydrogen phosphate (amino acid analysis grade).
5. McIlvaine buffer: 0.1 M citric acid, 0.2 M disodium phosphate (mixed in 79.45:20.55 ratio) (pH 3.0).

2.3 Materials for the Assay of Arginyltransferase Activity

1. Trizma base.
2. Hydrochloric acid (HCl).
3. Ethylenediaminetetraacetic acid (EDTA).
4. Magnesium chloride ($MgCl_2$).
5. Potassium chloride (KCl).
6. Sodium chloride (NaCl).
7. Sucrose.
8. 2-Mercaptoethanol.
9. Ammonium sulfate.
10. Bestatin.
11. Alumina powder.
12. Magnesium acetate.
13. DNase I.

14. Adenosine triphosphate (ATP).
15. ^{14}C-arginine (50 nCi/nmol = 1.85 kBq/nmol).
16. Trichloroacetic acid (TCA).
17. *E. coli* paste.
18. DEAE cellulose.
19. Hydroxyapatite.
20. Filter paper disk for assay (Whatman R 3MM 2.5 cm).
21. Bio-Rad protein assay kit.

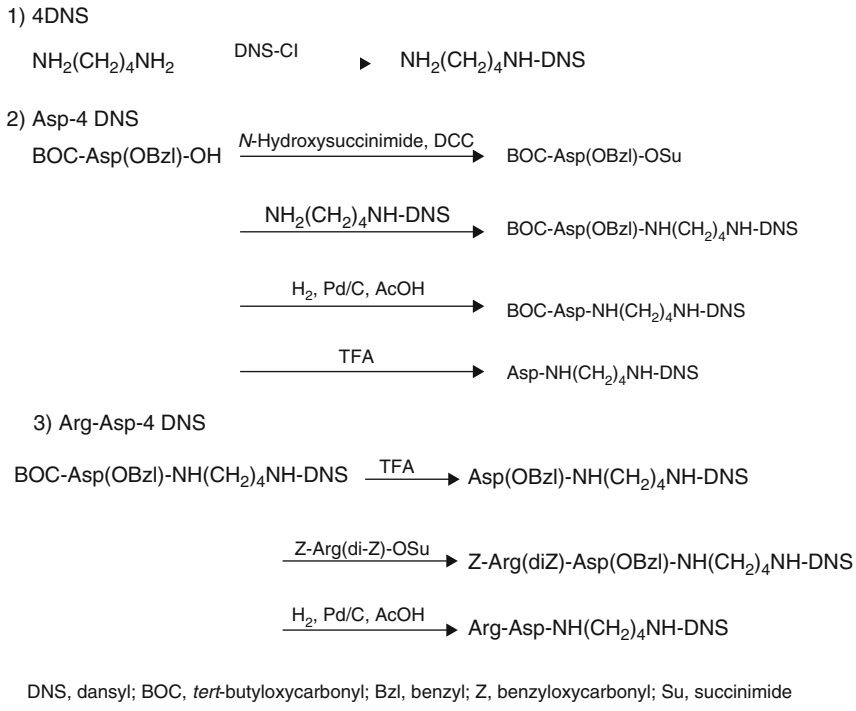
3 Methods

3.1 Chemical Syntheses of Fluorescent Substrate and Product

Synthetic routes of fluorescent compounds for the measurement of arginyltransferase activity are summarized in Fig. 1.

3.1.1 Synthesis of 4-Dansylamidobutylamine (4DNS)

1. Dissolve 1,4-diaminobutane (10 eq) in CH_2Cl_2 (10 mL/mmol).
2. Dissolve dansyl chloride (1 eq, DNS-Cl) in CH_2Cl_2:MeOH (1:1, 10 mL/mmol). Add the solution dropwise to the solution of



DNS, dansyl; BOC, *tert*-butyloxycarbonyl; Bzl, benzyl; Z, benzyloxycarbonyl; Su, succinimide

Fig. 1 Synthetic routes of the fluorescent compounds for arginyltransferase assay

1,4-diaminobutane. The reaction should be monitored by TLC ($CHCl_3$:MeOH:ammonia solution = 10:1:0.1) (*see* **Note 1**).

3. Evaporate the solvent and extract the residue with H_2O and $CHCl_3$ (*see* **Note 2**) and the $CHCl_3$ layer. After evaporation, subject the sample to a silica gel column chromatography using a solvent system of $CHCl_3$:MeOH:ammonia solution (10:1:0.1) (*see* **Note 3**).
4. Collect the fractions of 4DNS and evaporate them. Pure 4DNS is obtained as a yellow-green-colored oil (*see* **Note 4**).

3.1.2 Synthesis of N*-Aspartyl-4-Dansylamidobutylamide (Asp-4DNS)*

1. Dissolve *tert*-butyloxycarbonyl-L-aspartic acid β-benzylester (1 eq) and *N*-hydroxysuccinimide (1.6 eq) in dioxane (10 mL/mmol).
2. Add *N*,*N*′-dicyclohexylcarbodiimide (1.3 eq) dissolved in dioxane (10 mL/mmol) dropwise, and monitor the reaction on TLC (benzene/acetone = 5:1) for the formation of *tert*-butyloxycarbonyl-L-aspartic acid-α-succinimidyl-β-benzyl-diester. After filtration, evaporate the solvent and dissolve the residue in ethyl acetate. Remove the resulting precipitate by filtration (*see* **Note 5**). Evaporate the filtrate.
3. Dissolve the residue containing *tert*-butyloxycarbonyl-L-aspartic acid-α-succinimidyl-β-benzyl-diester in dioxane, and add it dropwise to the 4-dansylamidobutylamine (1 eq) dissolved in dioxane (10 mL/mmol). Monitor the *tert*-butyloxycarbonyl-L-aspartic acid-α-4-dansylamidobutylamido-β-benzylester on TLC (benzene/acetone = 5:1).
4. Evaporate the reaction mixture and dissolve the residue in benzene/acetone (20:1). Apply the solution to a silica gel column, and successively elute with benzene/acetone (10:1 and 5:1). Collect and evaporate the 5:1 eluate to obtain the protected Asp-4DNS.
5. Subject the protected Asp-4DNS to deprotection procedures. First, remove benzyl group by a catalytic reduction in AcOH with Pd/C (*see* **Note 6**). Purify the resulting compound once by silica gel column chromatography using a solvent system of $CHCl_3$:MeOH:AcOH (20:1:0.2). Second, remove *tert*-butyloxycarbonyl group with the treatment by TFA. Purify the resulting Asp-4DNS by silica gel column chromatography with the solvent systems of $CHCl_3$:MeOH:AcOH (4:1:0.3).
6. Collect and evaporate the major fluorescent fractions. Dissolve the residue in a small amount of ethanol. Add diethyl ether to this solution to obtain a precipitate of pure Asp-4DNS.

3.1.3 Synthesis of N-Arginylaspartyl-4-Dansylamidobutylamide (ArgAsp-4DNS)

1. Remove *tert*-butyloxycarbonyl group of the protected Asp-4DNS (Subheading 3.1.2, **step 4**) using TFA. Extract the de-protected product with 4 M NH_3 and chloroform to obtain the free form of Asp(OBzl)-4DNS.
2. Dissolve the Asp(OBzl)-4DNS (1 eq) in dioxane (50 mL/mmol), and add α-carboxyactivated and tribenzyloxycarbonyl-protected arginine (Z-Arg(di-Z)-OSu, 1 eq) to the solution. Stir at room temperature for 1.5 h.
3. Evaporate the dioxane and dissolve the residue in $CHCl_3$: MeOH:AcOH (25:1:0.4). Purify by silica gel column chromatography with the same solvent system. Collect the major fluorescent fractions containing the protected ArgAsp-4DNS, and recrystallize them from acetone.
4. Subject the protected ArgAsp-4DNS to usual deprotection procedure, a catalytic reduction in AcOH with Pd/C to remove benzyl and benzyloxycarbonyl groups simultaneously (*see* **Note 6**).
5. Purify the resulting preparation of ArgAsp-4DNS by silica gel column chromatography with the solvent system of $CHCl_3$: MeOH:AcOH (2:4:1). Collect the major fluorescent fractions and evaporate them to obtain pure ArgAsp-4DNS.

3.2 HPLC Analysis

1. Apparatus: Chromatopac C-R8A, system controller SCL-10AVP, autoinjector SIL-10ADVP, liquid chromatograph pump LC-10DVP, degasser DGU-14A, column oven CTO-10AVP, fluorescence detector RF-10AXL (Shimadzu).
2. HPLC system: a TSKgel-ODS80$_{TM}$ column (4.6 mm Φ × 150 mm) equilibrated with elution buffer (methanol/McIlvaine buffer (13:7) containing 15 mM sodium octanesulfonate). The flow rate is 0.8 mL/min, and the column oven temperature is 40 °C. The fluorescence is detected at an excitation wavelength of 333 nm and an emission wavelength of 536 nm.
3. Each analysis starts after the confirmation of a satisfactory resolution and a linearity of working curve using standard Asp4DNS, ArgAsp4DNS, and 4DNS of baseline separation (*see* **Note 7**). Determination is based on peak area.

3.3 Assay of Arginyltransferase Activity

This preparation is according to Zubay's publication [10].

3.3.1 Preparation of Arginyl-tRNA Synthetase from E. coli (See **Note 8**)

1. Mix *E. coli* paste (1 g) with distilled water (2 mL) and alumina powder (3 g). Grind the mixture using a mortar and pestle on ice for 30 min.
2. Add 6 ml of Tris-HCl buffer containing 10 mM magnesium acetate (pH 7.4). Add DNase I to the final concentration of 4 μg/mL.

3. Centrifuge the mixture at 17,000 × *g* for 20 min. Discard the pellet.
4. Centrifuge the supernatant at 78,000 × *g* for 150 min. Collect the upper two-thirds of the supernatant and discard the rest.
5. Dialyze the collected supernatant against 20 mM phosphate buffer containing 5 mM 2-mercaptoethanol (pH 7.7). The dialyzed supernatant is used as a source of arginyl-tRNA synthetase and can be stored in aliquots at −80 °C.
6. To determine the arginyl-tRNA synthetase activity, mix a reaction (total 100 μL) containing 20 μL of distilled water, 10 mM of Tris-HCl (pH 8.0), 5 mM of 2-mercaptoethanol, 9 mM of $MgCl_2$, 0.3 mM of ATP, 2 nM of ^{14}C-arginine (50 nCi/nM = 1.85 kBq/nM), 10 μL of arginyl-tRNA synthetase fraction, 1 mM of KCl, and 3 nM of α-lactalbumin. After incubation for an appropriate time at 37 °C, 40 μL each of the mixture is spotted on a filter paper disk and treated with cold or hot trichloroacetic acid (*see* **Note 9**). Incorporation of ^{14}C-arginine is counted by a liquid scintillation counter as arginyl-tRNA synthetase activity according to Mans and Novelli [11].

3.3.2 Preparation of Arginyltransferase from Hog Kidney as a Standard Enzyme

The preparation is according to the methods reported by Kato and Nozawa [12] and Soffer [13] (*see* **Note 8**).

1. Homogenize hog kidney in 20 mM Tris-HCl buffer (pH 7.8) containing 0.1 mM EDTA, 5 mM $MgCl_2$, 5 mM KCl, 0.25 mM sucrose, and 5 mM 2-mercaptoethanol using Polytron homogenizer on ice (1 g of wet tissue with 2 ml of homogenization buffer).
2. Centrifuge the homogenate at 20,000 × *g* for 20 min. Collect the supernatant (should be turbid).
3. Centrifuge the supernatant at 105,000 × *g* for 90 min. Discard the pellet.
4. Dialyze against 20 mM Tris-HCl buffer (pH 7.8) containing 0.1 mM EDTA, 5 mM $MgCl_2$, and 5 mM 2-mercaptoethanol.
5. Mix the supernatant with 40% saturated ammonium sulfate solution (*see* **Note 10**). Stir on ice for ~10 min until the formation of visible precipitate. Centrifuge at 20,000 × *g* for 30 min. Discard the pellet.
6. Mix the supernatant with 30% saturated ammonium sulfate solution. Stir and centrifuge as in **step 5**. Discard the supernatant. **Steps 5** and **6** result in the collection of the ammonium sulfate cut in the range of ~40–60%.
7. Dissolve the pellet in 20 mM Tris-HCl buffer (pH 7.8) containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol. Dialyze against the same buffer.

8. Adjust protein concentration in the dialyzed protein solution to 20 mg/mL with the same buffer. Precipitate at pH 5.2 by the addition of 0.1 M acetic acid.
9. Collect the precipitate by centrifugation at 20,000 × *g* for 30 min, and resuspend in 200 mM Tris-HCl (pH 7.8) containing 100 mM 2-mercaptoethanol. Dialyze the suspension against 10 mM Tris-HCl (pH 7.8) containing 100 mM 2-mercaptoethanol.
10. Apply the dialyzed solution to a DEAE cellulose column, and then elute stepwise with the dialysis buffer containing 200 and 400 mM NaCl.
11. Mix the 200 mM NaCl eluate with 75% ammonium sulfate, mix to form the precipitate, and collect it by centrifugation at 20,000 × *g* for 30 min. Dialyze against 10 mM potassium phosphate buffer (pH 7.8) containing 100 mM 2-mercaptoethanol. The dialyzed solution is used as arginyltransferase standard 1 (*see* **Note 11**).
12. Fractionate the dialyzed solution to a hydroxyapatite column equilibrated with 10 mM potassium phosphate buffer (pH 7.8) containing 100 mM 2-mercaptoethanol.
13. Elute with the gradient of 10 mM–1 M potassium phosphate buffer (pH 7.8) containing 100 mM 2-mercaptoethanol. The eluted fractions should contain two active peaks. Concentrate the second peak by ultrafiltration and use as arginyltransferase standard 2 (*see* **Note 12**).

3.3.3 Preparation of Crude Supernatants from Animal Tissues

1. Homogenize each tissue using the exact proportion of one part of wet tissue (g) to two parts of 20 mM Tris-HCl buffer (pH 7.8) containing 0.1 mM EDTA, 5 mM $MgCl_2$, 5 mM KCl, 0.25 mM sucrose, and 5 mM 2-mercaptoethanol (ml).
2. Centrifuge the homogenates at 105,000 × *g* for 90 min. Dialyze the supernatant before the experiment against 20 mM Tris-HCl buffer (pH 7.8) containing 0.1 mM EDTA, 5 mM $MgCl_2$, and 5 mM 2-mercaptoethanol (*see* **Note 13**).
3. The supernatant can be stored at −80 °C until use.

3.3.4 Assay of Arginyltransferase Activity in Crude Supernatants

1. Mix a reaction (total 100 μL) containing 10 mM of Tris-HCl (pH 8.0), 5 mM of 2-mercaptoethanol, 9 mM of $MgCl_2$, 0.3 mM of ATP, 2 nM of arginine, 10 μL of aminoacyl-tRNA synthetase fraction from *E. coli* (Arg-tRNA generating system), 1 mM of KCl, 10 nM of Asp-4DNS (arginine acceptor) (*see* **Note 14**), and 20 μL of crude supernatant and/or arginyltransferase standard (*see* **Note 12**).
2. Add bestatin to the incubation mixture to the final concentration of 0.1 mM as needed (*see* **Note 15**).

3. Incubate the reaction at 37 °C. To stop the reaction, add 100 μL of 20% TCA, and precipitate the protein component by centrifugation. For the analysis of arginylation, take an aliquot (40 μL), dilute 1:1 with the reaction buffer, and analyze by HPLC as described in Subheading 3.2 (*see* **Note 16**).

4 Notes

1. Samples on TLC should be detected by fluorescence lamp (254, 365 nm) or ninhydrin spray.
2. A large amount of 1,4-diaminobutane is removed by this extraction.
3. *N*,*N*′-Bis-dansyl-1,4-diaminobutane and a trace contamination of 1,4-diaminobutane are removed by this step.
4. Standard solution of 4DNS should be prepared by dissolving an aliquot in 0.01 M HCl.
5. This filtration is useful to remove dicyclohexylurea.
6. Dansyl group seems to interrupt the catalytic reduction, so Pd/C should be added repeatedly until the reaction is completed.
7. Actual chromatograms are seen in the references [8, 9].
8. All preparations should be treated in the cold room (4 ° C) or under cold conditions on ice.
9. For the hot TCA treatment, TCA solution should be boiled on a hot plate.
10. The use of saturated ammonium sulfate solution instead of a solid ammonium sulfate is important to prevent a partial inactivation of arginyltransferase activity.
11. To ensure accurate determination of arginyltransferase activity in crude supernatant, an addition-recovery of known activity of enzyme to the crude supernatant is important, assuming the presence of not only aminopeptidases or proteases but also RNase that decompose arginyl-tRNA.
12. The hydroxyapatite preparation from hog kidney is aminopeptidase-free. The pre-hydroxyapatite fraction is also usable as an arginyltransferase enzyme standard.
13. Arginyltransferase activities in rat tissues shown in the references [9] are not affected by the dialysis.
14. The apparent Km value of 30 μM for Asp-4DNS is calculated as described in the literature [8].
15. Bestatin is added for the prevention of the degradation of the product ArgAsp-4DNS by endogenous aminopeptidases

present in the biological samples, such as arginyl-tRNA synthetase preparations and crude supernatants.

16. Incubation time must be chosen after examination of apparent linearity of the enzymatic formation of ArgAsp-4DNS.

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Chapter 19

Identification of Arginylated Proteins by Mass Spectrometry

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Abstract

Here, we describe the method for the identification of arginylated proteins by mass spectrometry. This method has been originally applied to the identification of N-terminally added Arg on proteins and peptides and then expanded to the side chain modification which has been recently described by our groups. The key steps in this method include the use of the mass spectrometry instruments that can identify peptides with very high pass accuracy (Orbitrap) and apply stringent mass cutoffs during automated data analysis, followed by manual validation of the identified spectra. These methods can be used with both complex and purified protein samples and, to date, constitute the only reliable way to confirm arginylation at a particular site on a protein or peptide.

Key words Posttranslational modifications, Protein arginylation, Mass spectrometry

1 Introduction

Since the discovery of protein arginylation in 1963 [1, 2], many attempts have been made to identify proteins arginylated in vivo. These early studies relied mainly on the detection of radioactively labeled Arg and its incorporation into either protein or peptide-based test substrates or into crude intracellular mixtures which can then be fractionated and characterized. Using these methods, ATE1's specificity for N-terminal acidic residues, Asp and Glu, has been initially discovered, and several protein and peptide substrates of arginylation, have been identified, including ornithine decarboxylase [3–5], BSA, alpha-lactalbumin, thyroglobulin [6, 7], beta-melanocyte-stimulating hormone [8], insulin [9], and angiotensin II [8].

A breakthrough in the analysis of protein arginylation came with the recent development of mass spectrometry in application to posttranslational modifications. This method enabled high-throughput analysis of complex protein samples, enriched for arginylated proteins [10] or partially fractionated to analyze a particular intracellular structure or protein pool (e.g., nucleus, myofibrils, or platelet pellets [11–14]).

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This chapter describes the mass spectrometry-based procedure for the identification of protein arginylation, expanding on our originally published protocol for the identification of N-terminal arginylation [15].

2 Materials (*See* Note 1)

1. Protein sample for analysis (*see* **Note 2**).
2. Protein digestion solution: 1 mg/ml trypsin mass spectrometry grade (Promega, Cat#V5280) in 50 mM ammonium bicarbonate and 5 mM $CaCl_2$ (store in aliquots at -20oC; each aliquot after thawing can be used once) (*see* **Note 3** for other protease options).
3. Trichloroacetic acid (TCA) (Sigma, Cat#T9159) (*see* **Note 4**).
4. Acetone (Thermo Fisher, Cat#BP2403-4) (stored at -20oC).
5. Invitrosol LC/MS protein solubilizer (Invitrogen, Cat #MS10007).
6. Protein alkylation solution: 500 mM iodoacetamide (Sigma-Aldrich, Cat #I1149) in 100 mM ammonium bicarbonate (Sigma-Aldrich, Cat #11213) and 10 mM Tris(2-carboxyethyl)phosphine hydrochloride (Sigma-Aldrich, Cat #C4706), pH 8.5 (freshly made).
7. Peptide extraction buffer 5% formic acid (J.T.Baker, Cat#0129-01) (can be stored at room temperature for 1–4 months).
8. Buffer A (peptide loading buffer for liquid chromatography MS/MS): 5% acetonitrile (Sigma-Aldrich, Cat#00683), 0.1% formic acid in water (can be stored at room temperature for 1–4 months).
9. Buffer B (peptide elution buffer for liquid chromatography MS/MS): 100% acetonitrile, 0.1% formic acid in water (can be stored at room temperature for 1–4 months).
10. Buffer C (alternative peptide elution buffer for liquid chromatography MS/MS; *see* **Note 10**): 500 mM ammonium acetate, 100% acetonitrile, 0.1% formic acid in water (can be stored at room temperature for 1–4 months).
11. Calcium chloride (Sigma-Aldrich, Cat #12022).
12. Ammonium acetate (Sigma-Aldrich, Cat#73594).
13. 1 M dithiothreitol (DTT, Sigma-Aldrich, Cat# 43815) in water (stored at -20oC).
14. Mass spectrometer (LTQ-Orbitrap).
15. Database search software (SEQUEST, ProLuCID, DTASelect 2.0, deltaMassFilter).

3 Methods

3.1 Sample Preparation and Mass Spectrometry

1. Add 1:10 volume of 100% TCA solution to the sample.
2. Vortex briefly and incubate on ice for 30 min.
3. Centrifuge at 13000 g for 10 min.
4. Remove the supernatant.
5. Add 100% cold acetone to the protein pellet (acetone wash).
6. Centrifuge at 13000 g for 10 min.
7. Repeat acetone wash and subsequent centrifugation two more times.
8. Aspirate supernatant; air-dry the pellet (*see* **Note 5**).
9. Dissolve the TCA-precipitated protein sample in the protein solubilizer.
10. Add DTT to the final concentration of 10 mM, and incubate for 30 min at room temperature (*see* **Note 6**).
11. Add protein alkylation solution to the final concentration of 55 mM, and incubate for 20 min at room temperature in the dark (*see* **Note 7**).
12. Digest protein solution with trypsin (or other protease(s) as discussed in **Note 3**) by adding the solution to the protein sample at 1:100 weight ratio (i.e., 1 μg of trypsin per 100 μg of protein in the sample) (*see* **Note 8**).
13. Stop the digestion reaction by adding of 5% formic acid to the protein digestion solution. Collect the soluble fraction containing the extracted peptides (*see* **Note 9**).
14. Load the sample onto a reverse phase (RP) or strong cation exchange/RP MudPIT column, and elute with a linear 5%–100% gradient of acetonitrile (*see* **Note 10**).

3.2 Identification of Arginylated Peptides

1. Analyze the results by database searching. For N-terminal Arg identification, use ProLuCID [16] or an equivalent program (*see* **Note 11**). For side chain Arg identification, use the differential modification search with or without addition of 156.1011 (Arg), 170.1168 (monomethylated Arg), and 184.1325 (dimethylated Arg) on every Asp and Glu. The search results will yield the initial list of putative arginylated peptides (*see* **Note 12**).
2. Use DTASelect 2.0 algorithm [17–19] for automated data filtering of the search to eliminate the initial set of the false positives (*see* **Note 13**).
3. Use deltaMassFilter on the data from **Step 2** for high mass accuracy filtering. Use the p-value setting -p 0.0001 (*see* **Note 14**).
4. Manually analyze the identified arginylated peptides for possible mass ambiguities listed in Tables 1, 2 and 3. Discard the

Table 1
Mass ambiguities for arginylated peptides

Unmodified Arg (156.1011)											
-2 residue	−1 residue	−2 residue mass	−1 residue mass	Posttranslational modification (PTM)	PTM mass shift	Residue(s) + PTM mass	Delta mass (amu)	Delta mass in ppm	Ppm delta mass for peptides of mass 1000 amu	Ppm delta mass for peptides of mass 2000 amu	Ppm delta mass for peptides of mass 3000 amu
V	G	99.0684	57.0215	None		156.0899	−0.0112	−71.9534	−11.2320	−5.6160	−3.7440
A	G	71.0371	57.0215	Formylation	27.9949	156.0535	−0.0476	−305.1356	−47.6320	−23.8160	−15.8773
A	G	71.0371	57.0215	Dimethylation or ethylation	28.0313	156.0899	−0.0112	−71.9534	−11.2320	−5.6160	−3.7440
G	A	57.0215	71.0371	Formylation	27.9949	156.0535	−0.0476	−305.1356	−47.6320	−23.8160	−15.8773
G	A	57.0215	71.0371	Dimethylation or ethylation	28.0313	156.0899	−0.0112	−71.9534	−11.2320	−5.6160	−3.7440
G	V	57.0215	99.0684			156.0899	−0.0112	−71.9534	−11.2320	−5.6160	−3.7440
	V		99.0684	Non-specific alkylation	57.0215	156.0899	−0.0112	−71.9534	−11.2320	−5.6160	−3.7440
	L/I		113.0841	Carbamylation	43.0058	156.0899	−0.0113	−72.0687	−11.2500	−5.6250	−3.7500

N	114.0429	Acetylation	42.0106	156.0535	−0.0476	−305.0267	−47.6150	−23.8075	−15.8717
N	114.0429	Guanidination	42.0218	156.0647	−0.0364	−233.0669	−36.3820	−18.1910	−12.1273
N	114.0429	Trimethylation	42.0470	156.0899	−0.0112	−71.9406	−11.2300	−5.6150	−3.7433
D	115.0269	Amidine	41.0265	156.0535	−0.0476	−305.0651	−47.6210	−23.8105	−15.8737
Q	128.0586	Dimethylation or ethylation	28.0313	156.0899	−0.0112	−71.9406	−11.2300	−5.6150	−3.7433
R	156.1011	None		156.1011	0.0000	0.0000	0.0000	0.0000	0.0000

Mass shifts equal to Arg addition (+156.1011 Da) produced by different amino acid residues and posttranslational modifications in the positions adjacent and preceding the arginylated site. These ambiguities cannot be resolved by mass spectrometry and must be manually discarded

Delta mass = residue(s) + PTM mass shift - 156.1011

Delta mass in ppm = delta mass /156.1011*1000,000

ppm delta mass for peptides with mass of 1000 = delta mass/1000*1000000

ppm delta mass for peptides with mass of 1000 = delta mass/2000*1000000

ppm delta mass for peptides with mass of 1000 = delta mass/3000*1000000

Table 2
Mass ambiguities for monomethyl-arginylated peptides

Monomethylated Arg (170.1168)											
−2 residue	−1 residue	−2 residue mass	−1 residue mass	Posttranslational modification (PTM)	PTM mass shift	Residue (s) + PTM mass	Delta mass (amu)	Delta mass in ppm	Ppm delta mass for peptides of mass 1000 amu	Ppm delta mass for peptides of mass 2000 amu	Ppm delta mass for peptides of mass 3000 amu
A	*G	71.0371	57.0215	Acetylation	42.0106	170.0691	−0.0477	−280.2016	−47.6670	−23.8335	−15.8890
A	A	71.0371	71.0371	Dimethylation or ethylation	28.0313	170.1055	−0.0113	−66.3191	−11.2820	−5.6410	−3.7607
A	V	71.0371	99.0684			170.1055	−0.0113	−66.3191	−11.2820	−5.6410	−3.7607
*G	V	57.0215	99.0684	Methylation	14.0157	170.1056	−0.0112	−66.0252	−11.2320	−5.6160	−3.7440
*G	L/I	57.0215	113.0841			170.1055	−0.0113	−66.3427	−11.2860	−5.6430	−3.7620
S	T	87.03203	101.0477	Dehydration	−18.0106	170.0691	−0.0477	−280.4014	−47.7010	−23.8505	−15.9003
S	V	87.03203	99.0684	Deoxy	−15.9949	170.1055	−0.0113	−66.2310	−11.2670	−5.6335	−3.7557
	Q		128.0586	Acetylation	42.0106	170.0691	−0.0477	−280.1898	−47.6650	−23.8325	−15.8883
	Q		128.0586	Guanidination	42.0218	170.0804	−0.0364	−214.1470	−36.4300	−18.2150	−12.1433

Q	128.0586	Trimethylation	42.0470	170.1056	−0.0112	−66.0135	−11.2300	−5.6150	−3.7433
K	128.095	Acetylation	42.0106	170.1056	−0.0112	−66.1016	−11.2450	−5.6225	−3.7483
E	129.0426	Amidine	41.0265	170.0691	−0.0477	−280.2251	−47.6710	−23.8355	−15.8903
W	186.0793	Deoxy	−15.9949	170.0844	−0.0324	−190.5455	−32.4150	−16.2075	−10.8050
R	156.1011	Methylation	14.0157	170.1168	0.0000	0.0000	0.0000	0.0000	0.0000

Mass shifts equal to monomethylated Arg addition (+170.1168 Da) produced by different amino acid residues and posttranslational modifications in the positions adjacent and preceding the arginylated site. These ambiguities cannot be resolved by mass spectrometry and must be manually discarded
Mass difference = residue(s) + PTM mass shift - 170.1168
Mass difference in ppm = Mass difference /170.1168*1000,000
ppm Mass difference for peptides with mass of 1000 = Mass difference/1000*1000000
ppm Mass difference for peptides with mass of 1000 = Mass difference/2000*1000000
ppm Mass difference for peptides with mass of 1000 = mass difference/3000*1000000
The −1 and − 2 residues can be swapped
*G (57.0215) can be either G or alkylation

Table 3
Mass ambiguities for dimethyl-arginylated peptides

Dimethylated Arg (184.1325)											
−2 residue	−1 residue	−2 residue mass	−1 residue mass	Posttranslational modification (PTM)	PTM mass shift	Residue (s) + PTM mass	Delta mass (amu)	Delta mass in ppm	Ppm delta mass for peptides of mass 1000 amu	Ppm delta mass for peptides of mass 2000 amu	Ppm delta mass for peptides of mass 3000 amu
*G	A	57.02146	71.03711	Diethylation	56.06266	184.1212	−0.01117	−60.6737	−11.172	−5.586	−3.724
*G	P	57.02146	97.05276	Hydroxymethyl	30.01057	184.0848	−0.04762	−258.602	−47.617	−23.8085	−15.8723
*G	V	57.02146	99.06841	Dimethylation or ethylation	28.0313	184.1212	−0.01123	−60.9996	−11.232	−5.616	−3.744
*G	I/L	57.02146	113.0841	Methylation	14.0157	184.1212	−0.01119	−60.7498	−11.186	−5.593	−3.72867
*G	V	57.02146	99.06841	Dimethylation/ ethylation	28.0313	184.1212	−0.01123	−60.9996	−11.232	−5.616	−3.744
*G	Q	57.02146	128.0586	Amidation	−0.9848	184.0952	−0.03717	−201.844	−37.166	−18.583	−12.3887
*G	K	57.02146	128.095	Amidation	−0.9848	184.1317	−0.00075	−4.05143	−0.746	−0.373	−0.24867
A	A	71.03711	71.03711	Acetylation	42.01057	184.0848	−0.04762	−258.602	−47.617	−23.8085	−15.8723
A	P	71.03711	97.05276	Oxidation/ hydroxylation	15.9949	184.0848	−0.04763	−258.683	−47.632	−23.816	−15.8773
A	V	71.03711	99.06841	Methylation	14.0157	184.1212	−0.01118	−60.728	−11.182	−5.591	−3.72733

A	L/I	71.03711	113.0841			184.1212	−0.01124	−61.0213	−11.236	−5.618	−3.74533
A	N	71.03711	114.0429	Amidation	−0.9848	184.0952	−0.0372	−202.007	−37.196	−18.598	−12.3987
A	E	71.03711	129.0426	Deoxy	−15.9949	184.0848	−0.04761	−258.542	−47.606	−23.803	−15.8687
A	M	71.03711	131.0405	Oxoanaline	−17.9928	184.0848	−0.04762	−258.597	−47.616	−23.808	−15.872
S	P	87.03203	97.05276			184.0848	−0.04762	−258.602	−47.617	−23.8085	−15.8723
S	I/L	87.03203	113.0841	Deoxy	−15.9949	184.1212	−0.01122	−60.9398	−11.221	−5.6105	−3.74033
P	C	97.05276	103.0092	Deoxy	−15.9949	184.0671	−0.06536	−354.94	−65.356	−32.678	−21.7853
V	T	99.06841	101.0477	Deoxy	−15.9949	184.1212	−0.01122	−60.9127	−11.216	−5.608	−3.73867
	Q		128.0586	Diethylation	56.06266	184.1212	−0.01117	−60.6629	−11.17	−5.585	−3.72333
	K		128.095	Diethylation	56.06266	184.1577	0.02525	137.1296	25.25	12.625	8.416667
	W		186.0793	Didehydro	−2.01565	184.0637	−0.06875	−373.373	−68.75	−34.375	−22.9167
	R		156.1011	Dimethylation/ ethylation	28.0313	184.1324	0	0	0	0	0

Mass shifts equal to monomethylated Arg addition (+184.1325 Da) produced by different amino acid residues and posttranslational modifications in the positions adjacent and preceding the arginylated site. These ambiguities cannot be resolved by mass spectrometry and must be manually discarded

Mass difference = residue(s) + PTM mass shift - 184.1325

Mass difference in ppm = mass difference /184.1325*1000,000

peptides, for which mass ambiguities cannot be resolved even with the high mass accuracy data (e.g., preceding R, preceding I/L if urea was used in the sample, preceding V, and preceding GV/VG sequence).

5. Validate the arginylated peptide spectra manually by isotopic peak checking (*see* **Note 15**).
6. Check the ion fragmentation (MS/MS) spectrum for arginylated peptides in the Xcalibur Raw file using the Qual Browser program by manual viewing to identify the b ion series. Addition of N-terminal Arg onto peptides is expected to result in an altered pattern of fragment ions generated from the peptide's N-terminus (b ion series). Arginylated peptides usually have more prominent early b ions (corresponding to the lower mass fragments generated from the N-terminus) [20–23].

4 Notes

1. Since mass spectrometry usually requires highly pure reagents, the specific reagent grade is important for this procedure. For this reason, vendors and catalog numbers are given to each reagent where possible.
2. In general, any protein sample can be used for the analysis described below. It is recommended to analyze proteins in solution to achieve higher sequence coverage and minimal interference from possible chemical modifications during sample preparation. Note that the probability of detection of arginylated peptides increases with higher protein abundance and lower protein complexity. In addition, some purification methods may either enrich or select against the arginylated protein in the preparations. Finally, several types of conditions should be avoided during sample preparation, including urea (when used at any stage of sample preparation, e.g., during protein solubilization or 2D gel analysis, it results in non-specific carbamylation; carbamylation of Leu/makes them similar in mass to Arg) and Gly (common during immunoaffinity purifications or SDS PAGE and could theoretically result in non-specific addition of glycine onto the free amino groups of the protein's N-terminus).
3. Generally, the use of multiple proteases (e.g., a combination of trypsin, subtilisin, and elastase) increases sequence coverage during mass spectrometry. However, some of these proteases may destroy arginylated peptides by cutting immediately after the arginylated residue or producing peptides too short for detection. We found that trypsin is the optimal enzyme (highly

efficient and enabling the maximum sequence coverage during identification for the majority of proteins) and is compatible for arginylation detection.

4. TCA should not be stored dry. Prepare 100% solution immediately after arrival by adding water to the TCA jar so that the final volume of the solution equals the weight of TCA in the jar, e.g., 250 ml for 250 g package of TCA. Store at 4 °C.
5. TCA protein pellet can be stored at -20 °C for up to several months.
6. This step is necessary to reduce SH groups.
7. Iodoacetamide is highly unstable and light sensitive. Solution needs to be made fresh, and protein alkylation needs to be performed in the dark.
8. Longer digestion times, up to overnight, and higher enzyme ratios can be used with higher-complexity samples and lower-efficiency proteases.
9. Digested peptide solution at this stage can be concentrated by evaporation on the SpeedVac until only the insoluble peptide pellet remains and redissolving the pellet in 5% acetonitrile:0.1% formic acid. This step can improve the analysis but is not essential.
10. For single proteins and low-complexity samples, a one-step acetonitrile gradient is typically used. For the analysis of complex samples using Multidimensional Protein Identification Technology (MudPIT [24]), a 12-step gradient is recommended, as follows:

    Steps 1–11:

    - 1 min of 100% buffer A
    - 5 min of X% buffer C (the 5 min buffer C percentages (X) are 10, 15, 20, 25, 30, 35, 40, 45, 50, and 60%)
    - A 13 min 0–15% gradient of buffer B.
    - A 107 min 15–45% gradient of buffer B.
    - A 30 min 45–75% gradient of buffer B.
    - A 5 min 75–100% gradient of buffer B.
    - 5–20 min of 100% buffer A.

    Step 12:

    - 3 min of 100% buffer A
    - 20 min of 100% buffer C
    - A 10 min 0–15% gradient of buffer B.
    - A 107 min 15–70% gradient of buffer B.

11. In principle, any commercially available and/or user-developed programs for the identification of the addition of a fixed mass on a peptide's N-terminus could be used, such as SEQUEST [25], Mascot [26], X!Tandem [27], or OMSSA [28], but many factors should be accounted for in the development and use of such programs, and the validation steps described below should always be performed on the list of initial positives to identify the true arginylation targets. Based on our experience, ProLuCID [16] that we use in our analysis has better sensitivity and specificity for both modified and non-modified peptide identification compared to SEQUEST, Mascot, X!Tandem, and OMSSA. Thus, we recommend ProLuCID over other algorithms.
12. Depending on the sample preparation conditions, static modifications should be added into the search, e.g., for Cys in case of treatment with a reducing reagent (+ 57.02146).
13. For DTASelect2.0 filtering, we use false positive rate parameter (fp) defined as the ratio of the number of reverse (i.e., false) hits to the number of forward (i.e., true) hits that passed the DTASelect filtering. We filter with the false positive rate setting of 0.1% (fp 0.001). To ensure that the false positive rate is constant in the searches for modified and unmodified peptides, DTASelect2.0 applies separate filtering to modified and unmodified peptides, considering the possibilities of tryptic, half-tryptic, and non-tryptic peptides (−-modstat and --trypstat options of DTASelect2.0). One peptide serves as a requirement for the identification of a protein (−p 1) since we are looking for a specific modification feature of the protein rather than protein identification in the sample. Note that the high accuracy precursor mass information is not used in this step.
14. The deltaMassFilter program can effectively remove most simple ambiguities that result in a significant mass shift. For this filtering, the p-value for each peptide precursor mass is calculated as the delta mass (defined as the difference between the measured mass and the theoretical mass) for each modified peptide against the distribution of the delta masses of all the non-modified peptides. For each modified peptide, the delta mass p-value defines the chance that the observed mass corresponds to this particular peptide and not a highly similar one created by a similar mass shift as a result of another modification. A true hit (i.e., truly arginylated peptide) is assumed to have delta mass distribution similar to the distribution of the unmodified peptides, as evidenced by sufficiently high p-value.

15. For example, if the calculated mass (M) of the identified peptide is 1500, and the measured mass of the peptide is 1501, check the peak in the corresponding MS spectrum in the Raw file to confirm that the measured peak is indeed the M + 1 peak (which occurs if one of the carbons in the peptide is a heavy ^{13}C). If the measured mass of the peptide is 1500, then confirm that the peak in the corresponding MS spectrum is a monoisotopic peak. If this cannot be confirmed, eliminate the corresponding peptides as false positives.

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Chapter 20

Analysis of Arginylated Peptides by Subtractive Edman Degradation

Anna S. Kashina and John R. Yates III

Abstract

During the early studies of N-terminal arginylation, Edman degradation was widely used to identify N-terminally added Arg on protein substrates. This old method is reliable, but highly depends on the purity and abundance of samples and can become misleading unless a highly purified highly arginylated protein can be obtained. Here, we report a mass spectrometry-based method that utilizes Edman degradation chemistry to identify arginylation in more complex and less abundant protein samples. This method can also apply to the analysis of other posttranslational modifications.

Key words Edman degradation, Mass spectrometry, Arginylation

1 Introduction

Edman degradation is a classical method that has been used for N-terminal sequencing of proteins for decades and has proven instrumental in early studies of N-terminal arginylation. This method is efficient and reliable; however, it is best applied to pure and abundant protein samples, especially during analysis of such a posttranslational modification as arginylation, which often affects only a fraction of a protein and thus may result in ambiguous data.

During our recent analysis of posttranslational arginylation, we have devised a mass spectrometry-based procedure, which utilizes the Edman chemistry for sequential removal of the amino acid residues from a peptide's N-terminus, followed by mass spectrometry-based identification of the remaining peptide (with the subtraction of the remaining residue, leading to the name of the procedure) [1, 2]. This procedure combines the robustness of the Edman method with the modern advances in mass spectrometry and can be applied to N-terminal sequencing of any peptide without the same requirements for its abundance and purity as those of the original Edman method. Such analysis can be applied to both

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N-terminal and internal arginylation [3] and can be extended to the analysis of any other posttranslational modifications that do not prevent Edman reaction.

2 Materials

1. Arginylated peptide to be analyzed (lyophilized or dried using vacuum evaporator).
2. Phenyl isothiocyanate (PITC) (Pierce).
3. 50% aqueous pyridine.
4. 2:1 heptane/ethyl acetate solution.
5. Anhydrous trifluoroacetic acid (TFA).
6. n-Butyl acetate.
7. Buffer A (peptide loading buffer for liquid chromatography MS/MS): 5% acetonitrile (Sigma-Aldrich, Cat#00683), 0.1% formic acid in water (can be stored at room temperature for 1–4 months).
8. Buffer B (peptide elution buffer for liquid chromatography MS/MS): 100% acetonitrile, 0.1% formic acid in water (can be stored at room temperature for 1–4 months).
9. Mass spectrometer.
10. Database search software.

3 Methods

1. Dissolve the peptide in 50% aqueous pyridine to the concentration of ~1 mg/mL.
2. Add equal volume of 5% PITC in 50% pyridine.
3. Heat the mixture for 10 min at 50 °C.
4. Add equal volume of 2:1 heptane/ethyl acetate solution, and mix by vortexing for 10 s.
5. Centrifuge 5 min in a tabletop centrifuge at maximum speed. The solution should separate into the upper organic phase and the lower aqueous phase.
6. Remove the upper organic phase using a Pasteur pipette and discard.
7. Repeat the 1 heptane/ethyl acetate extraction of the lower aqueous phase by adding equal volume of 2:1 heptane/ethyl acetate solution and following **steps 4–6**. Discard the organic phase.
8. Lyophilize the aqueous phase.

9. Add 150 μL of anhydrous TFA, and cleave the sample by incubation for 5 min at 37 °C.
10. Lyophilize the sample.
11. Dissolve the peptide pellet in water to the original concentration of ~1 mg/mL (*see* **step 1**).
12. Add two volumes of n-butyl acetate and mix by vortexing for 10 s.
13. Centrifuge 5 min in a tabletop centrifuge at maximum speed. The solution should separate into the upper organic phase and the lower aqueous phase.
14. Remove the upper organic phase using a Pasteur pipette and discard.
15. Lyophilize the peptide and redissolve it in the original volume of buffer A.
16. Load the sample onto a reverse phase column, and elute with a linear 5%–100% gradient of acetonitrile (buffer A-buffer B) (*see* **Note 1**).

4 Note

1. Reduction of the peptide mass by the mass of Arg (156.1011) would indicate N-terminal arginylation. Reduction of the peptide mass by the size of Arg-Asp or Arg-Glu dipeptide would indicate side chain arginylation.

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Chapter 21

Transferase-Mediated Labeling of Protein N-Termini with Click Chemistry Handles

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Abstract

The *E. coli* aminoacyl transferase (AaT) can be used to transfer a variety of unnatural amino acids, including those with azide or alkyne groups, to the α-amine of a protein with an N-terminal Lys or Arg. Subsequent functionalization through either copper-catalyzed or strain-promoted click reactions can be used to label the protein with fluorophores or biotin. This can be used to directly detect AaT substrates or in a two-step protocol to detect substrates of the mammalian ATE1 transferase.

Key words N-terminus, N-end rule, Aminoacyl transferase, Protein modification, Chemoenzymatic reaction, Protein labeling, Click chemistry

1 Introduction

Protein N-terminal sequences impact cellular function by altering protein trafficking and stability [1]. Chemical reactions that can be used to detect and manipulate N-terminal sequences aid in deciphering the roles of those sequences, particularly if the reactions can be performed under conditions that maintain the native fold of the protein [2–4]. To achieve such "protein-friendly" reactions, we and others have exploited the substrate promiscuity of the *E. coli* leucyl phenylalanyl aminoacyl transferase (AaT) for N-terminal protein labeling [5–7]. AaT is the first enzyme in the prokaryotic N-end rule protein degradation pathway. Using aminoacyl tRNAs as substrates, AaT transfers a single Leu, Phe, or Met to the α-amine of an N-terminal Arg or Lys, after which the protein is recognized by ClpS and degraded by the ClpA/P protease machinery [8–10]. A somewhat similar pathway exists in eukaryotic cells, in which the arginyl transferase ATE1 transfers Arg from an aminoacyl tRNA to proteins bearing an N-terminal Asp, Glu, or cysteic acid (Cys*) [11, 12]. The ATE1 pathway is more complex than the AaT

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pathway for two reasons. First, N-terminal Arg modification results in ubiquitin ligase activity, which can lead to proteasome degradation [13, 14]. Second, Arg transfer to protein sidechains by ATE1 has also been observed; however, this will not be discussed here as these Arg modifications would be invisible to our detection method [15].

Tirrell and Sisido have shown that if AaT is presented with tRNAs bearing hydrophobic amino acids which resemble Leu, Phe, or Met, these amino acids will be transferred to appropriate N-termini (Fig. 1) [5, 6]. We have shown that AaT can accept a minimalist mimic of the 3′ end of aminoacyl tRNA, aminoacyl adenosine (Fig. 1) [7, 16]. A variety of unnatural amino acids can be transferred from adenosine donors (Zaa-A), which can be synthesized in three steps from Boc-protected amino acids. The sidechains of these amino acids can encompass a wide variety of functional groups, including fluorophores, photocrosslinkers, and Cys analogs for peptide ligation reactions. Here, we will focus on azide or alkyne amino acids that can be derivatized in a subsequent

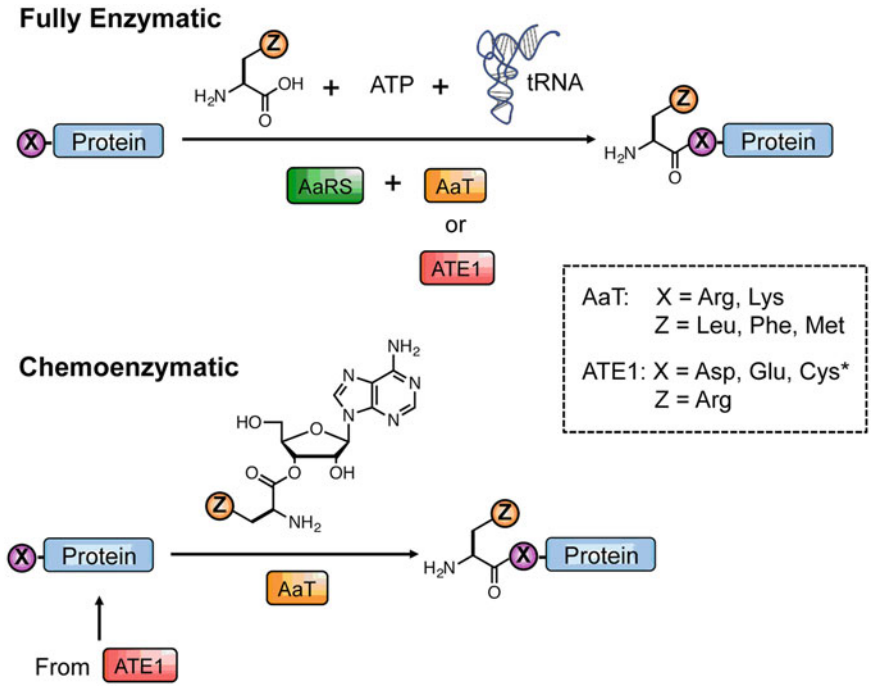

Fig. 1 Transferase-mediated N-terminal protein modification. Transferases (AaT or ATE1) recognize a specific N-terminal amino acid (X) and transfer another amino acid (Z) from a donor molecule. Top: In the fully enzymatic pathway that occurs in cells, the donor is an aminoacyl tRNA produced by an aminoacyl tRNA synthetase (aaRS), using a tRNA, an amino acid, and ATP. This pathway can be reconstituted in vitro with the components shown. Bottom: In the chemoenzymatic method, only a synthetic nucleic acid donor and transferase are required for labeling. Chemoenzymatic labeling has not been successfully demonstrated with ATE1, but ATE1 substrates can be labeled through a two-step sequence using AaT

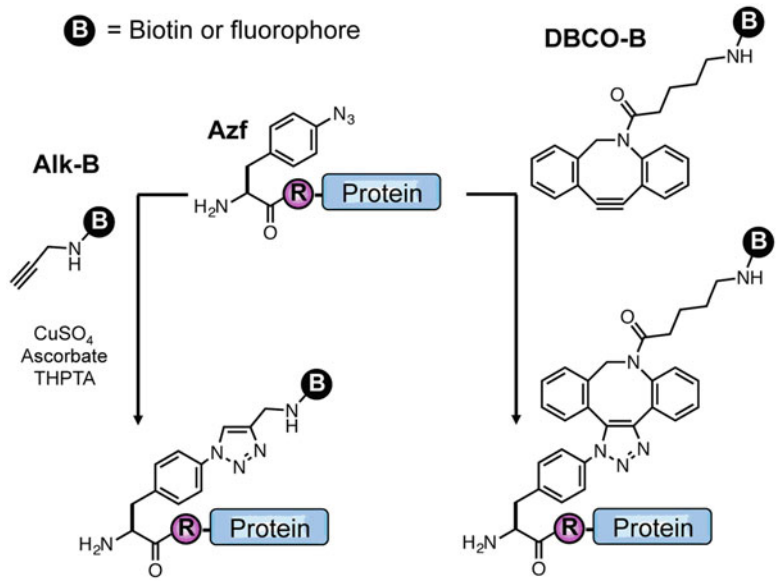

Fig. 2 Derivatization of transferase substrates by click reactions. After modification with an azide by AaT, the protein can be functionalized with labels such as biotin or fluorophores by reaction with linear or cyclic alkyne reagents. Reactions of azides with terminal alkynes (left) require catalysis by Cu^{I}, generated by sodium ascorbate reduction in the presence of a tris-(3-hydroxypropyltriazolylmethyl) amine (THPTA) ligand. Dibenzocyclooctyne (DBCO) reagents react by a strain-promoted mechanism and do not require additives

"click" reaction (Fig. 2) [17]. For example, azidophenylalanine (Azf) can be reacted with an alkyne bearing a fluorescent label or affinity tag, such as biotin. The derivatization can occur by either a copper-catalyzed click reaction with a terminal alkyne or a strain-promoted click reaction with a dibenzocyclooctyne (DBCO) [18, 19]. Many alkyne and DBCO reagents bearing fluorophores or biotin are sold for use as is, and novel reagents can easily be made by reaction of a fluorophore with a commercially available propargyl amine or DBCO-amine. Although no useful substrate promiscuity has yet been shown for ATE1, the complementary recognition motifs of AaT and ATE1 permit one to use AaT transfer reactions to label N-terminal Arg modifications resulting from ATE1 activity. Suspected ATE1 substrates can be subjected to a two-step reaction sequence of AaT transfer and subsequent labeling through a click reaction (Fig. 1). Since AaT has been shown to be extremely tolerant of amino acid variation at residues 2 and 3, this method should allow one to detect any protein that has been N-terminally arginylated by ATE1 [20].

Here, we describe protocols for labeling proteins based on their N-terminal sequences. These include the preparation of reagents: the recombinant expression and purification of AaT and ATE1 as well as three aminoacyl tRNA synthetases (aaRSs) that can be used

for the generation of tRNAs aminoacylated with natural or unnatural amino acids and the synthesis of Zaa-A donors. We also describe an HPLC-based assay that can be performed with reporter peptides to analyze transferase reaction yields and specificity with regard to either the N-terminal sequence or the Zaa-A donor. Finally, we provide protocols for labeling proteins in cell lysate through AaT modification and either copper-catalyzed or strain-promoted click reactions, using α-casein as an example.

2 Materials

All buffers must be prepared with 18 MΩ water purified with a Millipore system (Milli-Q).

2.1 Enzymes and Plasmids

1. pEG6 plasmid, containing His_{10}-tagged *E. coli* AaT under the control of the T7 promoter (Alexander Varshavsky, California Institute of Technology).
2. pAzfRS plasmid made from pCDNA3.1-RS_{Azf} (Thomas Sakmar, The Rockefeller University).
3. 3. pMet*RS plasmid, containing His_6-tagged *E. coli* Met*RS under the control of the T7 promoter (David Tirrell, California Institute of Technology).
4. Arginyltransferase (ATE1) and Arg-tRNA synthetase (RRS) (see expression and purification protocols in the chapter by Wang and Kashina in this volume).

2.2 Buffers for Protein Expression

1. 10X Tris buffer: 500 mM Tris, 3 M KCl. (Dissolve 60.57 g Tris base and 223.65 g KCl in 900 mL Milli-Q water. Adjust the pH to 8.0 with 6 M HCl, and bring the final volume to 1 L with Milli-Q water. Filter-sterilize the solutions using a 0.22 μm filter.)
2. Binding buffer 1: 50 mM Tris, 10 mM imidazole, 300 mM KCl, and 5 mM β-mercaptoethanol (BME), pH 8.0. (Dissolve 100 mL 10X Tris buffer and 681 mg imidazole in 800 mL Milli-Q water. Adjust the pH to 8.0 with 6 M HCl, and bring the final volume to 1 L with Milli-Q water. Add BME just prior to use. This buffer will be used to bind His-tagged proteins to Ni-NTA beads.)
3. Resuspension buffer 1: 50 mM Tris, 10 mM imidazole, 300 mM KCl, and 5 mM BME, pH 8.0, with 1:1000 His Tag protease inhibitor cocktail-to-buffer ratio (catalog number P8849; Sigma-Aldrich, St. Louis, MO, USA), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 10 units/mL DNAse1-Grade II. (Mix 200 μL His Tag protease inhibitor cocktail,

200 μL 1 M PMSF in DMSO, and 100 μL of DNAse1-Grade II into 20 mL 1X binding buffer 1. Make this buffer fresh each time.)

4. Wash buffer 1: 50 mM Tris, 50 mM imidazole, 300 mM KCl, 5 mM BME. (Mix 100 mL 10X Tris buffer with 3.40 g imidazole in 800 mL Milli-Q water. Adjust the pH to 8.0 with 6 M HCl, and bring the final volume to 1 L with Milli-Q water. Add BME just prior to use.)
5. Elution buffer 1: 50 mM Tris, 250 mM imidazole, 300 mM KCl, 5 mM BME. (Mix 100 mL 10X Tris buffer with 17.02 g imidazole in 800 mL Milli-Q water. Adjust the pH to 8.0 with 6 M HCl, and bring the final volume to 1 L with Milli-Q water. Add BME just prior to use.)
6. AaT storage buffer: 50 mM Tris, 30% glycerol, 120 mM ammonium sulfate, 5 mM BME. (Dissolve 6.06 g Tris base and 15.86 g ammonium sulfate into 600 mL Milli-Q water. Adjust the pH to 8.0 with 6 M HCl, and bring the volume to 700 mL with Milli-Q water. Bring the buffer to a final volume of 1 L with 300 mL glycerol. Add BME to a 5 mM concentration prior to use. Be sure to add 30% glycerol to the protein elutions that are dialyzed since glycerol will be unable to traverse the membrane. Also, if future reactions with the purified AaT need to exclude a reducing agent, the BME can be omitted without loss of activity.)
7. AzfRS/Met*RS storage buffer: 50 mM Tris, 50% glycerol, pH 7.5. Dissolve 6.06 g Tris base into 400 mL Milli-Q water. (Adjust the pH to 7.5 with 6 M HCl, and bring the volume to 500 mL with Milli-Q water. Bring the buffer to a final volume of 1 L with 500 mL glycerol. Be sure to add 50% glycerol to the protein elutions that are dialyzed since glycerol will be unable to traverse the membrane.)
8. Resuspension buffer 2: see Subheading 2.2.3 but with 5 mM imidazole.
9. Wash buffer 2: see Subheading 2.2.4 but with 15 mM imidazole.
10. Elution buffer 2: see Subheading 2.2.5 but with 50 mM imidazole.
11. ATE1/RRS storage buffer. Prepared as described in the chapter by Wang and Kashina in this volume.
12. 10X transfer buffer: 500 mM HEPES, 1.5 M KCl, 100 mM $MgCl_2$. (Dissolve 119.15 g HEPES, 111.83 g KCl, and 20.33 g $MgCl_2 \cdot 6H_2O$ in 900 mL Milli-Q water. Adjust the pH to 8.0 with 10 M NaOH, and bring the final volume to 1 L with Milli-Q water. Filter-sterilize the solutions using a 0.22 μm filter.)

2.3 Materials for Protein Expression

1. *E. coli* BL21-Gold (DE3) cells (catalog number 230132; Agilent Technologies).
2. SOC media (catalog number 15544034; Life Technologies).
3. LB agar plates (per liter of Milli-Q water: agar 15 g, tryptone 10 g, NaCl 10 g, and yeast extract 5 g) sterilized by autoclaving.
4. LB medium (per liter of Milli-Q water: tryptone 10 g, NaCl 10 g, and yeast extract 5 g) sterilized by autoclaving.
5. 1000X ampicillin stock: 100 mg ampicillin dissolved in 100 mL Milli-Q water, stored at −20 °C.
6. Ni-NTA superflow slurry (catalog number 30410; Qiagen).
7. Branson Sonifier 450 (catalog number 33995–309; VWR).
8. Bradford protein assay (catalog number B6916-500ML; Sigma-Aldrich).
9. Standard molecular biology equipment and supplies: spectrophotometer, orbital incubator, ultracentrifuge with fixed-angle rotor, microcentrifuge, standard dialysis tubing, magnetic stirrer, 20 mL disposable plastic columns, electrophoresis chamber and buffers, SDS-PAGE gels, and standard material for protein detection and staining.

2.4 Materials for Azf-a Synthesis and Chemoenzymatic Transfer Reactions

1. *N*-(*tert*-Butoxycarbonyl)-L-*p*-azidophenylalanine (Boc-Azf) (catalog number A-3570.0001; Bachem).
2. 5′-*O*-(4,4′-Dimethoxytrityl) adenosine (A-DMT) (non-catalog item; ChemGenes, Wilmington, MA, USA).
3. Chloroacetonitrile (catalog number C19651; Sigma-Aldrich).
4. *N,N,N*-Diisopropyl ethylamine (DIPEA) (catalog number 496219; Sigma-Aldrich).
5. *N,N,N,N*-Tetrabutylammonium acetate ($N(Bu)_4Ac$) (catalog number 335991; Sigma-Aldrich). Must be fresh or stored in aliquots in a drying oven as it absorbs water readily.
6. Trifluoroacetic acid (TFA; catalog number 299537-500G; Sigma-Aldrich).
7. HPLC buffers: HPLC buffer A consists of Milli-Q water with 0.1% TFA, and HPLC buffer B consists of acetonitrile (ACN) with 0.1% TFA.
8. Standard organic chemistry equipment and supplies: Round-bottom flasks, magnetic stirrer, separatory funnel, apparatus and materials for running and analyzing flash column chromatography, apparatus for rotary evaporation, Varian ProStar HPLC or equivalent instrument, and a Waters SunFire Prep C18-prep OBD column, 5 μm, 17x150 mm, or equivalent column.

2.5 Materials for Fully Enzymatic Transfer Reactions

1. *E. coli* total tRNA (catalog number R4251–2.5KU; Sigma-Aldrich).
2. Adenosine triphosphate (ATP; catalog number ICN15026605; Fisher).
3. Purified *E. coli* RRS (see chapter by Wang and Kashina in this volume).
4. Purified ATE1 (see chapter by Wang and Kashina in this volume).
5. Purified *E. coli* AaT (see Subheading 3.1).
6. Purified *E. coli* AzfRS (see Subheading 3.2).
7. Purified *E. coli* Met*RS (see Subheading 3.3).

2.6 Materials for HPLC Transfer Assays

1. XaaMcm or XaaAMC peptides. Synthesized using standard solid phase synthesis methods from Fmoc-protected amino acids and Fmoc-7-methoxycoumarinyl alanine (Mcm) (catalog number B-3740.1000; Bachem) or Fmoc-7-aminocoumarin-4-acetic acid (AMC) resin (catalog number 64513–01 or 64,513–05; AnaSpec, Fremont, CA, USA). AaT substrate LysAlaAMC (catalog number I-1260; Bachem) and other pre-made AMC peptides are commercially available.
2. α-Cyanohydroxycinnamic acid (CHCA) solution (catalog number C8982-10X10MG; Sigma-Aldrich). CHCA was dissolved in 50:50 Milli-Q water:ACN with 0.1% TFA. The CHCA solution is used to spot peptides for matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) analysis.
3. HPLC buffers: *see* Subheading 2.3.8.
4. An Agilent 1100 HPLC using a Waters Symmetry Shield C18 column or equivalent HPLC and analytical column.

2.7 Materials for Transfer Reactions in Cell Lysates

1. *See* Subheading 2.5 for materials.
2. L-*p*-Azidophenylalanine (Azf) (catalog number F-3075; Bachem).
3. L-γ-Azidohomoalanine (Aha) (catalog number F-4265; Bachem).
4. L-Homopropargylglycine (Hpg) (catalog number 1067; Click Chemistry Tools).
5. Standard molecular biology equipment and supplies: *see* Subheading 2.3.9.
6. Amicon Ultra 0.5 Centrifugal 10 kDa spin columns (catalog number UFC501096; Fisher).

2.8 Materials for Click Chemistry Labeling of Azide-Derived Proteins

1. Fluorescein 5,6-proparglyamide (Alk-Fl). Alk-Fl used here is not commercially available, but the functionally equivalent compound carboxyrhodamine 110-alkyne (TA106, Click Chemistry Tools) or Fluor 488-alkyne (761,621, Sigma-Aldrich) can be substituted in the protocol provided.
2. Cuprous sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) (catalog number C7631-250G; Sigma-Aldrich).
3. Sodium ascorbate (Ascorbate) (catalog number A7631-25G; Sigma-Aldrich).
4. Tris-(3-hydroxypropyltriazolylmethyl) amine (THPTA; catalog number 762342; Sigma-Aldrich).
5. Dibenzocyclooctyne tetramethylrhodamine (DBCO-TMR). DBCO-TAMRA (catalog number A131, Click Chemistry Tools) or DBCO-PEG4-Fluor 545 (catalog number 760773, Sigma-Aldrich) can be substituted in the protocol provided.
6. Standard molecular biology equipment and supplies: *see* Subheading 2.3.9.
7. Typhoon FLA 7000 laser scanner (catalog number 28–9558-09; GE Healthcare Life Sciences) or equivalent gel imager.

3 Methods

3.1 E. Coli *Leucyl Phenylalanyl Aminoacyl Transferase (AaT) Expression and Purification*

1. Transform the *E. coli* AaT pEG6 plasmid into *E. coli* BL21-Gold (DE3) cells using the heat shock method as follows. Combine 1 μL of plasmid DNA with 25 μL of BL21-DE3 cells in a 1.5 mL microcentrifuge tube, and incubate on ice for at least 30 min. Heat the tube in a water bath at 42 °C for 45 s. Place the cells on ice for 2 min. Add 450 μL SOC media to the cells, and incubate with shaking at a 45° angle for 1 h at 37 °C. Make a 1/10X dilution using 20 μL of the incubated cells and 120 μL SOC media. Plate cells from 1X and 1/10X stocks onto pre-warmed LB + Amp plates, and incubate overnight at 37 °C.
2. Prepare four 10 mL culture tubes with 5 mL each LB + Amp media. Pick four individual colonies from the LB + Amp plates, inoculate the 5 mL cultures, and incubate with shaking (250 rpm, 37 °C), staggering start times by 10 min. Grow for 2–3 h.
3. Check the OD_{600} of the cultures (1 mL culture, blanked against 1 mL Millipore water using disposable cuvettes). Choose the culture with OD_{600} closest to 0.5. This is the primary culture.
4. Inoculate 1 L LB + Amp in a 2 L sterile Erlenmeyer flask (secondary culture) with 1 mL primary culture, and grow

(275 rpm shaking, 37 °C) until $OD_{600} = 0.6$ (~ 3–4 h). Save 1 mL of the secondary culture for a glycerol stock by combining 1 mL secondary culture with 0.5 mL 60% glycerol, flash freezing, and storing at −80 °C. This glycerol stock can be used to inoculate future primary cultures (200 μL) rather than transforming a new batch of bacterial cells.

5. Add 1000 μL 1000X IPTG (1 M stock solution) to a 1 L culture to induce AaT overexpression with a final concentration of IPTG at 1 mM. Incubate for approximately 16 h at 25 °C with shaking at 250 rpm.
6. Centrifuge the secondary culture in 500 mL bottles (250 mL media in each) at 4225xg at 4 °C for 15 min. Pour off the supernatant, invert the bottles and tap them on paper towels to get rid of excess media, and place cell pellet on ice. Resuspend the pellet in 20 mL freshly prepared resuspension buffer 1 on ice by pipetting up and down with a serological pipet. Make sure that the pellet is completely resuspended. Pipet the solution into a 50 mL Falcon tube or similar container, and anchor with ice in a plastic container. Using a Branson Sonifier 450 or equivalent, sonicate the cell resuspension on ice, six times with the cycle: 1 min on, 1 min off. Sonicator settings: timer = hold, duty cycle percent = 30%, output control = 5. Transfer the cell suspension to 1.5 mL microcentrifuge tubes, and spin down at 14227xg, 4 °C, for 15 min. Collect the supernatant in 15 mL Falcon tubes and chill on ice.
7. Add 2 mL Ni-NTA superflow slurry to the collected supernatant, and shake on ice for 1 h. Purify AaT using a Ni-NTA column as follows. Pipet Ni slurry/cell supernatant into 20 mL disposable plastic column. Allow the suspension to flow through column. Wash the column with 20 mL binding buffer 1, being careful not to disturb the top layer. Wash the column with four 10 mL portions of wash buffer 1. Elute AaT with eight 2 mL portions of elution buffer 1. Incubate the elution volumes for 2 min with resin before allowing the solution to flow through.
8. Determine the purity of the elution fractions of AaT by 18% SDS-PAGE gel analysis. Combine these fractions and add glycerol to 30% of the final volume. If the protein concentration is low, according to gel analysis, the protein can be concentrated prior to glycerol addition (*see* **Note 1**). Dialyze pure protein fractions against 1 L transferase storage buffer overnight at 4 °C. The dialyzed AaT can be stored for several months at − 80 °C. Determine the final protein concentrations using a Bradford assay and bovine serum albumin (BSA) standard curve according to manufacturer instructions. The molecular weight of His-tagged AaT is 29 kDa.

3.2 Azidophenylalanine Synthetase (AzfRS) Expression and Purification

1. Express His_6-tagged AzfRS from the pAzfRS plasmid, a pET15b plasmid (catalog number 69661; EMD Millipore) with a modified *M. jannaschii* TyrRS inserted between the NdeI and XhoI cut sites, in *E. coli* BL21-Gold (DE3) cells. Perform AzfRS expression and purification, including plasmid transformation, cell growth and pelleting, lysis, and Ni-NTA purification using materials and procedures identical to those described for AaT in Subheading 3.1.
2. Once pure elution fractions of AzfRS, as determined by 18% SDS-PAGE gel analysis, are obtained, add glycerol to 50% of the final volume. Dialyze this stock against 1 L synthetase storage buffer overnight at 4 °C. The dialyzed AzfRS can be stored for several months at −80 °C. Determine protein concentrations using Bradford assay and BSA standard curve. The molecular weight of His-tagged AzfRS is 48.7 kDa.

3.3 Mutant Methionyl Synthetase (Met*RS) Expression and Purification

1. Express His_6-tagged *E. coli* Met*RS (an $L_{13}G$ mutant of wild-type *E. coli* MetRS) from the Met*RS plasmid, a variant of the pAJL-20 plasmid described in Link et al., in *E. coli* BL21-Gold (DE3) cells [21]. Perform Met*RS expression and purification, including plasmid transformation, cell growth and pelleting, lysis, and Ni-NTA purification using materials and procedures identical to those described for AaT in Subheading 3.1.
2. Once pure elution fractions of Met*RS, as determined by 18% SDS-PAGE gel analysis, are obtained, add glycerol to 50% of the final volume. Dialyze this stock against 1 L synthetase storage buffer overnight at 4 °C. The dialyzed Met*RS can be stored for several months at −80 °C. Determine protein concentrations using a Bradford assay and BSA standard curve. The molecular weight of His-tagged Met*RS is 13.7 kDa.

3.4 Aminoacyl Adenosine Donor Synthesis

An aminoacyl adenosine donor (Zaa-A) can be synthesized in three steps from the appropriate Boc-protected amino acid (Fig. 3). The synthesis of Azf-A starting from Boc-Azf (*see* **Note 2**) is described as a general procedure. A similar series of reactions can be used to synthesize Aha-A or Hpg-A starting from Boc-Aha or Boc-Hpg, respectively. Procedures are briefly summarized in a manner that should be accessible to one familiar with standard organic chemistry practices. Characterization of all compounds by mass spectrometry, ^{1}H NMR, and ^{13}C NMR has been reported previously [7].

1. Activation of the carboxylic acid to form *N*-Boc-Azf-cyanomethylester: add chloroacetonitrile, acting as solvent and reagent (5 mL), and diisopropylethylamine (DIPEA, 310 mg, 0.411 mL, 2.41 mmol) to Boc-pAzF (212 mg, 0.691 mmol) to a 10 mL round-bottom flask with a magnetic stir bar, and stir

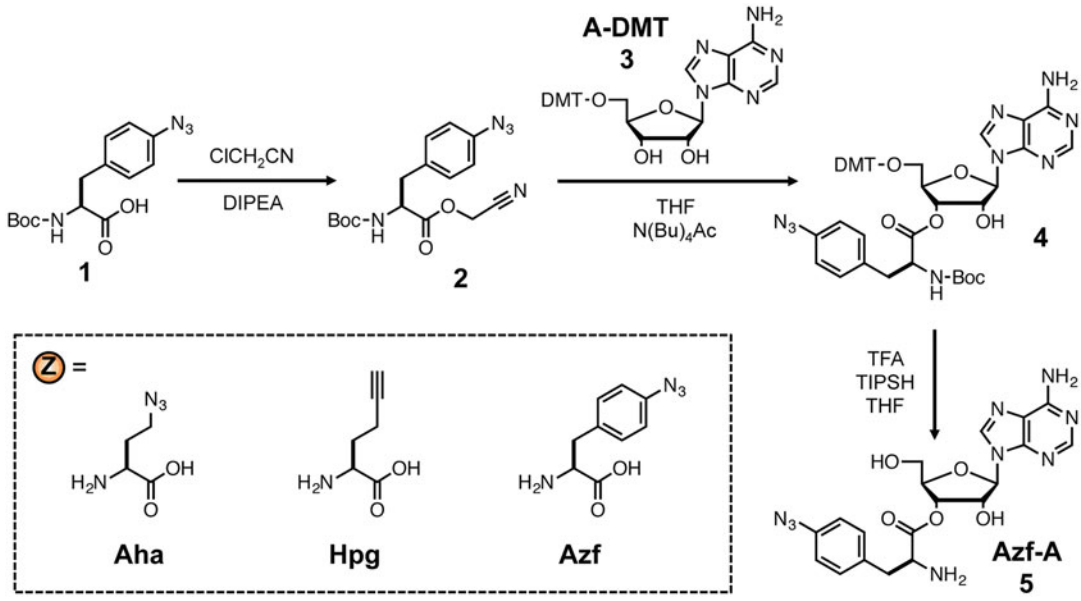

Fig. 3 Synthesis of aminoacyl adenosine donors. All reagents are commercially available as shown and in the corresponding Boc-protected forms of the amino acids

overnight at room temperature on a magnetic stir plate. Remove the solvent under reduced pressure via rotary evaporation, and purify *N*-Boc-Azf-cyanomethylester by SiO_2 flash chromatography (40–50% ethyl acetate in hexanes gradient). The R_f of *N*-Boc-Azf-cyanomethylester on thin layer chromatography (TLC) should be 0.5 in 50% ethyl acetate in hexanes.

2. Aminoacylation of protected adenosine to form *N*-Boc-Azf-adenosine-DMT-5′-hydroxyl. Add tetrahydrofuran (5 mL) to *N*-Boc-Azf-cyanomethylester (101 mg, 0.290 mmol), 5′--hydroxyl-dimethoxytrityl-adeonsine (A-DMT, 43 mg, 75 μM), and catalytic tetrabutylammonium acetate (N$(Bu)_4$Ac, 2 mg) in a 10 mL round-bottom flask with a magnetic stir bar. Purge air from the flask using Ar or N_2 gas, and seal the flask with a rubber septum. Stir the reaction overnight at room temperature on a magnetic stir plate. Remove the solvent under reduced pressure via rotary evaporation, and purify the product, *N*-Boc-Azf-adenosine-DMT-5′-hydroxyl, by preparative TLC, loaded in 100% ethyl acetate and eluted with 10% methanol in chloroform. The reaction typically produces ~45 mg of white solid product with ~70% yield. The R_f of *N*-Boc-Azf-adenosine-DMT-5′-hydroxyl should be 0.3–0.5 in ethyl acetate. On TLC, the product should appear as a double spot, corresponding to the 2′ and 3′ acylation sites.
3. To remove the protecting groups from Azf-A (*N*-Boc-Azf-adenosine-DMT-5′-hydroxyl), dissolve it in 1 mL TFA and

Table 1
Gradients used for HPLC purification of small molecules and peptides

Gradient # 1	Time (min)	Buffer A (%)	Gradient # 2	Time (min)	Buffer A (%)
15 mL/min	0:00	99	1 mL/min	0:00	99
	10:00	99		5:00	99
	40:00	65		10:00	70
	45:00	0		15:00	60
	50:00	0		20:00	0
	55:00	99		25:00	0
	60:00	99		27:00	99
				30:00	99

1 mL THF with four equivalents of a protecting group scavenger, triisopropylsilane (TIPSH), in a 5 mL round-bottom flask with a magnetic stir bar, and stir overnight at room temperature. At reaction completion, remove the solvent under reduced pressure via rotary evaporation (*see* **Note 3**). Extract the water-soluble product using a pipette as follows. Dissolve the residue in 1 mL each CH_2Cl_2 and water, draw the solution into a disposable glass pipette, and allow it to stand to separate the water and CH_2Cl_2 layers. Retain both the CH_2Cl_2 layer (bottom) and the water layer (top) in separate glass vials. Add 1 mL water to the retaining CH_2Cl_2 layer. Discard the CH_2Cl_2 layer (bottom), and retain the water layer (top). Re-extract the combined DCM layers with 1 mL of water. Combine the aqueous layers and dilute twofold with water. The product can be further purified via C18 reversed phase HPLC (Table 1, gradient 1). Both 2′ and 3′ acylated products should be collected (*see* **Note 4**).

3.5 Chemoenzymatic Peptide Transfer Assay

Peptide substrates for both the chemoenzymatic and fully enzymatic assays can take on a variety of forms, given the broad substrate tolerances of AaT and ATE1. Typically, we have used Mcm and AMC C-terminal chromophores (Fig. 4) since these can be monitored at 325 nm in the HPLC chromatogram where there is little interference from protein or RNA absorbance. Peptides of both types of sequences (e.g., ArgGlyMcm or GluAlaAMC) can be synthesized using standard Fmoc-based peptide synthesis techniques.

1. For a 125 μL scale reaction, mix the following components (final concentrations given, as stock concentrations may vary): aminoacyl adenosine donor Zaa-A (1 mM), purified AaT

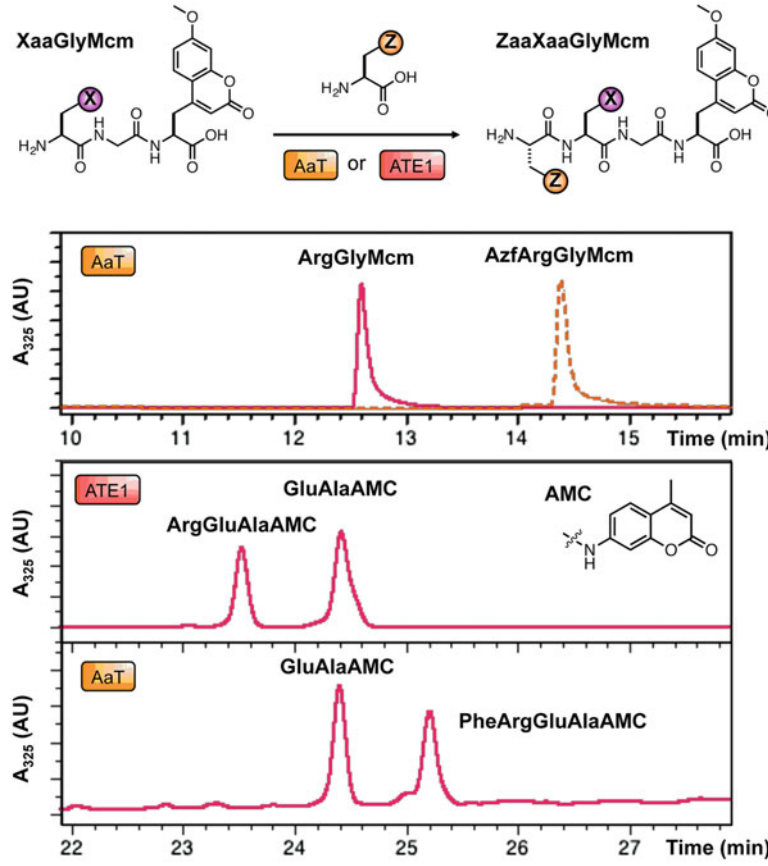

Fig. 4 Peptide transfer assay. Top: Peptides with varying N-terminal amino acids (Xaa) and an AMC or Mcm chromophore are used to analyze transferase substrate specificity. Amino acid Zaa can come from synthetic Zaa-A donors or AaRS-mediated tRNA activation. Middle: Overlay of HPLC chromatograms for a ArgGlyMcm peptide before and after transfer of Azf for 4 h using Azf-A donor and AaT. Bottom: HPLC chromatograms for GluAlaAMC peptide after transfer of Arg for 1 h using Arg, ATP, tRNA, ArgRS, and ATE1 and then transfer of Phe for 4 h using Phe-A donor and AaT

(2.26 μM), reporter peptide such as Arg-Gly-Mcm or Lys-Ala-Acm (100 μM), 12.5 μL 10X transfer buffer, and Milli-Q water to a final volume of 125 μL.

2. Incubate the reaction mixtures at 37 °C for 4 h (*see* **Note 5**). Quench the reaction with 375 μL 1% acetic acid. Remove the enzymes from the reactions by acetone precipitation. Dilute the reactions with four volumes of acetone (2 mL), and cool them at −20 °C for 1 h. Pellet the reactions at 14227xg at 4 °C for 20 min to separate precipitated protein. Transfer the supernatant to fresh 1.5 mL microcentrifuge tubes, and let them stand overnight at room temperature in a fume hood. After acetone evaporation, briefly dry the supernatant in a vacuum

centrifuge for 30 min to remove residual acetone. Dissolve the resulting solution in a total volume of 1.2 mL Milli-Q water, and analyze the reaction by injection on an HPLC (Table 1, gradient 2) to determine the transfer yield by integration of the absorbance intensities in the chromatogram monitored at 325 nm.

3. For product confirmation, HPLC fractions should be collected and analyzed by MS (*see* **Note 6**). For example, we performed MALDI MS by spotting peptide fractions in 1:1 CHCA at approximately 0.1 μM concentration. After drying on the benchtop at room temperature, MS data were obtained on a Bruker Ultraflex III TOF-TOF MALDI MS instrument using Bruker Peptide Standard I mix for mass calibration.

3.6 Fully Enzymatic Peptide Transfer Assay

1. For a 125 μL scale reaction, mix the following components (final concentrations given, as stock concentrations may vary): amino acid (Azf, Aha, or Hpg; 1 mM), ATP (2.5 mM), total *E. coli* tRNA (2 mg/mL), a synthetase (AzfRS, Met*RS, or ArgRS; 0.1 mg/mL), a transferase (AaT, 2.26 μM; or ATE1, 2.40 μM), and a reporter peptide (100 μM), 12.5 μL 10X transfer buffer, and Milli-Q water to a final volume of 125 μL. Like the chemoenzymatic method (Subheading 3.7), the reaction can be scaled up if desired.
2. Incubate the reaction mixtures at 37 °C for 30 min, and quench by adding 375 μL 1% acetic acid (*see* **Note 5**).
3. Perform acetone precipitation and subsequent analysis as described in Subheading 3.7.2.
4. If subsequent modifications are desired, either isolate the peptide products of a fully enzymatic reaction with Arg and ATE1 by collecting HPLC fractions, or take the crude mixture forward. After drying in a vacuum centrifuge, these peptides can be subjected to either fully enzymatic or chemoenzymatic AaT modification. For example, Fig. 4 shows a sequence in which Glu-Ala-AMC was partially converted to Arg-Glu-Ala-AMC using ATE1, and the mixture was treated with AaT, converting it to Phe-Arg-Glu-Ala-AMC in a second reaction.

3.7 Chemoenzymatic AaT Transfer to Proteins in Cell Lysates

1. For a reaction at the 110 μL scale, mix the following components (dry masses or final concentrations given): Azf-A (3 mM), AaT (2.6 μM), purified α-casein (0.012 mg or 0.12 mg), *E. coli* cell lysate (25 μL; *see* **Note 9** for lysate preparation procedure), 10X transferase buffer (4.2 μL), and Milli-Q water to 110 μL. For a reaction using an overexpressed protein in cell lysate, simply replace the α-casein and clarified *E. coli* lysate with the lysate containing the overexpressed protein (*see* **Note 7**). After mixing the other components, add

α-casein (or another target protein/lysate), and incubate the reaction at 37 °C for 2 h. After 1 h, add an additional equivalent (0.3 μmol) of Zaa-A. If proteins require partial denaturation to expose the N-terminus, denaturants or detergents may be included in the buffer (*see* **Note 8**).

2. For negative control reactions, replace a given reagent solution with an equivalent amount of Milli-Q water. For example, in a lysate-only control (used to identify non-specific Fl-Alk binding to background proteins in the cell lysate), Azf, AaT, and α-casein should be replaced with an equivalent amount of Milli-Q water. In a mock transferase reaction control (used identify off-target transfer to proteins other than the protein of interest), α-casein should be replaced with Milli-Q water.
3. Remove excess Azf-A by exchanging the reaction buffer four times with PBS using Amicon Ultra 0.5 Centrifugal 10 kDa spin columns and adjusting the final volume to 65 μL with PBS in Spectra/Por 1 dialysis tubing.

3.8 Fully Enzymatic AaT Transfer to Proteins in Cell Lysates

1. For a reaction at the 41.7 μL scale, mix the following components (dry masses or final concentrations are given, as stock concentrations may vary): Azf (1 mM), *E. coli* total tRNA (83.3 μg), ATP (2.5 mM), purified AzfRS (11.7 μg), AaT (5.8 μg), purified α-casein (0.002 or 0.02 mg), clarified *E. coli* lysate (3.33 μL), 10X transferase buffer (4.2 μL), and Milli-Q water to 41.7 μL. For a reaction using an overexpressed protein in cell lysate, simply replace the α-casein and clarified *E. coli* lysate with the lysate containing the overexpressed protein (*see* **Note 7**).
2. For negative control reactions, replace a given reagent solution with an equivalent amount of Milli-Q water (see Subheading 3.9.3).
3. Incubate the reaction mixtures at 37 °C for 2 h. Remove the amino acid by exchanging the reaction buffer four times with PBS using an Amicon Ultra 0.5 Centrifugal 10 kDa spin column. Adjust the final volume to 65 μL with PBS.

3.9 Copper-Catalyzed Click Labeling

1. To 41.7 μL of an Azf-modification reaction, add 1.6 μL of a 10 mM stock of Fl-Alk in PBS.
2. Form the Cu^{I}-THPTA catalyst by mixing 1.5 μL 80 mM $CuSO_4$, 12.5 μL 50 mM THPTA, and 9.5 μL 100 mM sodium ascorbate and incubating for 5 min at room temperature. The $CuSO_4$ and THPTA must be mixed together prior to the addition of sodium ascorbate. The catalyst mixture can be prepared on larger scales if multiple transfer reactions are to be subjected to click labeling.

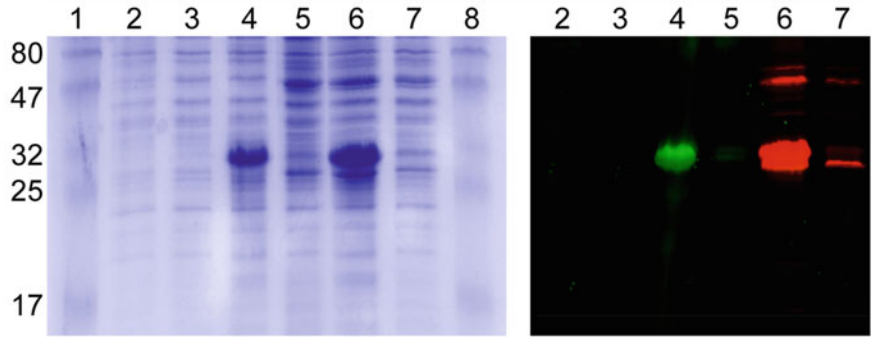

Fig. 5 Protein labeling by click reactions. Coomassie gel (left) lanes: (1) molecular weight (MW) markers (masses in kDa); (2) clarified *E. coli* lysate mixed with fluorescein alkyne (Alk-Fl); (3) clarified *E. coli* lysate reacted in a mock fully enzymatic AaT transfer reaction and copper-catalyzed Alk-Fl reaction with no α-casein present; (4) 0.1 mg α-casein labeled by transfer of Azf in a fully enzymatic AaT transfer reaction and copper-catalyzed Alk-Fl reaction; (5) 0.01 mg α-casein labeled by transfer of Azf in a fully enzymatic AaT transfer reaction and copper-catalyzed Alk-Fl reaction; (6) 0.1 mg α-casein labeled by transfer of Azf in a fully enzymatic AaT transfer reaction and strain-promoted DBCO-TMR reaction; (7) 0.01 mg α-casein labeled by transfer of Azf in a fully enzymatic AaT transfer reaction and strain-promoted DBCO-TMR reaction; (8) MW markers

3. Initiate the click reaction by adding 6.7 μL of the pre-mixed Cu^{I}-THPTA catalyst to the lysates and incubating at 37 °C for 1 h. Stop the reaction and remove excess Alk-Fl by exchanging the reaction buffer four times with PBS using Amicon Ultra 0.5 Centrifugal 10 kDa spin columns and adjusting the final volume to 65 μL with PBS.
4. Analyze the reaction by removing a 15 μL aliquot, combining it with 5 μL 4X LDS gel loading solution, heating this mixture to 95 °C for 1 min, and separating it on a 18% SDS-PAGE gel. Image the gel using a Typhoon FLA 7000 (GE Healthcare Life Sciences) or equivalent instrument. In Fig. 5, fluorescein fluorescence was detected using 473 nm excitation and a Y520 filter. Images were collected using a 100 μM pixel size. Total protein content was visualized on the same instrument using Coomassie Brilliant Blue staining (must be stained after fluorescent images are acquired).

3.10 Strain-Promoted Click Labeling

1. To 41.7 μL of an Azf-modification reaction, add 6.7 μL PBS and 1.6 μL of a 10 mM stock of DBCO-TMR in DMSO. Incubate the reaction at 37 °C for 1 h. Stop the reaction, and remove excess DBCO-TMR by exchanging the reaction buffer four times with PBS using Amicon Ultra 0.5 Centrifugal 10 KDa spin columns and adjusting the final volume to 65 μL with PBS.
2. Analyze the reaction by removing a 15 μL aliquot, combining it with 5 μL 4X LDS gel loading solution, heating this mixture to

95 °C for 1 min, and separating it on a 18% SDS-PAGE gel. Image the gel using a Typhoon FLA 7000 (GE Healthcare Life Sciences) or equivalent instrument. In Fig. 5, TMR fluorescence was detected using 532 nm excitation and an O580 filter. Images were collected using a 100 μM pixel size. Total protein content was visualized on the same instrument using Coomassie Brilliant Blue staining (must be stained after fluorescence images are acquired).

4 Notes

1. Prior to dialysis, protein concentrations can be increased by centrifugation in Amicon Ultra 0.5 Centrifugal 10 kDa spin columns.
2. Azf can be photo-excited to expel N_2 to form a nitrene, which can undergo subsequent ring expansion and radical reactions. These photochemical reactions are not prevalent under typical fluorescent room lighting, but if one suspects that such side reactions have occurred, they can be limited by dimming room lights or using Aha or Hpg labeling instead.
3. Extra precautions should be taken when evaporating an acid like TFA. A dry ice acetone trap should be used to condense the TFA vapors, and an oil-free Teflon pump should be used, if possible.
4. It has been determined that AaT preferentially uses the 3′ aminoacylated adenosine [22, 23]. However, in aqueous solution, the 2′ and 3′ aminoacylated adenosines interconvert, so additional purification to separate the isomers is unnecessary.
5. We have determined that the AaT ligation reaction efficiency can be increased using multiple doses of Zaa-A donor. Since the analog is susceptible to significant hydrolysis within 1 h, the additional doses of analog allow for more non-hydrolyzed substrate to be present for ligation.
6. In some instances, we have observed transfer of a second equivalent of amino acid to the protein's N-terminus, particularly with fully enzymatic transfer using AaT. This can be distinguished from single transfer by HPLC analysis for small peptides and by mass spectrometry analysis for full-sized proteins. If multiple transfers are suspected, limiting transfer reaction times can limit modification to a single transfer event. Multiple transfers can also be exploited to add multiple labels by click chemistry to increase the brightness of fluorescent labeling.
7. For the above protein-labeling protocols, bovine α-casein – a protein with the N-terminal sequence Arg-Pro-Lys that is

commercially available in large quantities – was used as a model protein. A diluted clarified *E. coli* cell lysate, described below, was used as the reaction medium (*see* **Note 9**). Azf was used as an exemplary amino acid, but the protocols are equally applicable to other unnatural amino acids prepared as Zaa-A donors for chemoenzymatic transfer or paired with an appropriate synthetase (Aha or Hpg with Met*RS) for fully enzymatic transfer.

8. Some proteins may be folded so that the N-terminal residues are not accessible for transferase modification. AaT-mediated transfer can be performed in the presence of up to 8 M urea (solution buffered to pH 7.5–8.0) with an IC_{50} of 5.0 M for transfer of Phe from Phe-A. Transfer from Phe-A can also be performed with up to 2 M guanidinium (solution buffered to pH 7.5–8.0) with an IC_{50} of 0.5 M. Fully enzymatic transfer using Met*RS is also possible in the presence of 1.0 M urea or 0.5 M guanidinium. No inhibition of AaT has been seen with the addition of up to 10% of Triton X-100. The impact of denaturants on ATE1-mediated transfer has not been explored, but since the primary interest is the discovery of physiologically relevant ATE1 substrates, this should be unnecessary.
9. For the above protocols, clarified *E. coli* lysate should be prepared as described in **steps 3.1.2** to **3.1.6**. To confer ampicillin resistance to *E. coli* BL21 cells if needed, cells prior to lysate preparation should be transfected with UC19 plasmid (using 1 μL pUC19 plasmid DNA introduced into *E. coli* BL21 cells using the heat shock method as described in Subheading 3.1.1). The chilled lysate 6 can be used in Subheadings 3.9 and 3.10.

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Chapter 22

Synthesis of Peptides and Proteins with Site-Specific Glutamate Arginylation

Marie Shimogawa, Yun Huang, Buyan Pan, and E. James Petersson

Abstract

Solid-phase peptide synthesis and protein semi-synthesis are powerful methods for site-specific modification of peptides and proteins. We describe protocols using these techniques for the syntheses of peptides and proteins bearing glutamate arginylation (E^{Arg}) at specific sites. These methods overcome challenges posed by enzymatic arginylation methods and allow for a comprehensive study of the effects of E^{Arg} on protein folding and interactions. Potential applications include biophysical analyses, cell-based microscopic studies, and profiling of E^{Arg} levels and interactomes in human tissue samples.

Key words Posttranslational modification, Glutamate arginylation, Protein semi-synthesis, Solid-phase peptide synthesis, Native chemical ligation

1 Introduction

Protein arginylation is an understudied posttranslational modification (PTM), catalyzed by arginyl-tRNA transferase enzyme 1 (ATE1), which transfers Arg from an aminoacyl tRNA to the protein N-terminus or to the side-chain carboxylates of Asp or Glu [1]. To study the effects of this PTM, the Kashina group has developed enzymatic arginylation methods using purified ATE1 and arginylated tRNA or tRNA fragments [2–6]. However, the in vitro enzymatic modification protocols pose major challenges in terms of yield and purification. ATE1 does not appear to have tight control over site-specificity of the modification, and the level of the modification can be low especially for protein substrates [3] – typically there is a mixture of differentially arginylated substrates. This makes it very hard to isolate a desired modification product, as these substrates often coelute on column chromatography. Preparing proteins with homogenous N-terminal arginylation can be achieved relatively simply by recombinant expression of the target protein with a cleavable N-terminal tag to eventually expose an

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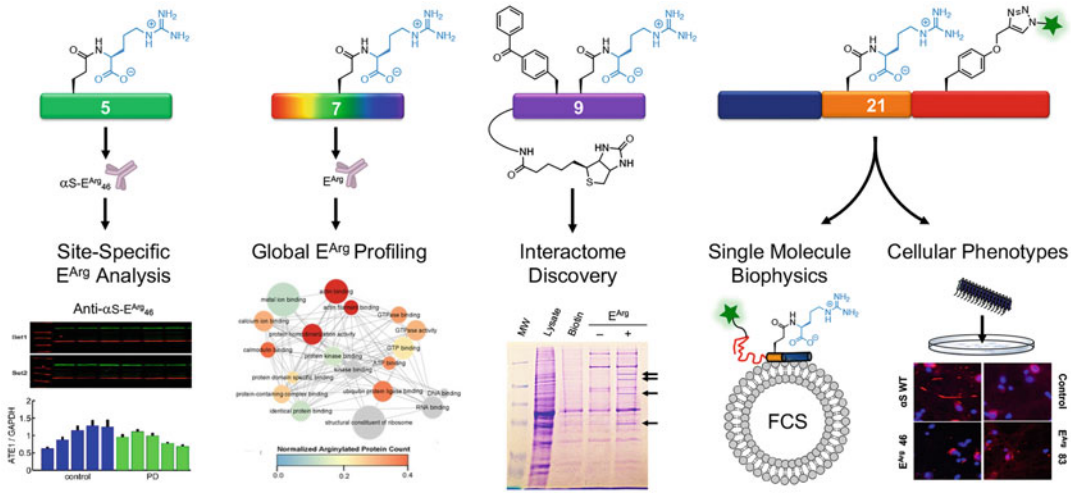

Fig. 1 Applications of synthetic E^{Arg} peptides and proteins

inserted, N-terminal arginine. Methods for studying N-terminal arginylation are discussed in, e.g., Chapters 8, 9, 10, 16, and 20 of this book. To generate proteins with side-chain arginylation homogenously incorporated, we developed a site-specific incorporation approach and performed semi-syntheses of alpha-synuclein (αS) with glutamate arginylation (E^{Arg}) at site 46, 83, or both, combined with an orthogonally installed fluorophore, which have been used to study the effects of the modification both in vitro [7] and in living cells [8, 9]. Additionally, we synthesized E^{Arg} peptides for generation of anti-arginylation antibodies, including those for specific E^{Arg} sites in αS [8] and pan-E^{Arg} antibodies (in progress) to assess global changes in arginylation.

Here, we describe protocols for synthesizing peptides and proteins bearing E^{Arg} at specific sites (Fig. 1). These include the syntheses of the E^{Arg} monomer unit and peptides, followed by protein semi-synthesis. The initial step is the synthesis of the E^{Arg} monomer unit, which contains protecting groups needed for Fmoc-based solid-phase peptide synthesis (SPPS). We then describe the syntheses of four different types of peptides: antigen peptides for generating antibodies specific to a protein arginylation site (**5**); antigen peptide libraries for generating a pan- E^{Arg} antibody (**7**), which recognizes arginylation regardless of the surrounding sequence; photo-cross-linkable peptides for E^{Arg}-dependent interactome studies (**9**); and peptide fragments to incorporate the PTM for use in protein semi-synthesis (**21**).

Semi-synthesis also involves cloning and the recombinant expression and purification of protein fragments for assembly through native chemical ligation (NCL) [10]. We included expression of a protein fragment bearing a "click" chemistry handle through unnatural amino acid mutagenesis [11] for site-specific

fluorescence labeling, which can be combined with NCL for efficient multiple labeling of proteins, as we have shown repeatedly for αS [7, 12–15]. As the last step of semi-synthesis, NCL assembles peptide/protein fragments into a full-length protein, followed by in situ desulfurization, converting a thiol-containing amino acid to a native amino acid (i.e., cysteine to alanine, penicillamine to valine). Such a protein could be used in fluorescence correlation spectroscopy (FCS) to monitor the interaction of the protein with another protein or macromolecule [7, 15]. More complex adaptations of these methods can be used to introduce a second fluorophore for Förster resonance energy transfer (FRET) experiments to probe conformational change, which our laboratory has done with other PTMs, like tyrosine phosphorylation [15]. The types of experiments enabled by this fluorescent labeling have been reviewed elsewhere, in the context of amyloid forming proteins like αS [16], as well as for protein folding more generally [17].

Taken together, these methods allow for a comprehensive study of the effects of this PTM on protein folding and interactions, from detailed biophysical studies to cell-based microscopy studies and even profiling of E^{Arg} levels and interactions in human tissue samples. While the examples are all provided in the context of αS, the methods are general and can be applied to any protein of interest that is amenable to semi-synthesis.

2 Materials

Use Milli-Q filtered (18 MΩ) water (MilliporeSigma; Burlington, MA, USA) to make all the buffers.

2.1 Arginylated Glutamate Monomer Synthesis

1. *N*-[(9*H*-Fluoren-9-ylmethoxy)carbonyl]-L-glutamic acid 1-allyl ester (Fmoc-Glu-OAll) (VWR; Radnor, PA, USA).
2. *tert*-Butyl *N*-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)-L-argininatehydrochloride (H-Arg(Pbf)-OtBu•HCl) (VWR).
3. Isobutyl chloroformate (IBCF) (Sigma-Aldrich; St. Louis, MO, USA).
4. *N*-Methylmorpholine (NMM) (Alfa Aesar; Tewksbury, MA, USA).
5. Phenylsilane (Sigma-Aldrich).
6. Tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) (Sigma-Aldrich).
7. Standard organic chemistry equipment and supplies: round-bottom flasks, magnetic stirrer, ice bath, apparatus and materials for extraction, drying agent, apparatus for argon atmosphere, Büchner funnel, apparatus, materials for running and analyzing flash column chromatography, and apparatus for rotary evaporation.

2.2 Peptide Synthesis (See Note 1)

1. 2-Chlorotrityl chloride resin (Sigma-Aldrich).
2. Fmoc-hydrazide (Chem-Impex; Wood Dale, IL, USA).
3. Methanol capping reagent: 5% (v/v) methanol in dimethylformamide (DMF).
4. Acetyl capping reagent: 5% (v/v) acetic anhydride in DMF.
5. Fmoc deprotection reagent: 20% (v/v) piperidine in DMF.
6. 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (EMD Millipore; Burlington, MA, USA).
7. Diisopropylcarbodiimide (DIC)(Sigma-Aldrich).
8. 7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP) (Chem-Impex).
9. *N*-hydroxybenzotriazole (HOBt) (TCI America; Portland, OR, USA).
10. *N,N*-Diisopropylethylamine (DIPEA) (EMD Millipore).
11. Fmoc-amino acids.
12. Fmoc-Glu(Arg(Pdf)-O*t*Bu)-OH (see Subheading 3.1.1).
13. 4-Pentynoic acid (Sigma-Aldrich).
14. Diazo-biotin-azide (Click chemistry tools; Scottsdale, AZ, USA).
15. Kaiser test reagents: reagent A, 80% (w/v) phenol in ethanol; reagent B, 5% (w/v) ninhydrin in ethanol; and reagent C, 2% (v/v) potassium cyanide in a 1 mM aqueous solution of pyridine.
16. Peptide synthesis apparatus: stir bars, fritted plastic syringes, stoppers, glass vials, tubing, vacuum pump, solvents, mass spectrometry instruments, and reagents for peptide cleavage.
17. HPLC buffers: HPLC buffer A consists of Milli-Q water with 0.1% TFA, and HPLC buffer B consists of acetonitrile (ACN) with 0.1% TFA.
18. C18 HPLC columns.
19. Lyophilizer.

2.3 Plasmids and Cloning

1. pTXB1-αS-intein-H_6 plasmid: containing α-synuclein (αS) with a C-terminal fusion to the *Mycobacterium xenopi* GyrA intein and C-terminal His_6 tag. Preparation of this plasmid was described previously [18]. Other inteins can be used; *see* **Note 2**.
2. pTXB1-αS-TAG_{114}-intein-H_6 plasmid: identical as the above plasmid, except the mutation at site 114. Preparation of this plasmid was described previously [19].
3. DNA oligomers for site-directed mutagenesis/deletions.

Primer sequences:

αS_{1-76}	Forward	5′-TGCATCACGGGAGATGCA-3’
	Reverse	5′- TGCTGTCACACCCGTCA-3’
αS_{91-140}-C_{91}	Forward	5′-TGCACTGGCTTTGTCAAAAAG-3’
	Reverse	5′-CATATGTATATCTCCTTCTTAAAGTTAAAC-3’

4. T100 thermocycler or its equivalent.
5. Q5 High-Fidelity 2X Master Mix (New England Biolabs; Ipswich, MA, USA).
6. DNAse/RNAse-free water for molecular biology.
7. DNA cleanup kit: DNA Clean & Concentrator – 5 (Zymo Research; Irvine, CA, USA) or its equivalent.
8. NanoQuant Plate (TECAN; Zürich, Switzerland) and SPARK (TECAN) plate reader or its equivalent for DNA quantification.
9. T4 DNA Ligase (New England Biolabs).
10. T4 Polynucleotide Kinase (PNK) (New England Biolabs).
11. 10x T4 DNA Ligase Buffer (New England Biolabs).
12. DpnI (New England Biolabs).
13. *E. coli* Dh5α competent cells (New England Biolabs).
14. SOC media (Life Technologies; Carlsbad, CA, USA).
15. LB agar plates (Agar 15 g, tryptone 10 g, NaCl 10 g, and yeast extract 5 g per liter of Milli-Q water) sterilized by autoclaving and supplemented with appropriate antibiotics once cooled down enough.
16. LB medium (tryptone 10 g, NaCl 10 g, and yeast extract 5 g per liter of Milli-Q water:) sterilized by autoclaving.
17. 1000x ampicillin stock: 100 mg ampicillin dissolved in 1 mL Milli-Q water, stored at −20 °C.
18. DNA miniprep kit: QIAprep Spin Miniprep Kit (50) (Qiagen; Germantown, MD, USA) or its equivalent.
19. Standard molecular biology equipment and supplies: centrifuges, dry bath, water bath.

2.4 Buffers for Protein Expression

1. Resuspension buffer: 40 mM Tris, pH 8.3. Dissolve 4.846 g Tris base in 900 mL Milli-Q water. Adjust the pH to 8.3 with 6 M HCl, and bring the final volume to 1 L with Milli-Q water. Filter-sterilize the solutions using a 0.22 μm filter. Each time dissolve 1 protease inhibitor tablet (Roche; Basel, Switzerland) into 20 mL of this just prior to use.

2. Equilibration/wash buffer 1: 50 mM HEPES, pH 7.5. Dissolve 11.92 g HEPES in 900 mL Milli-Q water. Adjust the pH to 7.5 with 5 M NaOH, and bring the final volume to 1 L with Milli-Q water. Filter-sterilize the solutions using a 0.22 μm filter.
3. Wash buffer 2: 50 mM HEPES, 5 mM imidazole, pH 7.5. Dissolve 11.92 g HEPES and 340.4 mg imidazole in 900 mL Milli-Q water. Adjust the pH to 7.5 with 5 M NaOH/6 M HCl, and bring the final volume to 1 L with Milli-Q water. Filter-sterilize the solutions using a 0.22 μm filter.
4. Elution buffer: 50 mM HEPES, 300 mM imidazole, pH 7.5. Dissolve 11.92 g HEPES and 20.42 g imidazole in 900 mL Milli-Q water. Adjust the pH to 7.5 with 6 M HCl, and bring the final volume to 1 L with Milli-Q water. Filter-sterilize the solutions using a 0.22 μm filter.
5. Thiazolidine deprotection buffer: 2 M guanidium, 200 mM phosphate, 30 mM TCEP, 100 mM methoxyamine, pH 4. Prepare this buffer freshly. Dissolve 1.91 g guanidine hydrochloride, 240 mg sodium phosphate monobasic, and 86.00 mg TCEP in 5 mL Milli-Q water. Adjust the pH to 4.0 with 5 M NaOH/6 M HCl, and bring the final volume to 10 mL with Milli-Q water.
6. Thiazolidine deprotection dilution buffer: 200 mM phosphate, 30 mM TCEP, 100 mM methoxyamine, pH 4. Prepare this buffer freshly. Dissolve 240 mg sodium phosphate monobasic and 86.00 mg TCEP in 5 mL Milli-Q water. Adjust the pH to 4.0 with 5 M NaOH/6 M HCl, and bring the final volume to 10 mL with Milli-Q water.

2.5 Protein Expression and Labeling

1. *E. coli* BL21-DE3 competent cells (New England Biolabs).
2. pDule-pCNF plasmid (Addgene #85494): encoding for a modified *Methanocaldococcus jannaschii* tyrosyl synthetase capable of charging a suppressor $tRNA_{CUA}$ with propargyltyrosine (PpY or π).
3. SOC media (Life Technologies).
4. LB agar plates. Per 1 liter of Milli-Q water, add 15 g Agar, 10 g tryptone, 10 g NaCl, and 5 g yeast extract. Sterilize by autoclaving, and supplement with appropriate antibiotics once cooled down to 37 °C or below.
5. LB medium. Per 1 liter of Milli-Q water, add 10 g tryptone, 10 g NaCl, and 5 g yeast extract. Sterilize by autoclaving.
6. 10X M9 salts. Per liter of Milli-Q water, add 60 g sodium phosphate dibasic, 30 g potassium phosphate monobasic, 5 g sodium chloride, and 10 g ammonium chloride.

7. Magnesium (II) sulfate stock: 1 M magnesium (II) sulfate. Dissolve 29.98 g solid magnesium sulfate into 250 mL Milli-Q water. Sterile filter through 0.22 μm bottle-top filter.
8. Iron (II) chloride stock: 15 mg/mL Iron (II) chloride. Dissolve 3.75 g solid iron (II) chloride into 250 mL 1 M HCl. Sterile filter through 0.22 μm bottle-top filter.
9. Zinc (II) chloride stock: 15 mg/mL zinc (II) chloride. Dissolve 3.75 g solid zinc (II) chloride into 250 mL Milli-Q water. Sterile filter through 0.22 μm bottle-top filter.
10. Calcium chloride stock: 0.01 M calcium chloride. Dissolve 0.275 g solid calcium chloride into 250 mL Milli-Q water. Sterile filter through 0.22 μm bottle-top filter.
11. Yeast extract stock: 10% w/v yeast extract. Dissolve 25 g yeast extract into 250 mL Milli-Q water. Autoclave on liquid cycle to sterilize. Store at 4 °C.
12. Glucose stock: 40% w/v glucose. Dissolve 100 g glucose (dextrose) into 250 mL Milli-Q water with stirring at room temperature. Sterile filter through 0.22 μm bottle-top filter.
13. M9 minimal media per 500 mL media: Make 1X M9 salts by adding 450 mL Milli-Q water to 50 mL 10X M9 salts, and sterilize by autoclaving. Once cooled down, add appropriate antibiotics, 1 mL magnesium sulfate stock, 0.5 mL ferrous chloride stock, 0.5 mL zinc chloride stock, 5 μL calcium chloride stock, 1 mL yeast extract stock, and 6.25 mL glucose stock. For other media, *see* **Note 4**.
14. 1000X ampicillin stock: 100 mg ampicillin dissolved in 1 mL Milli-Q water, stored at −20 °C.
15. 1000X streptomycin stock: 100 mg streptomycin dissolved in 1 mL Milli-Q water, stored at −20 °C.
16. 1000x IPTG stock: 1 M isopropyl β-d-1-thiogalactopyranoside (IPTG) in Milli-Q water, stored at −20 °C.
17. *O*-Propargyltyrosine (PpY or π): Synthesis of PpY, needed for site-specific click labeling, was previously described [20].
18. Nickel agarose resin (high density) (GoldBio; St. Louis, MO, USA).
19. Q700 sonicator (QSonica; Newtown, CT, USA) or equivalent.
20. β-Mercaptoethanol (BME) (Bio-Rad).
21. 2-Mercaptoethanesulfonate (MESNa) (Thermo Fisher Scientific; Waltham, MA, USA).
22. HPLC buffers: HPLC buffer A consists of Milli-Q water with 0.1% TFA and HPLC buffer B consists of acetonitrile (ACN) with 0.1% TFA.
23. C4 HPLC columns.

24. Lyophilizer (Labconco).
25. Atto 488-azide (Sigma-Aldrich). Stock solution: 20 mM Atto 488-azide. Dissolve 1 mg into 55.4 μL fresh DMSO. Store at −20 °C.
26. Copper (II) sulfate. Stock solution: 80 mM copper (II) sulfate. Dissolve 12.8 mg into 1 mL Milli-Q water. Store at −20 °C.
27. Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) (Sigma-Aldrich). Stock solution: 50 mM THPTA in Milli-Q water. Dissolve 21.7 mg into 1 mL Milli-Q water. Store at −20 °C).
28. Sodium ascorbate. Stock solution: 100 mM sodium ascorbate in degassed Milli-Q water. Dissolve 19.8 mg of sodium ascorbate into 1 mL of the degassed Milli-Q water.
29. Apparatus for argon atmosphere (balloons, syringes, needles, rubber stopper, etc.)
30. Standard molecular biology equipment and supplies: spectrophotometer, orbital incubator, ultracentrifuge with fixed-angle rotor, microcentrifuge, standard dialysis tubing, magnetic stirrer, 20 mL disposable plastic columns.

2.6 Native Chemical Ligation (NCL)

1. NCL buffer pH 3: 6 M guanidium, 200 mM phosphate, pH 3.0. Dissolve 5.74 g guanidine hydrochloride and 240 mg sodium phosphate monobasic into 5 mL water. Adjust pH as necessary and bring the final volume to 10 mL. Filter-sterilize the solutions using a 0.22 μm filter. *See* **Note 6** on pH.
2. NCL buffer pH 7: 6 M guanidium, 200 mM phosphate, pH 7.0. Dissolve 5.74 g guanidine hydrochloride and 240 mg sodium phosphate monobasic into 5 mL water. Adjust pH with 5 M NaOH and bring the final volume to 10 mL. Filter-sterilize the solutions using a 0.22 μm filter. *See* **Note 6** on pH.
3. Sodium nitrite. Stock solution: 500 mM $NaNO_2$ in water. Dissolve 10.3 mg $NaNO_2$ into 300 μL water. *See* **Note 6** on pH.
4. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Sigma-Aldrich). Stock solution for NCL/MES conversion: 500 mM TCEP, 6 M guanidium, 200 mM phosphate, pH 7.0. Dissolve 14.3 mg TCEP into 50 μL NCL buffer pH 7. Adjust pH with 5 M NaOH and bring the final volume to 100 μL. *See* **Note 6** on pH.
5. Stock solution for desulfurization: 1 M TCEP, 6 M guanidium, 200 mM phosphate, pH 7.0. Dissolve 28.6 mg TCEP into 40 μL NCL buffer pH 7. Adjust pH with 5 M NaOH and bring the final volume to 100 μL. *See* **Note 6** on pH.

6. 4-Mercaptophenylacetic acid (MPAA) (Chem-Impex). Stock solution: 500 mM MPAA, 6 M guanidium, 200 mM phosphate, pH 7.0. Dissolve 8.41 mg MPAA into 60 μL NCL buffer pH 7. Adjust pH with 5 M NaOH and bring the final volume to 100 μL. *See* **Note 6** on pH.
7. 2-Mercaptoethanesulfonate (MESNa) (Thermo Fisher Scientific). Stock solution: 500 mM MES, 6 M guanidium, 200 mM phosphate, pH 7.0. Dissolve 8.20 mg MESNa into 60 μL NCL buffer pH 7. Adjust pH if necessary and bring the final volume to 100 μL. *See* **Note 6** on pH.
8. Methyl thioglycolate (MTG) (Sigma-Aldrich). Stock dilution: Dilute 4.47 μL to 100 μL with NCL buffer pH 7. *See* **Note 6** on pH.
9. VA-044 (FUJIFILM Wako Chemical USA Corp.; Richmond, VA, USA). Stock solution: 200 mM VA-044 in degassed water. Dissolve 6.47 mg VA-044 into 100 μL degassed water.
10. Reduced glutathione (GSH) (Sigma-Aldrich). Stock solution: 1 M GSH, 6 M guanidium, 200 mM phosphate, pH 7.0. Dissolve 30.7 mg GSH into 50 μL NCL buffer pH 7. Adjust pH and bring the final volume to 100 μL. *See* **Note 6** on pH.
11. pH indicator paper for pH 6.4–8.0 range.
12. Apparatus for argon atmosphere (balloons, syringes, needles, rubber stopper, etc.).
13. Purified synthetic peptide fragments (see Subheading 3.2.3).
14. Purified, recombinant protein fragments (see Subheading 3.3.2., 3.3.3. and 3.3.4).
15. HPLC buffers: HPLC buffer A consists of Milli-Q water with 0.1% TFA, and HPLC buffer B consists of acetonitrile (ACN) with 0.1% TFA.
16. Lyophilizer.

3 Methods

3.1 Synthesis of the Fmoc-Glu(Arg(Pdf)-OtBu)-OH

Procedures are briefly summarized (see Fig. 2) in a manner that should be accessible to one familiar with standard organic chemistry practices. Characterization of all compounds by mass spectrometry, ^{1}H NMR, and ^{13}C NMR has been reported previously [7].

1. Coupling of Fmoc-Glu-OAll and Arg(Pbf)-O*t*Bu to form Fmoc-Glu(Arg(Pbf)-O*t*Bu)-OAll: Dissolve Fmoc-Glu-OAll **1** (1.77 g, 3.56 mmol) in 15 mL tetrahydrofuran (THF) in a round-bottom flask, stir in an ice-salt bath. Add 2 equiv. *N*-methylmorpholine (NMM) and 1 equiv. isobutyl chloroformate (IBCF) and stir for 15 min. Add 1 equiv. Arg(Pbf)-

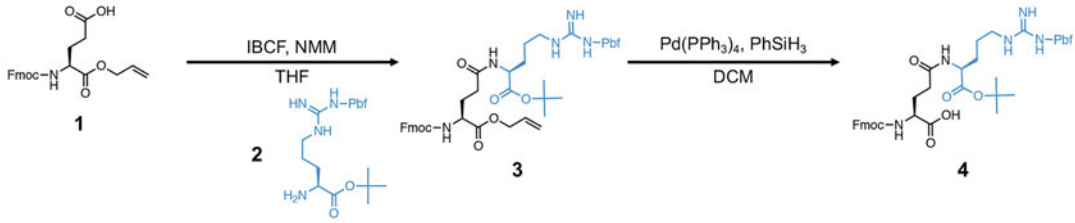

Fig. 2 Synthesis of Fmoc-Glu(Arg(Pdf)-OtBu)-OH (**4**)

O*t*Bu **2**, stir for 1 h, then warm gradually to room temperature, and let stir overnight. Filter the reaction over a Büchner funnel, and wash it 2x with 5% $NaHCO_3$ and then with brine. Dry the extract over $MgSO_4$, filter, and concentrate to an oil under reduced pressure. The R_f of Fmoc-Glu(Arg(Pbf)-O*t*Bu)-OAll **3** on thin-layer chromatography (TLC) should be 0.66 in 5:2:1 ethyl acetate: hexanes: methanol +0.5% acetic acid.

2. Deprotection of allyl group to form Fmoc-Glu(Arg(Pbf)-O*t*Bu)-OH: Redissolve Fmoc-Glu(Arg(Pbf)-O*t*Bu)-OAll **3** from step 1 in 5 mL of dichloromethane (DCM) degassed with argon. Add 1 equiv phenylsilane ($PhSiH_3$) and 2.5% mol tetrakis(triphenylphosphine)palladium(0) catalyst ($Pd(PPh_3)_4$), and stir until the solution turns black. Wash the reaction with 2x with 5% $NaHCO_3$, and back extract the aqueous layer with 5 mL DCM. Wash combined organic layers with brine and dry with $MgSO_4$, filter, and evaporate to dryness. Purify by column chromatography on silica (5:2:1 ethyl acetate: hexanes: methanol combined with 0.125% acetic acid). Azeotrope at least four times with toluene in ethyl acetate to remove as much acetic acid as possible to prevent acetylation side reactions during peptide synthesis. Purified product is obtained as an off-white powder: The R_f of Fmoc-Glu(Arg(Pbf)-O*t*Bu)-OH **4** on TLC should be 0.28 in 5:2:1 ethyl acetate: hexanes: methanol + 0.5% acetic acid. *See* **Note 7**.

3.2 Peptide Synthesis

3.2.1 Peptide Synthesis for Site-Specific E^{Arg} Antibody Generation (Anti-Arginylated αS)

We describe a procedure for synthesizing two arginylated peptides targeting αS E^{Arg}_{46} (CVGSKTKE^{Arg}GVVH, **5**) or αS E^{Arg}_{83} (CAVAQKTVE^{Arg}GAG, **6**) on the 30 μmol scale using standard Fmoc-based solid-phase peptide synthesis strategy. The structure of the peptides can be found in Fig. 3. Characterization of the product by MALDI and analytical HPLC can be found in Fig. 4a, b and are previously published [8]. *See* **Note 8**.

1. To a fritted plastic syringe, add 20 mg 2-chlorotrityl resin (100–200 mesh, 1.5 mmol/g), a stir bar, and v1:1 dichloromethane (DCM): dimethylformamide (DMF). Make sure the resin is submerged. Swell resin by stirring for at least 30 min.

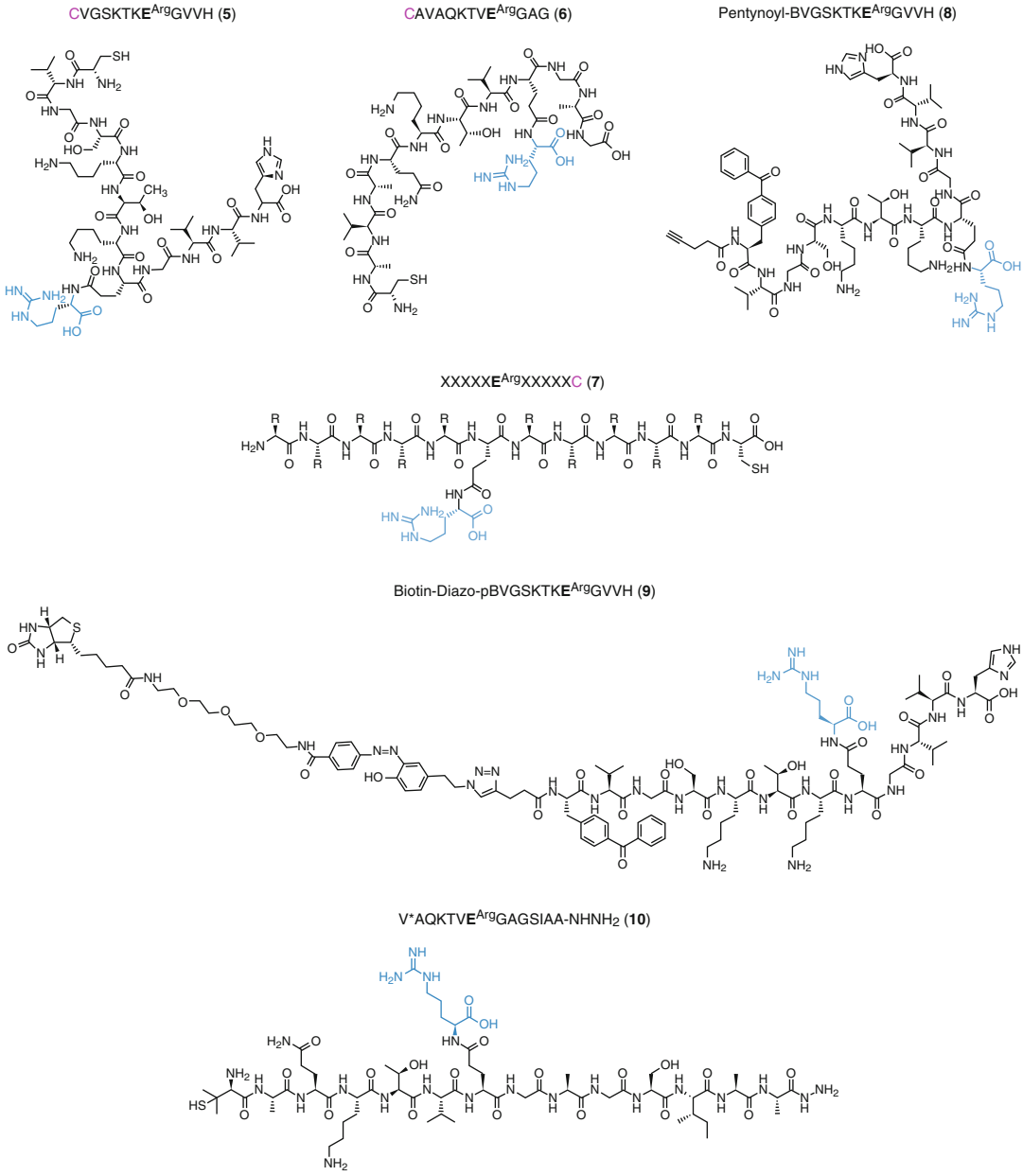

Fig. 3 Arginylated peptide sequences/structures

2. Remove the DCM/DMF by vacuum filtration and wash the resin three times with DMF.
3. Dissolve the first Fmoc-amino acid (2 equiv) in DMF at a concentration of 0.15 M. Add *N,N*-diisopropylethylamine (DIPEA, 4 equiv) to this, and pre-activate the solution by gentle mixing. Add the solution to the resin and stir for 15 min at room temperature. Remove the mixture from resin

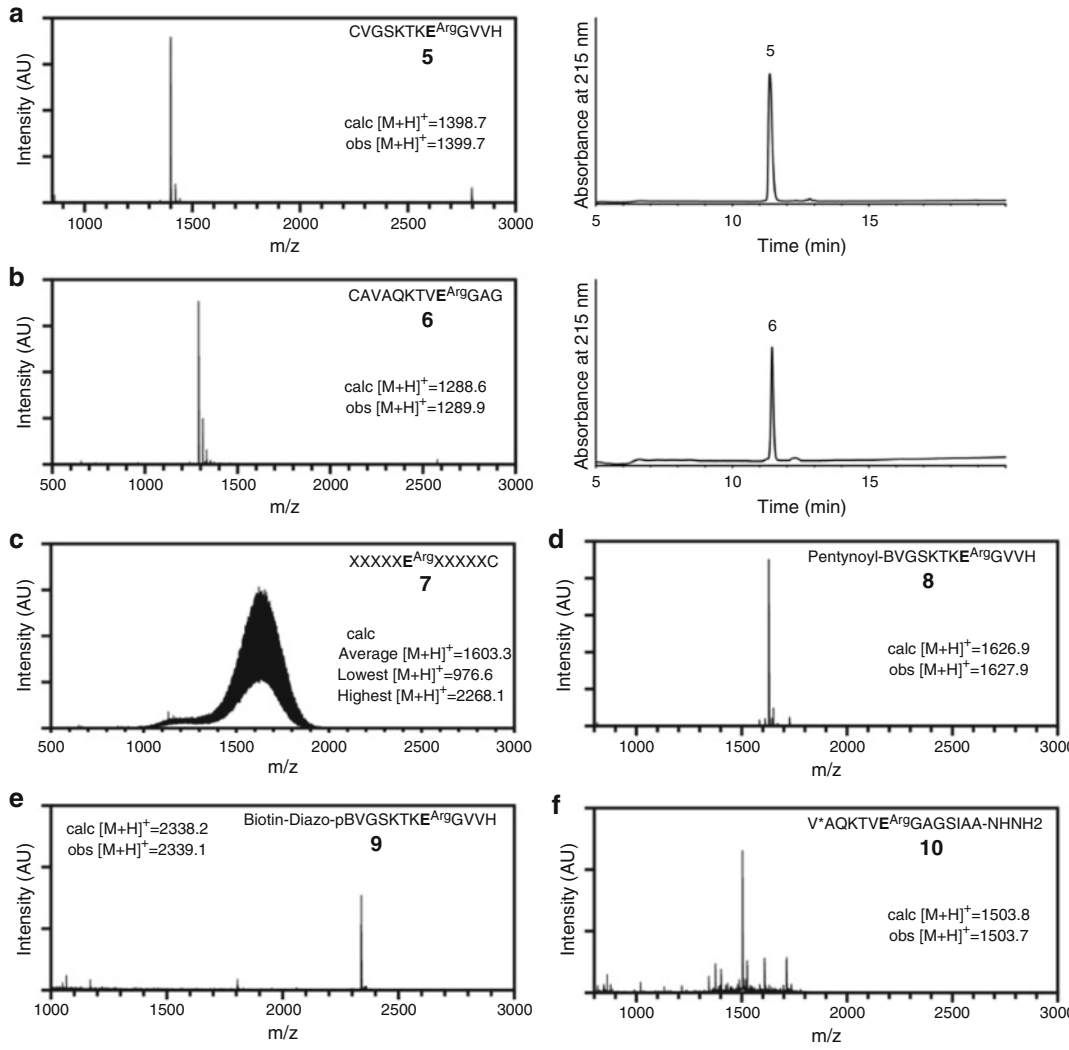

Fig. 4 Characterization of arginylated peptides: (**a**, **b**) MALDI-MS and analytical HPLC (gradient, 2%–60% B over 30 min) of product peptide CVGSKTKE^{Arg}GVVH (**5**, **a**) and CAVAQKTVE^{Arg}GAG (**6**, b); (**c**) MALDI-MS of product peptide library XXXXXE^{Arg}XXXXXC (**7**); (**d**–**f**) MALDI-MS of product peptide pentynoyl-BVGSKTKE^{Arg}GVVH (**8**, d); Biotin-diazo-pBVGSKTKEArgGVVH (**9**, e); and V*AQKTVE^{Arg}GAGSIAA-acyl hydrazide (**10**, f; αS_{77-90}-V*$_{77}$E$^{Arg}_{83}$-NHNH$_2$)

by vacuum filtration, and wash the resin three times with DMF, three times with DCM, and three times with DMF.

4. Add 5% methanol in DMF to cap unreacted sites on the resin. Gently stir for 10 min. Remove the mixture from resin by vacuum filtration, and wash the resin three times with DMF, three times with DCM, and three times with DMF.
5. Remove the Fmoc group by adding 20% piperidine/DMF and stir for 5 min. Collect the solution by pushing air through.

Repeat this once. Wash the resin three times with DMF, three times with DCM, and three times with DMF.

6. Perform Kaiser test for positive control. For Kaiser test: To a small glass tube, add one drop of reagent A, reagent B, and reagent C and sample resin (at least a few particles). Resuspend well, and heat the glass tube at 100–110 °C for 2–5 min. You should see a color change from yellow to dark blue (positive). If you see yellow (negative), remake the Kaiser test reagents and test again.
7. Quantify the amount of coupled amino acid by the Fmoc content in the deprotection solution using the absorbance at 301 nm (extinction coefficient: 7800 cm^{-1} M^{-1}; Fmoc quantification).
8. Couple amino acids to elongate the peptide. Monitor completion of couplings by Kaiser test. For standard amino acids, dissolve Fmoc-amino acid (4 equiv) at a concentration of 0.2–0.3 M and HBTU (3.8 equiv) in DMF. Add DIPEA (8 equiv) and pre-activate the amino acid by gentle mixing. Add the activated mixture to the deprotected peptidyl resin and stir for 30 min. Perform washes with DMF and DCM between each coupling. Deprotect Fmoc groups by adding 20% piperidine in DMF twice and stirring for 5 min each. For the coupling of Fmoc-Glu(Arg(Pbf)-OtBu)-OH, activate 2 equiv. of Fmoc-Glu(Arg(Pbf)-OtBu)-OH in DMF with 1.9 equiv. HBTU and 4 equiv. DIPEA, and then mix with the resin for 1–2 h at 37 °C. Perform Kaiser test after every coupling. Add fresh reagents or extend coupling time if color change is observed.
9. After further elongation, wash the resin with DCM 4 times. Prepare 2 mL cleavage solution comprised of 90% TFA, 5% TIPS, and 5% DCM and cool on ice. Pour 0.5 mL of the solution onto the resin on ice. The rest is stored at 4 °C. Stir it for 30 min at room temperature.
10. Filter and collect the solution into a scintillation vial. Repeat this twice (i.e., 1.5 h cleavage in total). Wash the syringe with the remaining cleavage solution and pool into the scintillation vial.
11. Add the cleavage solution dropwise into tenfold volume of ice-cold diethyl ether in a Falcon tube. Mix this vigorously and allow the substrate to precipitate on dry ice for 5–10 min.
12. Centrifuge the tube for 5 min at 4 °C (4000Xg), and remove the supernatant by decantation. To further get rid of cleavage cocktail, add 10 mL of ice-cold diethyl ether to each pellet, vortex vigorously, allow the substrate to precipitate on dry ice for 10 min, and centrifuge the tubes again. Repeat this once.

13. Remove the supernatant by decantation and dry the crude product overnight in a fume hood.
14. Dissolve the pellet in an acetonitrile-water mixture (final acetonitrile % v/v < 20% to ensure column binding upon HPLC injection) and filter using a chemically compatible system. Purify by reverse-phase HPLC over a C18 column, using 0.1% TFA in water/0.1% TFA in acetonitrile as mobile phase.
15. Check the purity by analytical HPLC and confirm identity by mass spectrometry (Fig. 4a, b). Combine pure fractions, flash-freeze in liquid nitrogen, and lyophilize.

3.2.2 Peptide Library Synthesis for Pan-E^{Arg} Antibody Generation

We describe a procedure for synthesizing the arginylated peptide library XXXXXE^{Arg}XXXXXC (X: mixture of 19 non-cysteine natural amino acids, methionine is replaced with norleucine to prevent oxidation issues) on the 100 μmol scale for generation of antibodies recognizing arginylation, regardless of the surrounding sequence. Our method utilizes Fmoc-based solid-phase peptide synthesis strategy and an isokinetic mixture reported previously [21, 22]. *See* **Note 9**. The general structure of the peptide library can be found in Fig. 3. Characterization of the product by MALDI can be found in Fig. 4c.

1. Swell 167 mg 2-chlorotrityl resin in v1:1 DCM/DMF by stirring for at least 30 min in a fritted plastic syringe. Couple the first amino acid Fmoc-Cys(Trt)-OH, cap unreacted sites, and remove Fmoc group as described in Subheading 3.2.1.1. After Fmoc quantification, resuspend resin in 2 mL v1:1 DCM/DMF, adjust resin amount to 100 μmol by volume, and transfer to a glass vessel.
2. Make isokinetic mixture. Weigh 19 natural amino acids (240 equiv. total per residue – 2400 equiv. in total; norleucine instead of methionine for better stability; Table 1) following the ratio determined previously [21]. Dissolve each amino acid into DMF, so the concentration is 0.3 M. Dissolve 32.4 g HOBt (2400 equiv) into combined amino acid solution. Split equally to make 10 aliquots. Flash-freeze and store them at − 20 °C.
3. Perform coupling with isokinetic mixture. To a thawed aliquot, add DIC (240 equiv., 3.716 mL) and activate the cocktail by mixing gently. Pour the solution onto the resin and stir well for an h at room temperature. Perform Kaiser test and washes with DMF/DCM/DMF.
4. Add 20% piperidine and stir well for 5 min. Remove the solution by vacuum filtration. Repeat this once. Perform Kaiser test to confirm deprotection. Perform washes with DMF/DCM/DMF.

Table 1
Components of isokinetic mixture

Reagent	Equiv	MW (g/mol)	Weight for 1 mmol scale(g)
Fmoc-l-Ala-OH	8.16	311.33	2.540
Fmoc-l-Arg(Pbf)-OH	15.6	648.77	10.121
Fmoc-l-Asn(Trt)-OH	12.48	596.67	7.446
Fmoc-l-Asp(tBu)-OH	8.4	411.45	3.456
Fmoc-l- Gln(Trt)-OH	12.72	610.7	7.768
Fmoc-l-Glu(tBu)-OH	8.64	425.47	3.676
Fmoc-Gly-OH	6.96	297.31	2.069
Fmoc-l-His(Trt)-OH	8.4	619.71	5.206
Fmoc-l-Ile-OH	41.76	353.41	14.758
Fmoc-l-Leu- OH	11.76	353.41	4.156
Fmoc-l-Lys(Boc)-OH	14.88	468.54	6.972
Fmoc- l-Phe-OH	6	387.43	2.325
Fmoc-l-Pro-OH	10.32	337.37	3.4817
Fmoc-l-Ser(tBu)-OH	6.72	383.44	2.577
Fmoc-l-Thr(tBu)-OH	11.52	397.46	4.579
Fmoc-l-Trp(Boc)-OH	9.12	526.58	4.802
Fmoc-l- Tyr(tBu)-OH	9.84	459.53	4.5218
Fmoc-l-Val-OH	27.12	339.39	9.204
Fmoc-l-Nle-OH	9.12	353.41	3.223
19 nat AA total	240		

5. Repeat 3 and 4 four times. Transfer the resin to a plastic fritted syringe.
6. Couple Fmoc-Glu(Arg(Pbf)OtBu)-OH (2 equiv) and deprotect Fmoc group as described in Subheading 3.2.1.1. Transfer the resin back to a glass vessel.
7. Repeat 3 and 4 five times. Transfer the resin to a plastic fritted syringe.
8. Wash the resin with DCM four times. Prepare 4 mL cleavage solution (Reagent K: 82.5% v/v TFA, 5% w/v phenol, 5% v/v water, 5% v/v thioanisole, and 2.5% v/v 1.2-ethanedithiol), and cool on ice. Pour 1 mL of the solution onto the resin on ice. The rest is stored at 4 °C. Perform cleavage and precipitation as described in Subheading 3.2.1.1. Dry the crude product overnight in a fume hood.

9. Prepare an acetonitrile-water mixture for lyophilization. Dissolve each pellet, flash-freeze the solution and lyophilize. We got 52% yield starting from the first amino acid, based on the average mass. MALDI characterization of the product library is shown in Fig. 4c.

3.2.3 Peptide Synthesis for Cross-Linking and Pull-Down Applications

We describe a procedure for synthesizing an arginylated peptide for photo-cross-linking and pull-down applications, Biotin-Diazo-pBVGSKTKE^{Arg}GVVH (B = 4-benzoyl-L-phenylalanine, p = pentynoyl), on the 3 μmol scale (initial peptide synthesis on 30 μmol scale). Our method includes Fmoc-based solid-phase peptide synthesis strategy to synthesize pBVGSKTKE^{Arg}GVVH, followed by a click reaction to conjugate to a PEG linker bearing biotin. The structure of the peptides can be found in Fig. 3. Characterization of the product by MALDI can be found in Fig. 4d, e. *See* **Note 10.**

1. Swell 20 mg 2-chlorotrityl resin in v1:1 DCM/DMF by stirring for at least 30 min. Perform the first amino acid coupling and subsequent elongation as described in Subheading 3.2.1, until formation of BVGSKTKE^{Arg}GVVH-resin.
2. Add 4-pentynoic acid (5 equiv) activated by HBTU (4.5 equiv) and DIPEA (10 equiv) in DMF to the resin. Mix for 30 min.
3. Wash the resin 3 times with DMF and 3 times with DCM.
4. Cleave the peptides from resin by treating with a cleavage cocktail (90% TFA, 5% TIPS, 5% DCM) for 1.5 h. Pool the cleavage solution into cold ether, collect the precipitate, and purify it by reverse-phase HPLC using a C18 column and 0.1% TFA in water /0.1% TFA in acetonitrile as mobile phase. Confirm identity of pentynoyl BVGSKTKE^{Arg}GVVH by mass spectrometry (Fig. 4d), pool pure fractions, flash-freeze, and lyophilize.
5. Dissolve pentyl-BVGSKTKE^{Arg}GVVH 5 mg (3.1 μmol) in 400 μL 100 mM sodium phosphate buffer, pH 7. Dissolve diazo-biotin-azide 2 equiv. (4.4 mg) in 100 μL DMSO, and add to the peptide.
6. Mix 50 μL 100 mM $CuSO_4$ and 50 μL 100 mM THPTA (tris-hydroxypropyltriazolylmethylamine), and add to the above mixture.
7. Add 1 mg sodium ascorbate (1.8 equiv., 5.7 μmol) and incubate at room temperature for 4 h.
8. Purify by reverse-phase HPLC over a C18 column. Use 0.1% TFA in water /0.1% TFA in acetonitrile as mobile phase.
9. Confirm identity by mass spectrometry (Fig. 4e) and purity by analytical HPLC. Pool pure fractions and lyophilize.

3.2.4 Peptide Synthesis for Arginylated Protein Semi-Synthesis

We describe a procedure for synthesizing arginylated peptide fragment V*AQKTVE^{Arg}GAGSIAA-hydrazide (αS_{77-90}- V*$_{77}$E$^{Arg}{}_{83}$-hydrazide) for semi-synthesis of arginylated αS (αS-E$^{Arg}{}_{83}$) on the 30 μmol scale using standard Fmoc-based solid-phase peptide synthesis strategy (V*: penicillamine). Hydrazide functions as a thioester surrogate [23], while penicillamine does as a valine precursor [24]. The structure of the peptide can be found in Fig. 3. Characterization of the product by MALDI can be found in Fig. 4f and was published previously [7].

1. Swell 200 mg 2-chlorotrityl resin in v1:1 DMF/DCM for at least 30 min. Wash the resin three times with DMF, three times with DCM, and three times with DMF.
2. Dissolve Fmoc-hydrazine (1 equiv.; 25.4 mg) and DIPEA (2 equiv.; 34.8 μL) in DMF. Derivatize the resin by reacting overnight at room temperature. Perform washes with DMF/DCM/DMF.
3. Cap unreacted sites with 5% methanol in DMF, stirring for 10 min. Perform washes with DMF/DCM/DMF.
4. Couple the first amino acid using 4 equiv. Fmoc-protected amino acid at a concentration of 0.2–0.3 M, HBTU (3.8 equiv) and DIPEA (8 equiv). Add the activated mixture to the resin and stir for 30 min. Perform washes with DMF/DCM/DMF.
5. Cap unreacted sites by stirring with 5% acetic anhydride for 10 min. Wash the resin three times with DMF, three times with DCM, and three times with DMF.
6. Deprotect Fmoc groups by adding 20% piperidine in DMF twice and stirring for 5 min each. Perform Kaiser test for positive control and Fmoc quantification. From here on, prepare coupling reagents based on this quantification.
7. Couple amino acids and remove Fmoc group for subsequent elongation as described in Subheading 3.2.1.1. For coupling Fmoc-Pen(Trt)-OH, stir 4 equiv. amino acid at a concentration of 0.4 M with 4 equiv. 7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP), 4 equiv. HOBt, and 8 equiv. DIPEA at 60 °C for 1 h. Double or triple couple.
8. Cleave the peptide from the resin. Prepare cleavage cocktail (95% TFA, 2.5% TIPS, 2.5% H_2O), and agitate for 1 h at room temperature. Pool the cleavage solution into cold ether, collect the precipitate, and purify by reverse-phase HPLC over a C4 column. Use 0.1% TFA in water /0.1% TFA in acetonitrile as mobile phase.
9. Confirm identity by mass spectrometry (Fig. 4f) and purity by analytical HPLC.

10. Pool pure fractions and lyophilize the product. We got 28% yield, calculated from hydrazine derivatization (**step 6**).

3.3 Protein Expression for Arginylated Protein Semi-Synthesis

3.3.1 Production of Plasmids Coding αS Fragment-Intein Constructs

We describe a procedure for deletion PCR on a plasmid containing full-length αS to generate protein fragments used for NCL.

1. Perform deletion PCR. Mix the following together in a PCR tube on ice in this order: 23 μL nuclease-free water, 25 μL Q5 High-Fidelity 2X Master Mix, 1 μL of pTXB1-αS-intein-H_6 plasmid (< 1 μg/mL), and 0.5 μL each of forward/reverse primer (50 μM).
2. Set the tubes in a thermocycler and run the following program:

Step	Number of cycles	Temperature	Duration
1	1	98 °C	0:30 min
2	20	98 °C 70 °C (−0.5 °C/cycle) 72 °C	0:10 min 0:30 min 4:00 min
3	15	98 °C 60 °C 72 °C	0:10 min 0:30 min 4:00 min
4	1	72 °C	2:00 min
5	1	4 °C	Hold

3. Clean up the PCR reactions using a DNA cleanup kit. Quantify DNA concentration using the NanoQuant Plate and a plate reader to check the reactions.
4. Circularize the PCR product by phosphorylating 5′ end of each DNA strand and ligating it with the 3′ end, using T4 PNK and T4 DNA ligase, respectively. Mix the following together on ice: 5 μL nuclease-free water, 1 μL 10X T4 DNA ligase buffer, 2 μL purified PCR product, 0.5 μL DpnI, 0.5 μL T4 DNA ligase, and 1 μL T4 PNK.
5. Incubate the reaction at 37 °C for 60 min.
6. Cool the reaction on ice for a few minutes, and add 50 μL competent Dh5α cells. Incubate for 15 min.
7. Heat shock the cells at 42 °C for 45 sec and keep them back on ice for 2 min.
8. Add 400 μL SOC media and incubate cells for 1 h at 37 °C with 250 rpm shaking.
9. Plate 200 μL cell suspension onto an agar plate supplemented with 0.1 mg/mL ampicillin, and incubate overnight at 37 °C.

10. Pick single colonies and grow each in a culture tube with 5 mL LB supplemented with ampicillin. Incubate cells for overnight at 37 °C with 250 rpm shaking.
11. Isolate cells by centrifuging at 4000 g for 20 min and discarding media. Purify DNA using a miniprep kit.
12. Quantify DNA concentration using the NanoQuant Plate and a plate reader. Submit samples from each colony for sequencing to check identity. Keep the product at −20 °C.

3.3.2 Production of N-Terminal Protein Fragment

We describe a procedure for recombinant expression and purification of N-terminal protein fragment. The protein is fused to a C-terminal intein tag, which allows for transthioesterification upon addition of excess thiol. To prevent hydrolysis of the thioester, prechill all the buffers before use. Characterization of the product by MALDI was previously published [7].

1. Transform plasmid containing αS_{1-76} fused to a polyhistidine-tagged GyrA intein from *Mycobacterium xenopi* (Mxe), following the procedure in Subheading 3.3.1., into BL21(DE3) competent cells.
2. Pick single colonies to inoculate primary cultures in 5 mL LB media supplemented with 0.1 mg/mL ampicillin. Grow at 37 °C with 250 rpm shaking for 3–6 h until the culture gets cloudy.
3. Prepare LB media for secondary culture by autoclaving and supplementing 0.1 mg/mL ampicillin. Inoculate secondary cultures at 1.5–2%.
4. Incubate secondary culture at 37 °C in a shaker at 250 rpm until optical density (OD) reaches ~0.6. Induce expression of the gene of interest with 1 mM IPTG.
5. Grow the culture in the shaker-incubator at 18 °C overnight.
6. Split culture into bottles and centrifuge the culture at 4000 rpm, 20 min, 4 °C, using GS3 rotor. Prepare resuspension buffer (see Subheading 2.4.) and cool on ice.
7. Discard the media and resuspend cell pellets were in the resuspension buffer. Combine resuspended cells into a metal cup.
8. Sonicate cells in the cup in an ice bath (5 min, 1 s ON, 1 s OFF).
9. Centrifuge the resulting lysate at 14,000 rpm, 25 min, 4 °C, using SS34 rotor.
10. Set up a Ni-NTA affinity column to purify αS_{1-76} fused to GyrA intein. Equilibrate 3 mL bed volume Ni-NTA resin by flowing through 10 mL equilibration buffer (see Subheading 2.4). Plug the bottom of the column, resuspend with isolated supernatant, and incubate at 4 °C for 1 h. Prechill buffers for wash and elution (see Subheading 2.4).

11. Transfer the slurry onto the column and let go the flow-through. Wash the resin with 15 mL wash buffer 1 and then with 20 mL wash buffer 2. Add 10–12 mL elution buffer and collect eluted protein of interest.
12. Remove excess imidazole by dialyzing into 3 L of 20 mM Tris pH 7.5 buffer overnight at 4 °C.
13. Dissolve 200 mM sodium 2-mercaptoethanesulfonate (MESNa) into dialyzed solution and incubate overnight with agitation at 4 °C for transthioesterification.
14. Remove excess MESNa by dialyzing cleaved proteins into 3 L of 20 mM Tris, pH 7.5 buffer overnight at 4 °C.
15. Set up a second Ni-NTA column to remove the free intein from the sample. Equilibrate 3 mL bed volume Ni-NTA resin as described in 10 and resuspend it with dialyzed, cleaved proteins before incubation at 4 °C for 1 h.
16. Transfer the slurry onto the column and collect the flow-through, which should include the protein of interest. Elute with 10 mL elution buffer in case transesterification did not work.
17. Purify the protein fragment-thioester by RP-HPLC over a C4 column.
18. Check the identify of fractions by MALDI-MS, combine desired products, and lyophilize. We got 24.1 mg per 1 L of *E. coli* culture.

3.3.3 Production of C-Terminal Protein Fragment (no Labeling)

We describe a procedure for recombinant expression and purification of C-terminal protein fragment without labeling. Expression of proteins with N-terminal cysteine in *E. coli* results in its production with the initiator methionine, which is cleaved in vivo by endogenous methionine aminopeptidase (MAP). The exposed N-terminal cysteine reacts with endogenous aldehydes to form thiazolidine adducts. Our protocol uses methoxyamine for deprotection of thiazolidine adducts. *See* **Note 11.**

1. Transform plasmid containing αS_{91-140}-C_{91} fused to a polyhistidine-tagged GyrA intein from Mxe into BL21(DE3) competent cells, grow culture, and express the gene of interest, following the procedure in Subheading 3.3.2.
2. Follow the identical purification procedure up to running the first Ni-NTA affinity column, as in Subheading 3.3.2.
3. Elute the protein of interest and cleave off intein by incubation with 200 mM β-mercaptoethanol (βME) on a rotisserie overnight at room temperature.

4. Remove excess imidazole and βME by dialyzing the cleaved protein of interest into 20 mM Tris, pH 8 buffer overnight at 4 °C.
5. Run a second Ni-NTA column as described in Subheading 3.3.2. to remove the free intein from the sample.
6. Purify the protein fragment and its thiazolidine adducts by RP-HPLC over a C4 column.
7. Check the identify of fractions by MALDI-MS, combine desired products, and lyophilize. We got 12.2 mg of the adducts per 1 l of *E. coli* culture.
8. Prepare buffers for thiazolidine deprotection (see Subheading 2.4). Redissolve the lyophilized peptides into the deprotection buffer and incubate at 37 °C for a few h.
9. Check the reaction hourly by MALDI-MS.
10. Dilute the reaction with thiazolidine deprotection dilution buffer (see Subheading 2.4.) so that guanidium concentration is less than 1 M for HPLC injection.
11. Purify the product by RP-HPLC over a C4 column.
12. Check the identify of fractions by MALDI-MS, combine desired products, and lyophilize. This reaction typically gives quantitative yield.

3.3.4 Production of Fluorescently Labeled C-Terminal Protein Fragment

We describe a procedure for recombinant expression and purification of C-terminal protein fragment with a site-specific fluorescent labeling. Unnatural amino acid mutagenesis via amber codon suppression is used to incorporate *o*-propargyltyrosine (PPY, π) at a desired position and produce C-terminal fragments for site-specific labeling with Atto 488. Labeling with Atto 488-azide via copper-catalyzed azide-alkyne cyclization is performed before thiazolidine deprotection, since we found that the labeling condition simultaneously deprotects most thiazolidine derivatives and oxidizes some portion of N-terminal cysteine. The detailed information of this can be found in our published work [7].

1. Co-transform the following plasmids into BL21(DE3) competent cells: i) the plasmid containing αS_{91-140}-$C_{91}TAG_{114}$ fused to a polyhistidine-tagged GyrA intein from Mxe and ii) pDule-pCNF plasmid. Plate 200–400 μL cell suspension onto an agar plate supplemented with 0.1 mg/mL ampicillin and 0.1 mg/mL streptomycin.
2. Pick single colonies to inoculate primary cultures in 5 mL LB media supplemented with 0.1 mg/mL ampicillin and 0.1 mg/mL streptomycin. Grow at 37 °C with 250 rpm shaking for 3–6 h until the culture gets cloudy.

3. Prepare M9 minimal media as described above by autoclaving and supplementing 0.1 mg/mL ampicillin. Inoculate secondary cultures at 1–2%.
4. Incubate secondary culture at 37 °C in a shaker at 250 rpm until optical density (OD) reaches ~0.6–0.8.
5. Add π (220 mg/L) to the media and incubate for 10 min.
6. Induce expression of the gene of interest with 1 mM IPTG.
7. Grow the culture in the shaker-incubator at 18 °C overnight.
8. Follow the identical purification procedure up to purifying the protein fragment and thiazolidine adducts by HPLC following lyophilization, as in Subheading 3.3.3. We got 5.8 mg of this per 1 L of *E. coli* culture.
9. Prepare for labeling with Atto 488-azide via copper-catalyzed azide-alkyne cyclization. Degas 20 mM Tris pH 8 buffer for at least 10 min. Redissolve the fragment in 20 mM Tris pH 8. Make sodium ascorbate stock solution and thaw other stock solutions.
10. Prepare catalytic mixture consisting of 2 equiv. $CuSO_4$, 10 equiv. Tris(3- hydroxypropyltriazolylmethyl)amine (THPTA), and 20 equiv. sodium ascorbate. Let it sit for 10 min.
11. Add the catalytic mixture to the protein along with 2 equiv. fluorophore.
12. Check product formation by MALDI-MS.
13. Purify labeled fragments by HPLC over a C4 column.
14. Check the identify of fractions by MALDI-MS. Combine desired products or pyruvate-derived thiazolidines and lyophilize.
15. Prepare buffers for thiazolidine deprotection (see Subheading 2.4). Redissolve the lyophilized, pyruvate-derived thiazolidines into the deprotection buffer and incubate at 37 °C for a few h.
16. Check reaction progress, purify, and lyophilize as in Subheading 3.3.3. The yield from **8** we got was 59%.

3.4 Native Chemical Ligation for Arginylated Protein Semi-Synthesis

We describe a procedure for three-part ligation to synthesize αS with glutamate arginylation at site 83, unlabeled (Fig. 5) or with an orthogonally installed fluorophore (Fig. 6). Reaction traces by analytical HPLC and characterization of the purified products by MALDI and/or analytical HPLC can be found in Fig. 7. Further characterization of crude reaction can be found in our published work [7].

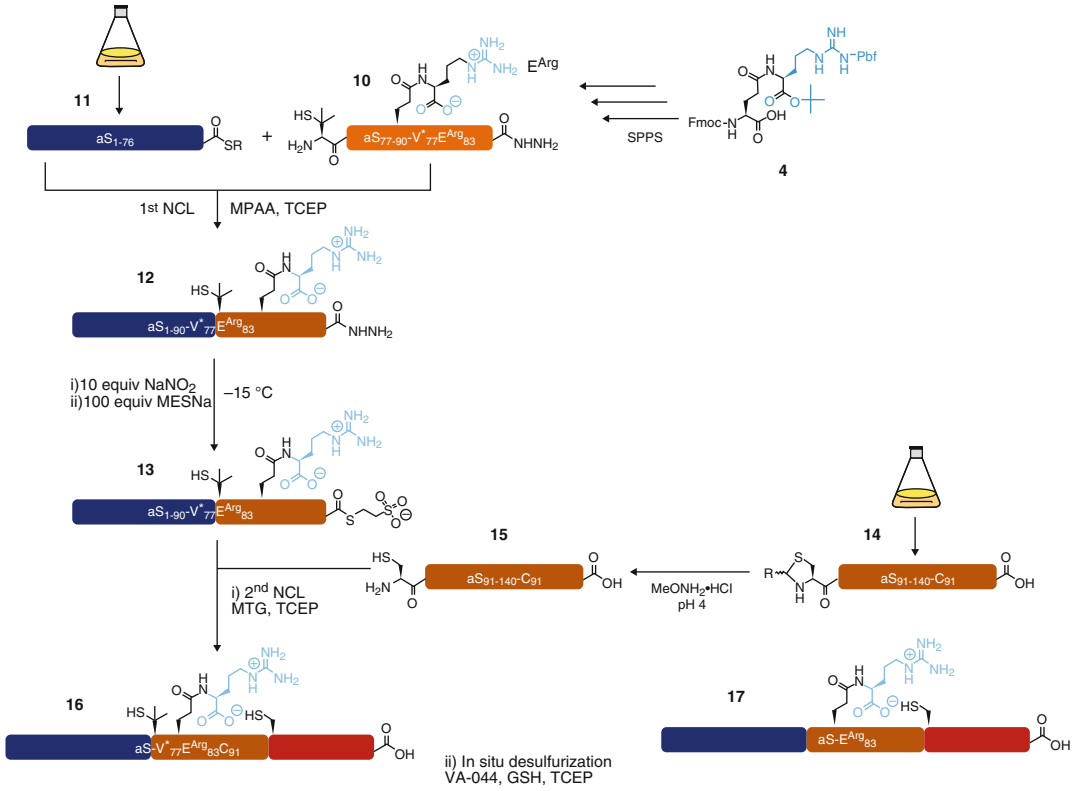

Fig. 5 Semi-synthesis of αS-E^{Arg}_{83}

3.4.1 NCL 1: Ligation Between $\alpha S_{1–76}$-Thioester and $\alpha S_{77–90}$- $V^*_{77}E^{Arg}_{83}$-Hydrazide

1. Degas pH 7 NCL buffer. Redissolve $\alpha S_{1\text{-}76}$-MES thioester (7.2 mg; 789 nmol; 1 equiv) and $\alpha S_{77\text{-}90}$- $V^*_{77}E^{Arg}_{83}$-hydrazide (5.8 mg; 3.16 μmol; 4 equiv) in NCL buffer pH 7 (see Subheading 2.6.) to the final concentration of 1–3 mM. Prepare stock solution of MPAA and TCEP (see Subheading 2.6). *See* **Note 6** on pH. *See* **Note 12** on equivalents.
2. Add MPAA to 100 mM and TCEP to 40–50 mM final concentrations. Adjust reaction pH to 6.8–7.0.
3. Incubate the reaction at 37 °C with agitation at 500 rpm for overnight. Monitor the reaction by MALDI-MS, and supplement TCEP as necessary.
4. Reduce the reaction with a few μL of the TCEP stock, and let it sit at room temperature for ~ 10 min before purification by RP-HPLC over a C4 column.
5. Check the identify of fractions by MALDI-MS, combine desired products, and lyophilize. We got 61% yield from this NCL. MALDI characterization of the product can be found in Fig. 7a

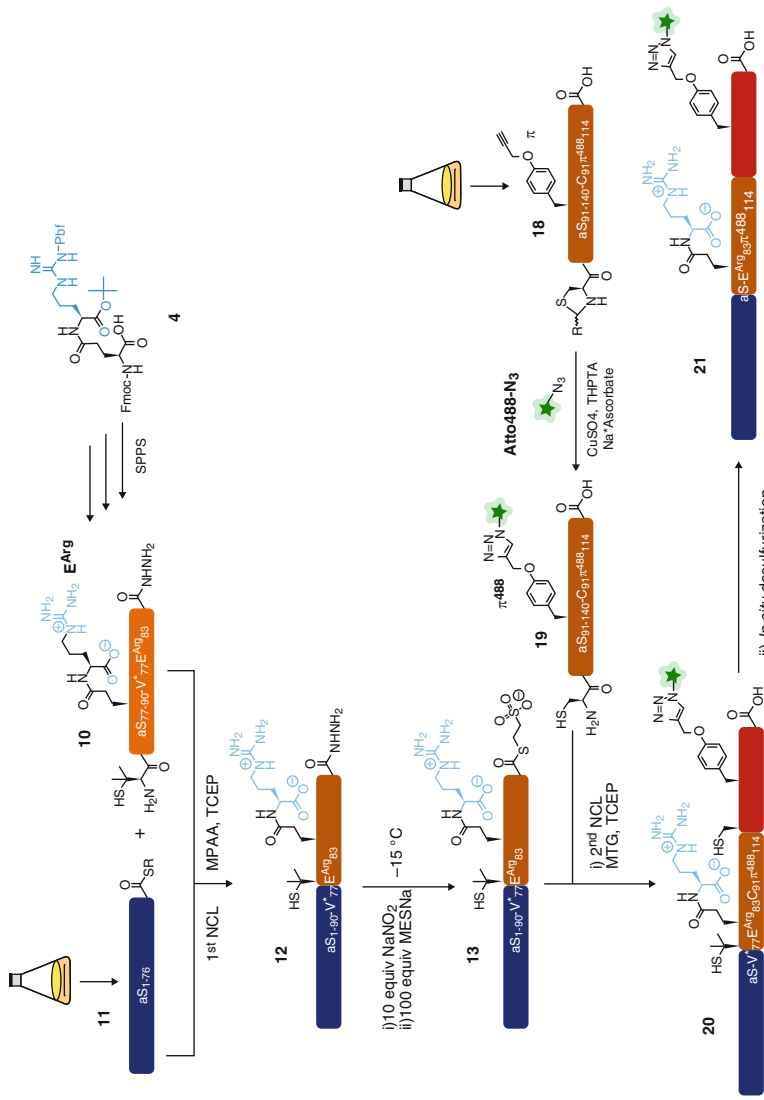

Fig. 6 Semi-synthesis of αS-$E^{Arg}_{83}\pi^{488}_{114}$ (with fluorescent labeling)

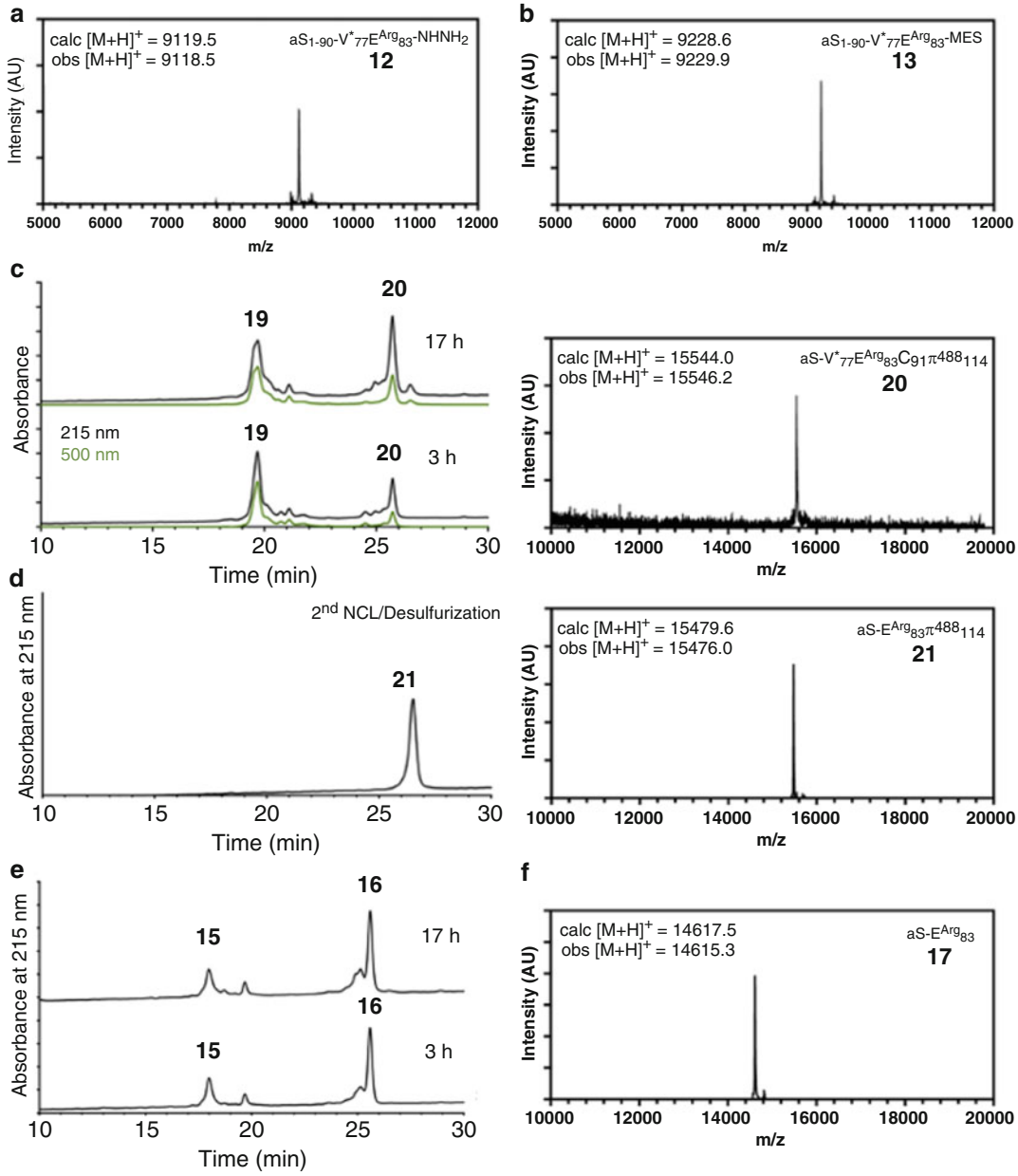

Fig. 7 Synthesis of αS-E$^{Arg}_{83}$ and αS-E$^{Arg}_{83}\pi^{488}_{114}$: (**a**) MALDI-MS of product αS_{1-90}-V*$_{77}$E$^{Arg}_{83}$-NHNH$_2$ (6) resulting from NCL between αS_{1-76}-MES (**11**) and αS_{77-90}-V*$_{77}$E$^{Arg}_{83}$-NHNH$_2$ (**10**); (**b**) MALDI-MS of conversion of intermediate **12** to αS_{1-90}-V*$_{77}$E$^{Arg}_{83}$-MES (**13**); (**c**) analytical HPLC (gradient, 10–50% B over 30 min) and MALDI-MS of MTG-mediated NCL between αS_{1-90}-V*$_{77}$E$^{Arg}_{83}$-MES (**13**) and αS_{91-140}-C$_{91}\pi_{488}$^{114} (**19**) to give αS- V*^{77}E^{Arg83}C$^{91}\pi^{488}_{114}$ (**20**); (**d**) analytical HPLC and MALDI-MS of in situ desulfurized product αS-E$^{Arg}_{83}\pi^{488}_{114}$ (**21**); (**e**) analytical HPLC (gradient, 10–50% B over 30 min) of MTG-mediated NCL between αS_{1-90}-V*$_{77}$E$^{Arg}_{83}$-MES (**13**) and αS_{91-140}-C$_{91}$ (**15**) to give αS- V*^{77}E^{Arg83}C^{91} (**16**); (**f**) MALDI-MS of in situ desulfurized product αS-E$^{Arg}_{83}$ (**17**)

3.4.2 NCL 2: Ligation Between $\alpha S_{1–90}$-$V^*_{77}E^{Arg}_{83}$-Hydrazide and $\alpha S_{91–140}$-C_{91}/$\alpha S_{91–140}$-$C_{91}\pi^{488}{}_{114}$ Followed by Desulfurization, Using MPAA as a Thiol Additive

The second ligation involves thioesterification of $\alpha S_{1–90}$-$V^*_{77}E^{Arg}_{83}$-hydrazide, ligation of the resulting thioester with $\alpha S_{91–140}$-C_{91}/ $\alpha S_{91–140}$-$C_{91}\pi^{488}{}_{114}$, and desulfurization of the ligation product αS- V^*_{77} $E^{Arg}_{83}C_{91}$ / $\alpha S_{91–140}$- V^*_{77} $E^{Arg}_{83}C_{91}\pi^{488}{}_{114}$ (i.e., conversion of cysteines and penicillamines used in ligation to the respective native alanines and valines). We describe two strategies for this: Here, the use of 4-mercaptophenylacetic acid (MPAA) as a thiol additive allows thioesterification and NCL without purifying the intermediate thioester, thanks to its quenching ability of excess sodium nitrite. The ligation product needs to be purified before desulfurization reaction due to radical quenching ability of MPAA [25]; in Subheading 3.4.3, the use of methyl thioglycolate (MTG) as a thiol additive allows NCL and desulfurization reaction in a one-pot manner, due to its poor ability of radical quenching [26]. As MTG thioester is not very stable and MTG is not potent in quenching oxidation agents, the acyl hydrazide starting material is converted to a MES thioester and purified. MES thioester goes through in situ conversion to MTG thioester once NCL is set up. Since the latter strategy only requires HPLC purification of full-length protein once, it is potent especially when the NCL yield is low.

This section describes NCL 2 using MPAA as a thiol additive. An alternative procedure using MTG as a thiol additive is described in Subheading 3.4.3.

1. Dissolve the product of NCL1, or the peptide acyl-hydrazide (111 nmol), in pH 3 NCL buffer (see Subheading 2.6.) for a final concentration of 2–3 mM and chill to −15 °C in an ice-salt bath. Make $NaNO_2$ stock solution (see Subheading 2.6). *See* **Note 6** on pH. *See* **Note 12** on equivalents.
2. For hydrazide to azide conversion, add 10 equiv. $NaNO_2$ and agitate by magnetic stirring for 15 min at −15 °C. Degas pH 7 NCL buffer and make MPAA and TCEP stock solution (see Subheading 2.6).
3. Add 40–50 equiv. MPAA to the mixture and then the partner peptide (150 nmol). Warm the reaction to room temperature and adjust the pH to 6.8–7.0.
4. Add TCEP to 40 mM final concentration and incubate the reaction at 37 °C with agitation at 500 rpm for a few h. Monitor product formation by MALDI-MS.
5. Purify the product by RP-HPLC over a C4 column.
6. Check the identify of fractions by MALDI-MS, combine desired products, and lyophilize. We got 32% yield over two steps.

7. Degas pH 7 NCL buffer and redissolve the purified NCL product in pH 7 NCL buffer to the final concentration of 0.4–0.8 mM.
8. Make stock solution of glutathione (GSH), TCEP, or radical initiator VA-044 (see Subheading 2.6).
9. Mix the NCL product with GSH, TCEP, and VA-044 to the final concentration of 100 mM GSH, 250 mM TCEP, and 20 mM radical initiator VA-044. Incubate in an argon-purged tube at 37 °C overnight.
10. Monitor product formation by MALDI-MS.
11. Purify the product by RP-HPLC over a C4 column.
12. Check the identify of fractions by MALDI-MS, combine desired products, and lyophilize. We got 4.8% yield over three steps (unlabeled construct), calculating from the peptide acyl hydrazide.

3.4.3 NCL 2: Ligation between αS_{1-90}-$V^*_{77}E^{Arg}_{83}$-Hydrazide and αS_{91-140}-C_{91}/ αS_{91-140}-$C_{91}\pi^{488}{}_{114}$ Followed by Desulfurization, Using MTG as a Thiol Additive

1. Dissolve the product of NCL1, or the peptide acyl-hydrazide (5.10 mg; 481 nmol), in pH 3 NCL buffer (see Subheading 2.6.) for a final concentration of 2–3 mM and chill to −15 °C in an ice-salt bath. Make $NaNO_2$, MESNa, and TCEP stock solutions (see Subheading 2.6). *See* **Note 6** on pH. *See* **Note 12** on equivalents.
2. For hydrazide to azide conversion, add 10 equiv. $NaNO_2$ and agitate by magnetic stirring for 15 min at −15 °C.
3. Add 100 equiv. MESNa to the mixture. After 10 min, warm the reaction to room temperature and adjust the pH to 6.8–7.0.
4. Add TCEP to 40 mM final concentration, check the pH and incubate the reaction at room temperature for 30 min. Monitor product formation by MALDI-MS.
5. Purify the MES thioester product by RP-HPLC over a C4 column.
6. Check the identify of fractions by MALDI-MS, combine desired products, and lyophilize. MALDI characterization of this product can be found in Fig. 7b.
7. Degas NCL buffer pH 7. Redissolve the purified intermediate, bearing C-terminal MES thioester (unlabeled - 1.90 mg; 177 nmol, labeled - 2.10 mg; 196 nmol), together with its ligation partner (unlabeled - 0.85 mg; 152 nmol, labeled - 1.05 mg; 162 nmol) to a 2 mM final concentration. Make a stock dilution of MTG and a stock solution of TCEP (see Subheading 2.6).
8. Add 100 equiv. MTG and supplement with TCEP to a 40 mM final concentration. Adjust pH to 6.8–7.0.

9. Incubate the reaction at 37 °C with agitation at 500 rpm for a few h. Monitor product formation by MALDI-MS and analytical HPLC (Fig. 7c: labeled construct; Fig. 4e: unlabeled construct).
10. Prepare for one-pot desulfurization. Dilute NCL reaction mixture to the final protein concentration of 0.4–0.8 mM. Prepare stock solution of VA-044, GSH, and TCEP (see Subheading 2.6).
11. Mix the NCL reaction mixture with GSH, TCEP, and VA-044 to the final concentration of 100 mM GSH, 250 mM TCEP, and 20 mM radical initiator VA-044. Incubate in an argon-purged tube at 37 °C overnight.
12. Monitor product formation by MALDI-MS.
13. Purify the product by RP-HPLC over a C4 column.
14. Check the identify of fractions by MALDI-MS, combine desired products, and lyophilize. For unlabeled construct, we got 61% yield over two steps from MES thioester. For the labeled construct, we got 29% yield over two steps. Characterization of the labeled product by MALDI and/or analytical HPLC can be found in Fig. 7d (labeled) and Fig. 4f (unlabeled).

4 Notes

1. It is important that peptide synthesis reagents are dry. For reagents that need to be stored in a fridge or a freezer, leave the bottles out for at least 30 min before opening them so that they can warm to prevent condensation.
2. Other inteins such as DnaE inteins could alternatively be used [27, 28]. For each protein of interest, it is recommended that expression yield and cleavage efficiency are compared between different intein fusions.
3. As alternative alkyne-bearing unnatural amino acids, pyrrolysine analogues, such as N^{ε}-butynyloxycarbonyllysine [29] or N^{ε}-*o*-ethinyl-benzyloxycarbonyl-lysine [30], can be incorporated using an orthogonal pyrrolysyl-tRNA synthetase or its mutant.
4. Unnatural amino acid mutagenesis could also be done using autoinducing media or LB media. The temperature for protein expression could be tuned, as well. The choice of media should be made based on previous results with the same incorporation machinery or individual yield optimization for each protein of interest.

5. A lot of other thiol additives have been used in NCL [10]. MPAA is the most used due to its affordability and ease of handling, but other aryl thiols such as 4-hydroxythiophenol or 3-mercaptobenzyl sulfonate could be used as an alternative. For alkyl thiols, 2,2,2-trifluoroethanethiol (TFET) could be used instead of MTG. MESNa thioesters are primarily used for storage purposes because of the high stability. The choice of additive can be determined by preferred reactivity of the thioester but could also be affected by availability, handling preferences, or the retention time of the NCL components/products.
6. To adjust the pH of stock solutions for NCL or NCL reaction, add small volumes of NaOH at a time, vortex, and check pH. Repeat this until you get the ideal pH. It is important to not basify the solution more than necessary. In the case of the NCL reaction, excessively high pH will result in faster hydrolysis of thioester. Also, too much addition of NaOH and compensation with HCl can result in unnecessarily high salt concentration that may affect the solubility of reaction components.
7. When working up step 2, the presence of palladium may lead to the formation of an emulsion layer, depending on the scale. Filtering the reaction through Celite helps get rid of palladium and suppress emulsion.
8. Peptide synthesis can be stopped at different points. For short-term stoppage, coupling reactions can be let go overnight or resin can be stored at room temperature after washes following either coupling or capping, but storing peptides with deprotected termini should be avoided. For long-term storage, perform extensive washes with DCM and vacuum for at least 30 sec to dry out resin and keep it in −20 °C. Before resuming the synthesis, swell resin in v1:1 DMF/DCM for at least 30 min and perform washes. On another note, especially for synthesis of longer peptides, keep resin loading up to 40% of the capacity to prevent interchain association, which may cause aggregation.
9. Other approaches for randomizing sequence during peptide synthesis include the split-and-pool approach [31], the spot-synthesis approach [32], and the tea-bag approach [33]. Factors to determine an appropriate method for each application would be labor intensity, reproducibility, the preferred size of the library, or the synthesis scale.
10. Biotin or the cross-linker could be attached at a different position of the peptide. Optimization of linker length is necessary to make sure that biomolecules are pulled down and to achieve proximity sensing.

11. In vivo cleavage of N-terminal residues by MAP or other aminopeptidases is dependent on sequence context [34] and potentially structural conformation, meaning that cleavage does not work as described above all the time. If you have troubles, you could change the ligation site or alternatively encode an N-terminal tag that can be cleaved off during purification process to expose N-terminal cysteine. The alternative method will also bypass thiazolidine deprotection step.
12. Adding excess C-terminal fragment will often speed up ligation and improve the yield, especially when the thioester peptide is prone to circularization or hydrolysis. The excess C-terminal peptide can be recovered after the reaction unless desulfurization is performed without purifying the ligation product.

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Chapter 23

Reconstitution of the Arginyltransferase (ATE1) Iron-Sulfur Cluster

Verna Van and Aaron T. Smith

Abstract

As global regulators of eukaryotic homeostasis, arginyltransferases (ATE1s) have essential functions within the cell. Thus, the regulation of ATE1 is paramount. It was previously postulated that ATE1 was a hemoprotein and that heme was an operative cofactor responsible for enzymatic regulation and inactivation. However, we have recently shown that ATE1 instead binds an iron-sulfur ([Fe-S]) cluster that appears to function as an oxygen sensor to regulate ATE1 activity. As this cofactor is oxygen-sensitive, purification of ATE1 in the presence of O_2 results in cluster decomposition and loss. Here, we describe an anoxic chemical reconstitution protocol to assemble the [Fe-S] cluster cofactor in *Saccharomyces cerevisiae* ATE1 (*Sc*ATE1) and *Mus musculus* ATE1 isoform 1 (*Mm*ATE1–1).

Key words Arginylation, Arginyltransferases, Iron-sulfur clusters, Anoxic reconstitution, Chemical reconstitution, Ferrozine assay

1 Introduction

Arginylation is the enzymatic addition of the amino acid arginine to a protein, and this posttranslational modification (PTM) is becoming recognized as essential in maintaining eukaryotic cellular homeostasis [1–3]. Because of its significance, it is also critical to understand the regulation of this key PTM. Recent studies have shown that arginylation is regulated by, and responds to, oxidizing small molecules such as dioxygen (O_2) and nitric oxide (NO). The sensing of NO is tied to the ability of this small molecule to oxidize the thiol(ate) Cys side chain to sulfinic acid, which serves as a recognizable side chain substrate for ATE1 [4]. Additionally, arginylation regulates a pVHL-independent O_2-sensing pathway by arginylation of hypoxia-inducible factor α (HIF1α) in yeast [5], plants, and mammals [6]. Most recently, our lab has discovered a new regulatory paradigm in which ATE1 itself is a sensor of O_2 that is mediated by the presence of an [Fe-S] cluster; this cluster is

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coordinated by four Cys residues that have been shown to be essential to activity of the enzyme [7, 8]. Among other critical and notable observations, the identity of the [Fe-S] cluster is O_2-sensitive: when reconstituted under anoxic conditions, the cluster is [4Fe-4S] in its composition, whereas once exposed to O_2, the cluster transmutes into a [2Fe-2S] composition through a [3Fe-4S] intermediate composition. These observations are strikingly similar to bacterial [Fe-S] cluster proteins that are either known to or putatively sense O_2 [9, 10]. In this protocol, we outline the steps for successful anoxic reconstitutions of *Saccharomyces cerevisiae* ATE1 (*Sc*ATE1) and *Mus musculus* ATE1 isoform 1 (*Mm*ATE1–1). The process begins with the transfer of the reagents, proteins, and materials into the anoxic environment of a glove box. Iron is then added to the samples in a stepwise manner, followed by the stepwise addition of a sulfide donor in a manner that minimizes precipitation of insoluble iron sulfide and protein aggregation. Excess iron sulfide is then removed from the samples by buffer exchange and/or gel filtration. The [Fe-S] cluster binding is assessed by electronic absorption spectroscopy, and iron quantitation is determined by the spectrophotometric ferrozine assay. This protocol leads to successful incorporation of a [4Fe-4S] into recombinant forms of *Sc*ATE1 and *Mm*ATE1–1, which can be used for further downstream analyses.

2 Materials

2.1 Chemical Reconstitution

1. Anaerobic chamber (operating at $\leq$5 ppm O_2).
2. Mettler Toledo ME54TE analytical balance.
3. Thermo Scientific Heraeus Pico 17 microcentrifuge with a 24 x 1.5/2.0 mL ClickSeal rotor.
4. Eppendorf ThermoMixer® C.
5. Cary 60 UV-Vis spectrophotometer.
6. Degassed water obtained from a Milli-Q water system.
7. Reconstitution buffer: 50 mM Tris (pH 7.5), 100 mM KCl, 5% (v/v) glycerol, 10 mM DTT.
8. 100 mM $FeCl_3$ solution: Measure 27.0 mg of $FeCl_3 \bullet 6H_2O$ into a clean 1.5 mL Eppendorf tube. Add 1.00 mL of water. Make fresh each time (*see* **Note 1**).
9. 100 mM Na_2S solution: Measure 24.0 mg of $Na_2S \bullet 9\ H_2O$ into a clean 1.5 mL Eppendorf tube. Add 1.00 mL of reconstitution buffer. Make fresh each time (*see* **Note 1**).
10. 2.0 M KCl solution: Dissolve 37 g of KCl in 250 mL deionized water.

11. Corning® Costar® Spin-X® centrifuge tube filters (0.22 μm pore size).
12. MilliporeSigma™ Amicon™ Ultra-0.5 centrifugal filter units 30 kDa molecular weight cutoff (MWCO).
13. VWR® two-sided disposable UV-transparent plastic cuvettes or a reusable quartz cuvette, both with 1 cm pathlengths.
14. Eppendorf tubes.
15. Spatula or scoopula.
16. Recombinant *Sc*ATE1 and/or *Mm*ATE1–1, expressed and purified from *Escherichia coli*, as described in [7].

2.2 Ferrozine Assay

1. Cary 60 UV-Vis spectrophotometer.
2. Thermo Scientific Heraeus Pico 17 microcentrifuge with a 24 × 1.5/2.0 mL ClickSeal rotor.
3. VWR® two-sided disposable UV-transparent plastic cuvettes or a reusable quartz cuvette, both with 1 cm pathlengths.
4. Degassed water obtained from a Milli-Q water system.
5. 50% (m/v) trichloroacetic acid (TCA).
6. 75 mM sodium ascorbate solution: Dissolve 15 mg sodium ascorbate in 1.00 mL deionized water. Make fresh each time.
7. 10 M ammonium acetate solution: Dissolve 77 g ammonium acetate in 100 mL deionized water.
8. 100 mM ferrozine: Dissolve 49.2 mg ferrozine in 1.00 mL deionized water (*see* **Note 2**).

3 Methods

3.1 Bringing Materials into Anaerobic Chamber

1. Bring large volumes of solutions, chemicals, and plastics into the glove box well in advance. Let large volumes of solutions, chemicals, and plastics equilibrate with the atmosphere of the glove box for several days to several weeks (*see* **Note 3**).
2. Bring small volumes of solutions such as proteins into the glove box the night before planning reconstitution. Let the small volumes of solutions such as proteins equilibrate with the atmosphere of the glove box overnight while shaking at lowered temperatures (6–8 °C) (*see* **Note 3**).

3.2 Chemical Reconstitution of Protein

1. Use Table 1 to calculate the final volume of the reaction for a protein concentration of 100 μM. Then, use the calculated final volume to determine the volume of each component to add according to each final concentration. For $FeCl_3$ and Na_2S, divide the volume by 4 (*see* **Note 4**).

Table 1
Sample table outlining volumes for reconstitution mixtures. The volumes of both $FeCl_3$ and Na_2S are typically divided by 4, and the resulting volumes are used for stepwise addition of each solution

	Initial concentration	Final concentration	Volume
Protein		100 μM	
$FeCl_3$	10 mM	400 μM	
Na_2S	10 mM	400 μM	
KCl	2 M	300 mM	
Buffer	–	–	
		Final volume:	

2. Make fresh 1.00 mL stocks of 100 mM $FeCl_3$ and Na_2S (*see* **Note 1**).
3. Make 1 mL of 1:10 dilutions of both the 100 mM $FeCl_3$ and Na_2S.
4. In a clean 1.5 mL Eppendorf tube, add your protein, buffer, and KCl (in that order). Put the tube on the ThermoMixer (*see* **Note 5**).
5. Add $FeCl_3$ to the mixture stepwise in four parts, with 10–15 min of shaking at 300 rpm at 6–8 °C between each addition (*see* **Notes 6** and **7**).
6. Add Na_2S to the mixture stepwise in four parts, with 10–15 min of shaking at 300 rpm at 6–8 °C between each addition (*see* **Notes 6** and **7**).

3.3 Removal of Excess Iron Sulfide

1. Once both the iron- and sulfide-containing solutions have been added, incubate the reaction with shaking at 300 rpm at 6–8 °C for approximately 2 h to allow for equilibration.
2. After 2 h, spin down the mixture in a microcentrifuge at 21,100 x*g* at 4 °C for 10 min to pellet any precipitate.
3. Pipet the supernatant into a fresh Costar® spin filter, and spin at 1500 x*g* at 4 °C for 10 min to filter the protein solution of particulates.
4. Transfer 400 μL of the spun-down protein into a 0.5 mL Millipore™ spin concentration and spin at 9600 x*g* at 4 °C for 10 min.
5. Repeat as necessary until all the protein is concentrated to ≈100 μL.
6. Buffer exchange the protein to remove excess iron sulfide by adding ≈400 μL of fresh reconstitution buffer, pipetting up and down to dilute, and spinning at 9600 x*g* at 4 °C for 10 min.

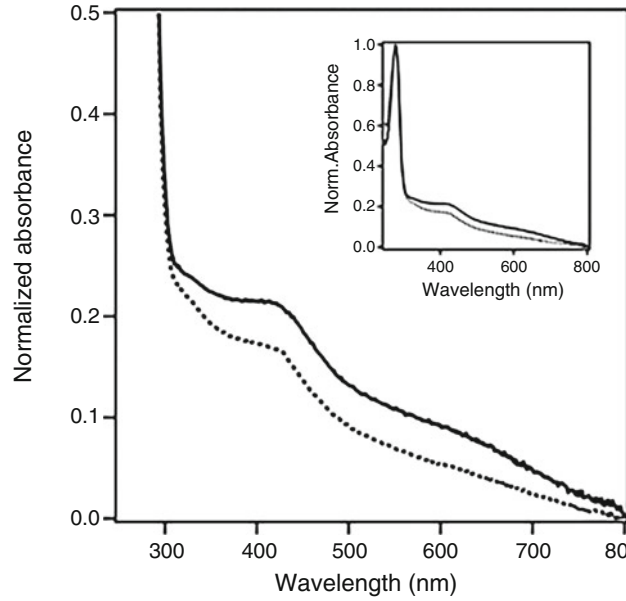

Fig. 1 Electronic absorption spectra of anoxically reconstituted *Sc*ATE1 (solid line) and *Mm*ATE1–1 (dashed line) demonstrate the presence of a [4Fe-4S] cluster. A broad absorption envelope centered approximately at 405 nm is common for [4Fe-4S] clusters. Inset: The full absorption spectra of both proteins are shown and are normalized to the most intense protein absorption peak at 280 nm (A_{280})

7. Repeat at least four times (*see* **Note 8**).
8. Protein can then be analyzed directly, or the protein can be incubated on the ThermoMixer® overnight with 300 rpm shaking at 6–8 °C.
9. If the protein is equilibrated overnight, an additional filtration step using a Costar® spin filter is recommended prior to use.

3.4 Electronic Absorption Spectroscopy and the Ferrozine Assay to Assess Cluster Loading

1. Make a 1:10 dilution of the sample to examine via electronic absorption. The protein is typically a golden brown color after [Fe-S] cluster incorporation (Fig. 1), and the electronic absorption spectrum typically exhibits a broad absorption maximum centered around 405 nm (Fig. 2).
2. Thaw a 100 mM ferrozine stock. Make a fresh 1:10 stock with deionized water.
3. Make fresh sodium ascorbate stock.
4. Calculate and measure out 12.5 nmol of protein; make a note of this volume (designated here as "X").
5. Denature the protein by adding 0.2X of 50% TCA; vortex the tube to mix (*see* **Note 9**).

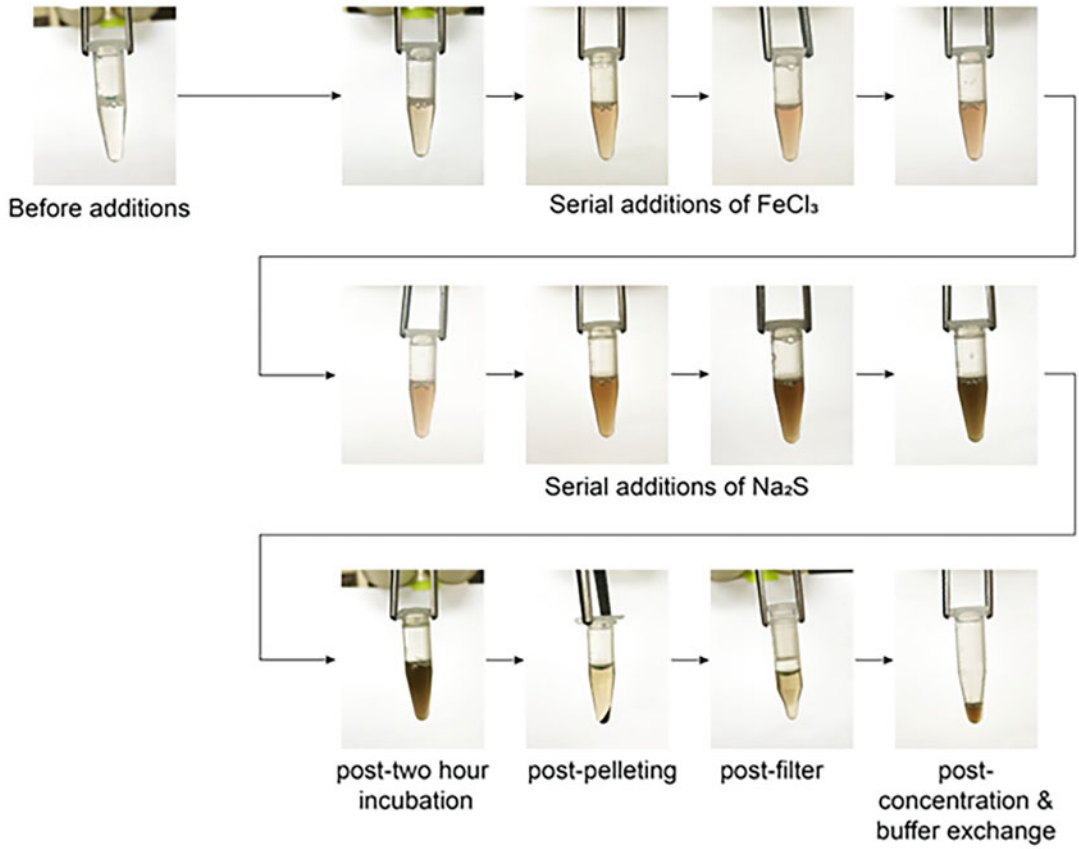

Fig. 2 Diagram of the anoxic reconstitution workflow coupled with photographs demonstrating color changes to the protein solution (here *Sc*ATE1) upon serial Fe^{3+} additions, serial S^{2-} additions, and the post-reconstitution processing of sample

6. Spin sample down at 21,100 x*g* for 10 min at room temperature.
7. In newly labeled tubes, prepare assay samples according to Table 2.
8. After the precipitated protein has been pelleted, transfer the supernatant to the corresponding assay sample (*see* **Note 10**) and vortex.
9. Use the spectrophotometer to read absorbance at 562 nm (A_{562}), blanked against the control.
10. Calculate the molar equivalents of Fe(II) per polypeptide with the conversion factor: Absorbance of 1 at $A_{562} \approx 25$ nmole of Fe(II) (based on a molar extinction coefficient, ε, of 28 mmol L^{-1} cm^{-1}) [7, 11].

Table 2
Table outlining component volumes for both control and sample reactions, where "X" denotes the volume of the sample containing 12.5 nmole of protein

	Control	Sample
Deionized water	500 – X (μL)	500 – X (μL)
75 mM sodium ascorbate	20 μL	20 μL
10 mM ferrozine	20 μL	20 μL
Saturated ammonium acetate	120 μL	120 μL
Supernatant	–	X
Buffer	X	–

4 Notes

1. Pay attention to the colors of the solutions. To get a better view of the solution color, hold the tube up to eye level with one hand, with a paper towel, Kimwipe, or white paper behind it. The stock solution of $FeCl_3$ is a yellow, translucent color and will be lighter, depending on the concentration. If you observe red-orange precipitation, remake the solution. The Na_2S stock solution should be colorless. If you see that the Na_2S solution is a gray color, remake the solution.
2. The ferrozine may take a while to dissolve. This 100 mM stock of ferrozine may be frozen and stored at −20 °C in the dark for future assays. The stock solution should be a deep yellow color.
3. For larger volumes of buffers, degas prior to bringing the bottles into the anaerobic chamber. Once in the anaerobic chamber, remove the cap and stir with a stir plate at least overnight.
4. The iron and sulfide sources are added separately and slowly (stepwise) to limit precipitation of iron sulfide, which is easily observed as a black precipitate. The order and the amount of time between each addition can be adjusted and may require optimization for different proteins.
5. When mixing the solutions for anoxic reconstitution, it is helpful to prechill the buffer and KCl stock in 1.5 mL Eppendorf tubes, since some proteins are temperature-sensitive. In our hands, *Sc*ATE1 is much more temperature sensitive than *Mm*ATE1–1.
6. After each addition of $FeCl_3$ and Na_2S, gently flick the bottom of the tube to mix. This step facilitates mixing of the solution better than just the use of a ThermoMixer, and this process is gentler than vigorous vortexing.

7. The color of the reconstituted sample should change over time. By the end of all four additions of $FeCl_3$, the solution should be a light pink-orange color. As the Na_2S is added, the mixture should transition to a golden brown color indicative of the [Fe-S] cluster. You may see some black precipitate in the mixture from insoluble iron sulfide that will settle at the bottom of the tube. The colored solutions should be clear; if the solution looks cloudy, protein precipitation may be occurring, and the buffer conditions may need to be adjusted. In particular, we have found that *Mm*ATE1–1 is sensitive to salt and may require a higher concentration of salt than *Sc*ATE1.
8. Gel filtration across a Sephadex column, such as a PD-10 column to perform the iron and sulfide removal is an alternative; however, the protein will need to be concentrated afterwards due to dilution.
9. Upon addition of 50% TCA, you should be able to confirm that the protein has been precipitated by visualization of an opaque, white solution that turns into a white pellet after centrifugation.
10. Upon addition of the supernatant, the sample should turn a shade of magenta. Qualitatively, deeper colored samples contain more iron, while lighter samples have less iron.

Acknowledgments

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Chapter 24

N-Terminal Arginylation Pull-down Analysis Using the R-Catcher Tool

Taewook Seo, Goeun Han, and Hyunjoo Cha-Molstad

Abstract

Protein arginylation is a unique and under-explored posttranslational modification, which governs many biological functions and the fate of affected proteins. Since ATE1 was discovered in 1963, a central tenet of protein arginylation is that arginylated proteins are destined for proteolysis. However, recent studies have shown that protein arginylation controls not only the half-life of a protein but also various signaling pathways. Here, we introduce a novel molecular tool to elucidate protein arginylation. This new tool, termed R-catcher, is derived from the ZZ domain of p62/sequestosome-1, an N-recognin of the N-degron pathway. The ZZ domain, which has been shown to strongly bind N-terminal arginine, has been modified at specific residues to increase specificity and affinity for N-terminal arginine. R-catcher is a powerful analysis tool allowing researchers to capture the cellular arginylation patterns under various stimuli and conditions, thereby identifying potential therapeutic targets in numerous diseases.

Key words Arginylation, R-catcher, Pull-down

1 Introduction

Protein arginylation is typically mediated by an enzyme named arginyltransferase 1 (ATE1) in a ribosome-independent manner. ATE1 can conjugate an arginine residue to exposed N-terminal aspartic acid, glutamic acid, or oxidized cysteine residue generated by enzymatic action [1]. Recent studies indicate that protein arginylation occurs constitutively or in an inducible manner in response to cellular stresses, and its pattern may differ depending on cell types. Therefore, to determine the role of arginylation in various diseases, it is vital to identify the change in the arginylation pattern induced by the disease state, thus providing potential disease biomarkers. Unfortunately, there were no molecular tools or methods to comprehensively detect cellular arginylated proteins prior to our study published in 2021, since previous methods utilizing antibodies directed against either Nt-Arg-Asp or Nt-Arg-Glu [2] cannot

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detect NT-Arg-Cys or NT-Arg-X generated through endoproteolytic cleavage.

R-catcher is a robust and economical tool that can comprehensively and reproducibly detect cellular arginylated proteins. In addition, it can be stably expressed in cells to catch arginylated proteins in vivo. R-catcher is derived from the ZZ domain of p62/sqstm1, which binds Nt-Arg of BiP induced in response to cytosolic foreign DNA or proteasomal inhibition [3–5]. Binding analysis has revealed that the p62-ZZ domain possesses an unusually high binding affinity for Nt-Arg, although it also binds other type-1 basic N-degrons, such as Lys and His, as well as type-2 bulky/aromatic N-degrons, such as Tyr, Trp, and Phe [4]. Therefore, we exploited the ZZ domain's high affinity for Nt-Arg to build a molecular tool to precisely capture arginylated proteins in cells.

We started with the p62-ZZ domain fragment 83–175, which has similar N-degron binding characteristics as full-length p62. First, this fragment was minimized to 122–175 (p62-$ZZ_{122–175}$), which exhibited a significant binding affinity for only Nt-Arg and Nt-Tyr. Then, using the insights from two studies that solved x-ray crystal structures of the p62-ZZ domain complexed with Nt-Arg peptides [6, 7], we substituted N132 for D132. This substitution significantly lowered the affinity of p62-$ZZ_{122\text{-}175}^{N132D}$ for Nt-Tyr to undetectable levels in pull-down assays, whereas the fragment's affinity for Nt-Arg was significantly increased [8]. Next, we optimized pull-down assays by adding a small Twin-Strep-tag to the N-terminus of p62-$ZZ_{122\text{-}175}^{N132D}$, generating the final R-catcher tool with a molecular weight of approximately 9 kDa.

R-catcher exhibits a high binding affinity for Nt-Arg with a K_D of 2.85 μM, at least 20 times higher than any other N-terminal residue as judged by our ITC analysis. R-catcher also successfully pulled down known arginylated model substrates, including R-nsP4, R-RGS4, R-BiP, and R-PDI expressed in cells. Using HeLa cells treated with MG132 plus thapsigargin (TG), a condition previously seen to induce the arginylation of ER proteins [3, 4], we coupled R-catcher pull-down with LC-MS/MS analysis, which led to the identification of 59 known and putative arginylated proteins. As we predicted, novel ATE1-dependent and independent arginylated ER proteins were identified. Analyzing these proteins and their interactions with other proteins from our pull-down revealed their involvement in many critical biological processes. Therefore, R-catcher represents a valuable tool to unlock the arginylome, revealing novel disease targets.

2 Materials

1. *Escherichia coli* BL21(DE3) cells.
2. 2X YT media or LB media for bacterial growth.
3. IPTG.
4. PMSF (phenylmethylsulfonyl fluoride) and protease inhibitor cocktail (e.g., Roche, 5056489001 or equivalent).
5. R-catcher lysis buffer: 50 mM Tris–HCl pH 8.0, 300 mM NaCl, 10% glycerol.
6. R-catcher elution buffer: 50 mM Tris–HCl pH 8.0, 300 mM NaCl, 250 mM imidazole, 10% glycerol.
7. R-catcher conjugation buffer: 100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA (Please add this buffer after R-catcher elution buffer if possible).
8. Plasmid DNA for expression R-catcher (pETDUT1 R-catcher (Twin strep-His-$ZZ_{122\text{-}175}^{N132D}$)).
9. Ultrasonic homogenizer.
10. Purification system (AKTA series or other FPLC purification systems).
11. Strep-Tactin XT resin (IBA, 2-5030-025).
12. RA and AR dipeptides (BACHEM).
13. Bestatin.
14. Rotator (360° rotation).
15. Rocking shaker.
16. Pull-down assay buffer: 20 mM HEPES pH 7.9, 200 mM KCl, 10% glycerol, 0.05% Tween 20.
17. D-biotin.
18. Hypotonic buffer: 20 mM HEPES pH 7.6, 200 mM KCl, 10% glycerol, 0.01% NP-40.
19. 4–20% gradient TGX mini precast gel (e.g., Biorad, 4561094)
20. Silver staining kit (ELPIS Biotech, EBP-1051).
21. Invitrosol™ LC/MS Protein Solubilizer Kit (e.g., Thermo Fisher Scientific, MS10007).

3 Methods

3.1 Expression and Purification of R-Catcher

1. Transform pETDUT Twin-Strep-His6-$ZZ_{122\text{-}175}^{N132D}$ (R-catcher: WT or D129A) into BL21(DE3) competent cells.
2. The next day, inoculate six to eight colonies into 20 mL 2X YT media in a 200 mL flask.
3. Culture cells at 37 °C for 3–4 h, rotating at 200 rpm.

4. Measure the OD of the culture. Then, dilute the culture to an OD600 of 0.01 to a final volume of 1 L in a 2 L flask using 2X YT media.
5. Incubate the culture at 37 °C, rotating at 200 rpm until the OD600 is 0.5 ~ 0.6, and then add 1 mM IPTG to the culture.
6. Incubate the culture at 18 °C at 200 rpm, overnight. The next day, harvest the cells when the OD600 is 1.8.
7. Weight the cell pellet. Add 10 mL of lysis buffer per gram of cell pellet.
8. Thoroughly resuspend the cell pellet and add 100 μM PMSF.
9. Lyse cells with sonication using the following cycle: 30 amplitude 5-s pulse followed by a 15-s rest, repeat for 20 min.
10. Centrifuge the sample at 15,000 × g for 30 min to pellet cell debris.
11. Filter the supernatant through a 0.22 μm syringe filter into a fresh tube.
12. Inject the filtered sample into a His-NTA purification column (AKTA or Ni-NTA resin).
13. Wash the column with 100 mL of 10 mM imidazole-containing buffer to remove nonspecific bindings.
14. Elute the R-catcher protein with slowly increasing concentrations of imidazole at a flow rate of 1 mL/min to a final concentration of 250 mM (Fig. 1a) (*see* **Note 1**).
15. Measure the protein concentration using a Bradford assay, or check purity with coomassie blue staining (Fig. 1b).
16. Store the purified R-catcher protein at −20 °C (*see* **Note 2**).

3.2 Immobilization of R-Catcher on Resin

1. Pipette out 120 μL of Tactin resin (50% slurry) into a 1.5 mL tube. Spin the resin at 1500 × g for 3 min. Discard the supernatant. Resuspend the resin in 1 mL of R-catcher conjugation buffer. Incubate at 4 °C with rotation for 10 min. Repeat three times.
2. Add 300 μg of purified R-catcher protein into the 1 mL resuspended resin (*see* **Note 3**).
3. Incubate R-catcher immobilized resin for at least 3 h with gentle rotation at 4 °C.
4. Wash the R-catcher immobilized resin three times with 1 mL of R-catcher conjugation buffer.
5. Spin the resin at 1500 × g for 3 min and discard the supernatant. Resuspend the resin in 1 mL pull-down assay buffer, and incubate for 1 h with gentle rotation at 4 °C.
6. Spin the resin at 1500 × g for 3 min and discard the supernatant. Add 60 μL of pull-down assay buffer to make a 50% slurry.

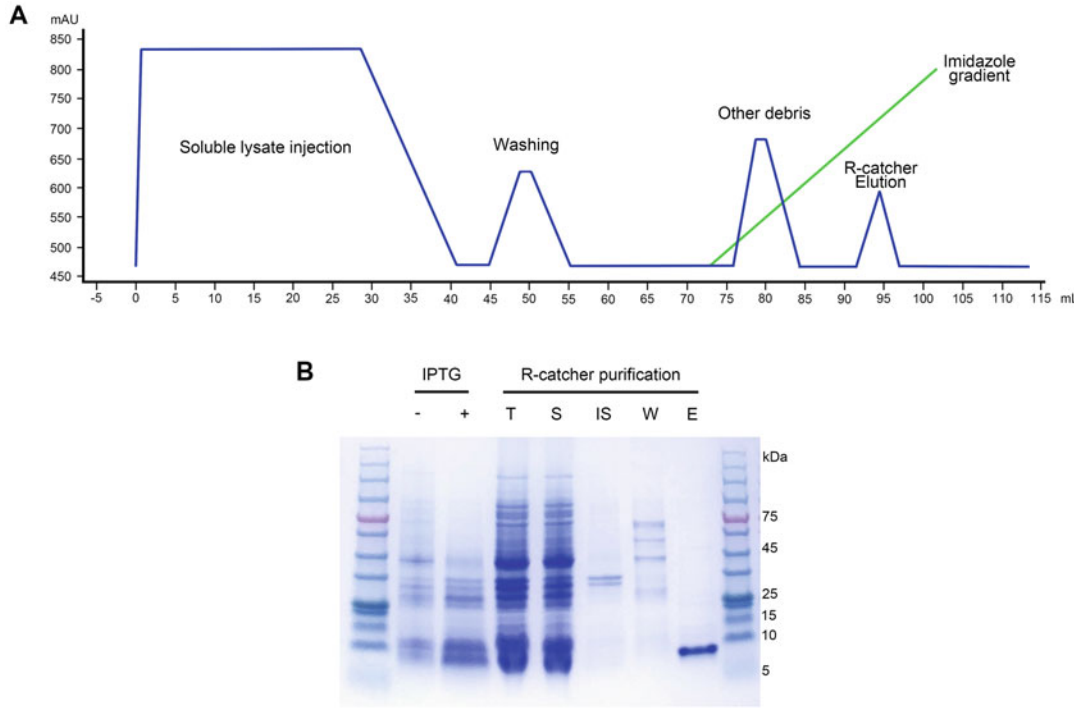

Fig. 1 Purification of R-catcher recombinant proteins. (**a**) R-catcher purification chromatogram. Soluble fraction from BL21(DE3) lysates apply to an AKTA FPLC machine with His-trap HP column. Washing step used 20 mM (8% from final 250 mM imidazole) imidazole. The imidazole gradient increased from 0% to 100%; the second peak contains R-catcher proteins. (**b**) Coomassie staining analysis of R-catcher purification. R-catcher is 9 kDa. 4–20% gradient SDS-PAGE gel used for separation

3.3 Preparing Cell Lysates for R-Catcher Pull-down

3.3.1 Induction and Analysis of Endogenous Arginylated Proteins

1. Culture HeLa cells in 10 cm culture plates in DMEM with 10% FBS under standard conditions.
2. When the cells reach 80% confluency, add 10 μM MG132 plus 100 nM thapsigargin and incubate for 24 h to induce arginylated proteins.
3. Wash cells with 10 mL ice-cold DPBS.
4. Harvest cells with a 10 cm scraper in 10 mL PBS and transfer to a 15 mL conical tube.
5. Spin down the cells at 2500 × g for 5 min.
6. Aspirate the supernatant and resuspend in 5× volume of hypotonic buffer and transfer to a 1.5 mL microcentrifuge tube.
7. Freeze and thaw three times.
8. Centrifuge at 13,000 × g to pellet cell debris for 20 min.
9. Transfer the supernatant to a fresh 1.5 mL microcentrifuge tube.
10. Measure the protein concentration using a Bradford assay.

11. Transfer 250 μg of proteins into 60 μL R-catcher conjugated resin prepared in Subheading 3.2 (*see* **Note 4**).
12. Add pull-down assay buffer to a final volume of 1 mL (*see* **Note 5**).
13. Incubate at least 3 h with gentle rotation at 4 °C.
14. Spin down the resin and wash three times with 1 mL pull-down assay buffer.
15. Add 1× volume of 2× sample loading buffer (60 μL) and boil 10 min at 100 °C.
16. Separate eluted proteins in an SDS-PAGE.
17. Perform a Western blot using the desired antibodies.

3.3.2 Preparation and Analysis of Exogenous Arginylated Proteins

1. Transfect plasmid DNA encoding arginylated model substrates into cells using lipofectamine 2000 following manufacturer's instructions. Then, 18 h later, treat cells with 5 μM bortezomib for an additional 24 h (*see* **Note 6**).
2. Harvest and lyse cells as described in Subheading 3.3.1, **steps 4–10**, to prepare target proteins (*see* **Note 7**).

3.4 R-Catcher Pull-down Assay with Peptide Competition

1. Prepare 50–350 μg of total protein lysates depending on the expression level of target proteins (prepared in Subheading 3.3.2) (*see* **Note 8**).
2. Incubate 60 μL of R-catcher conjugated resin (prepared in Subheading 3.2) with 25 mM RA or AR dipeptide in 1 mL pull-down assay buffer with gentle rotation for 1 h at 4 °C.
3. Spin down beads and carefully aspirate the supernatant. The pellet at this point contains R-catcher conjugated resin blocked with dipeptides.
4. Add protein lysates prepared in step 1 to the pellet from step 3 (R-catcher conjugated resin blocked with dipeptides) in 1 mL pull-down assay buffer containing 40 μM bestatin plus either 25 mM RA or 25 mM AR dipeptide (*see* **Note 9**).
5. Incubate the sample with gentle rotation for 3 h at 4 °C.
6. Then, spin down the resin at 1500 × g for 3 min.
7. Carefully aspirate the supernatant and wash the resin three times with 1 mL pull-down assay buffer.
8. The elution methods may differ, depending on the analysis methods used. *See* Subheading 3.5.

3.5 Analysis of R-Catcher Pull-down Proteins

1. Add 60 μL 2× sample loading buffer into 60 μL resin (packed volume).
2. Boil the sample for 10 min at 100 °C.

3.5.1 Western Blotting

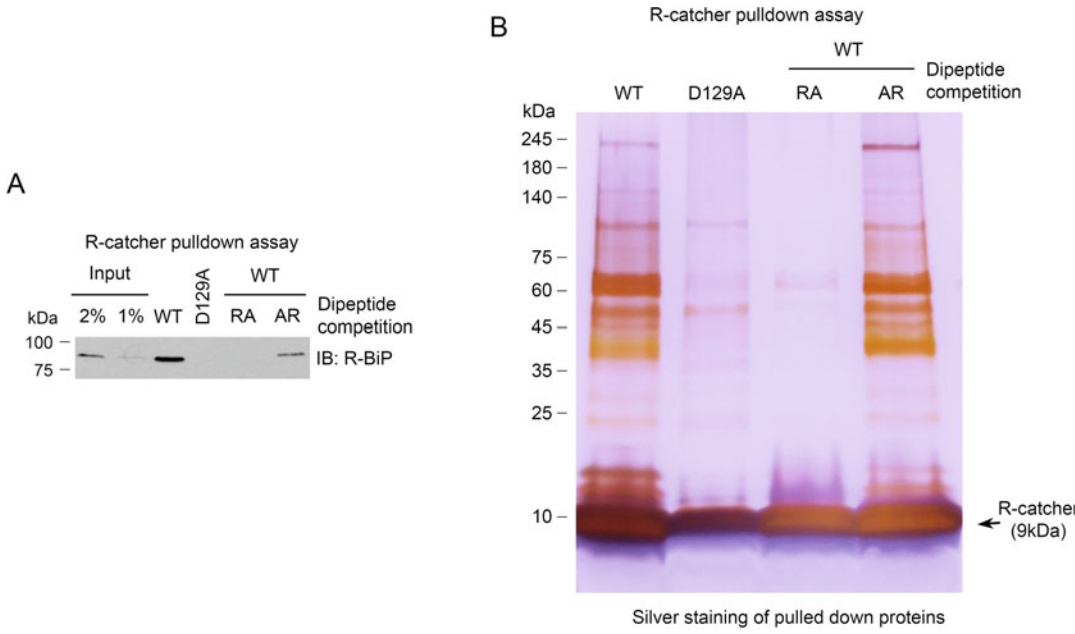

Fig. 2 Analysis of arginylated protein. (**a**) Arginylated-protein analysis using a Western blot. Labels are abbreviated; WT (wild type R-catcher), D129A (R-catcher D129A mutant), RA (Arg-Ala dipeptide), AR(Ala-Arg dipeptide). R-BiP(arginylated-BiP proteins) antibody ordered from AbFrontier (AR05MA0001). (**b**) Silver staining result of R-catcher pull-down assay. Putative arginylated protein could be considered the intersection between WT (R-catcher wild type) and AR (Ala-Arg) bands. The arrow indicated R-catcher bands

3. Separate eluted proteins onto an 8–15% SDS-PAGE, depending on the molecular weight of target proteins.
4. Use suitable antibodies to detect proteins of interest (Fig. 2a).

3.5.2 Silver Staining

1. Add 300 μL (5× volume of resin volume) R-catcher elution buffer to 60 μL resin.
2. Incubate samples in rocking shaker at 2000 rpm for 10 min (*see* **Note 10**).
3. Spin down the resin at 1500 × g for 3 min.
4. Transfer eluents into fresh 1.5 mL microcentrifuge tubes (*see* **Note 11**).
5. Dilute the sample with an appropriate volume of 2× sample loading buffer.
6. Separate eluted proteins onto 4–20% gradient SDS-PAGE.
7. Avoid gel contact with any iron materials (*see* **Note 12**).
8. Stain gels using the silver staining kit following the manufacturer's instruction (Fig. 2b).

3.6 R-Catcher Pull-down Assay for LC/MS Analysis

3.6.1 Resin Preparation

1. Immobilize 25 mg of a purified R-catcher protein on 2.5 mL Tactin resin (packed volume). Add R-catcher conjugation buffer to a final volume of 25 mL. Repeat the process for the mutant D129A negative control.
2. Incubate at 4 °C with gentle rotation overnight.
3. Wash the resin three times with 25 mL pull-down assay buffer. For each wash, rotate at 4 °C for 5 min. Spin at 845 × g for 3 min.
4. Resuspend resin in 5 mL pull-down assay buffer to make a 50% slurry.
5. Divide the R-catcher resin into three 15 mL conical tubes (each tube: 1.5 mL 50% slurry).
6. Block the resin with 25 mM RA or AR peptides in 13 mL pull-down assay buffer containing 40 μM bestatin. Incubate 1 h at 4 °C with gentle rotation.
7. Spin the resin at 845 × g for 3 min at 4 °C.
8. Discard the supernatant and store the resin on ice.

3.6.2 Cell Lysate Preparation

1. Lyse cells in Hypotonic Buffer.
2. Add 32 mg of cell lysates to 2.5 mL D129A conjugated resin (packed volume). Bring the final volume to 25 mL using pull-down assay buffer and incubate for 3 h at 4 °C with rotation to preclear nonspecific bindings.
3. Spin the resin at 845 × g for 3 min at 4 °C.
4. Transfer the supernatant into a fresh 50 mL conical tube (precleared sample).
5. Wash the D129A resin three times with 25 mL of pull-down assay buffer and store at 4 °C for later use.

3.6.3 R-Catcher Pull-down of Arginylated Proteins

1. Add 10 mg of the precleared sample made in Subheading 3.6.2 to 0.75 mL R-catcher conjugated resin (packed volume) in 12 mL of pull-down assay buffer for each sample (no competition, RA competition, AR competition). Add 25 mM RA or AR dipeptides and 40 μM bestatin to the RA and AR competition samples.
2. Incubate 3 h with gentle rotation at 4 °C.
3. Spin the resin at 845 × g for 3 min at 4 °C. Then, wash the resin three times with 10 mL pull-down assay buffer.
4. Add 10 mL pull-down assay buffer containing 10 mM biotin and rotate for 1 h at 4 °C to elute bound proteins (*see* **Note 13**).
5. Spin the resin at 845 × g for 3 min at 4 °C. Then, transfer the supernatant to a 20 kDa MW cutoff dialysis membrane.

6. Dialyze in 2.5 L ddH2O containing 2% SDS + 1% β-mercaptoethanol for 1 h at 4 °C to remove excess R-catcher protein. Repeat this step with overnight incubation.
7. Then, dialyze in 5 L ddH_2O for 1.5 h at 4 °C to remove SDS and β-mercaptoethanol. Repeat this step.
8. Transfer samples from the dialysis membrane into a 50 mL conical tube and lyophilize the sample.
9. Dissolve lyophilized proteins in 100 μL Invitrosol LC/MS protein solubilizer.

4 Notes

1. In this step, if you start with a high imidazole concentration, R-catcher protein may precipitate, causing low yield. If you use the AKTA purification system, set the elution buffer gradient 1–100% for 40 min. One hundred percent means 250 mM imidazole.
2. Frozen purified R-catcher protein is stable for up to 1 month at −20 °C and up to 6 months at −80 °C.
3. Recombinant R-catcher protein has a Twin-Strep-tag on its N-terminus. For conjugating R-catcher proteins with resin, we want to recommend using the Strep-Tactin XT agarose resin (IBA, 2-5030-025). You do not need to remove imidazole from the purified R-catcher at this step. Our results have shown that imidazole does not affect the binding of Twin-Strep to Tactin resin.
4. When D129A is used as a negative control, the D129A purification method is the same as R-catcher purification.
5. If the lysate volume exceeds 100 μL, add an equal volume of 2× pull-down buffer to avoid diluting the pull-down buffer. Then, bring the volume up to 1 mL using 1× pull-down buffer.
6. Exogenous proteins contain a C-terminal tag (usually c-Myc tag) for detection. Since arginylated proteins typically have a short half-life in cells, cells expressing a target protein need to be treated with a proteasome inhibitor such as 5 μM bortezomib or MG132 to obtain a high protein expression.
7. Lysis conditions may require slight modification, depending on the subcellular localization of target proteins.
8. Using an excessive volume of cell extracts can interfere with R-catcher binding to arginylated proteins. Protein concentrations needed for the pull-down assay must be optimized depending on the target protein. Typically, 250 μg is used as a starting point.

9. Competition with RA dipeptide is utilized as a means to confirm protein arginylation. If the binding of a target protein is competed out by RA dipeptide but not by AR dipeptide, the protein is assumed to possess Nt-Arg.
10. Avoid 360° rotation to elute bound proteins due to insufficient mixing of the resin with the elution buffer in a 1.5 mL tube, resulting in lower elution efficiency.
11. Removing D-biotin from your eluted sample using the Amicon filtration system may improve silver staining results.
12. Iron or magnetic materials can cause adverse effects on silver staining. Always use clean plastic or glass wear while wearing nitrile gloves to avoid them.
13. Bound proteins on D129A resin (0.75 mL) will also be eluted in this step.

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Chapter 25

Identification of Protein Arginylation by Encapsulated N-Terminal Peptide Enrichment Method

Shinyeong Ju and Cheolju Lee

Abstract

Mass spectrometric analysis of N-terminal peptides reveals altered amino acid sequences at the protein's N-terminus and the presence of posttranslational modifications (PTM). Recent advancement in enriching N-terminal peptides facilitates the discovery of rare N-terminal PTMs in samples with restricted availability. In this chapter, we describe a simple, single-stage oriented N-terminal peptide enrichment method that helps the overall sensitivity of N-terminal peptides. In addition, we describe how to increase the depth of identification, to use software to identify and quantify N-terminally arginylated peptides.

Key words N-terminal arginylation, N-terminal peptide enrichment, Mass spectrometry, Proteomics, N-terminomics, iNrich

1 Introduction

Protein N-terminal arginylation (Nt-arginylation) is a very rare phenomenon, and therefore, it is challenging to find a peptide with such a modification through conventional bottom-up proteomics, due to the technical limitation of liquid chromatography-coupled mass spectrometry (LC-MS) technology on which current proteomics relies heavily [1]. Although antibodies that recognize N-terminal arginylation are available in Western blotting, whether the antibodies can also be used in affinity chromatography to enrich Nt-arginylated peptides is unknown [2, 3]. Thus, LC-MS after chemical enrichment of N-terminal peptides is the most appropriate method for identifying Nt-arginylation at present, despite the method's lack of selectivity for Nt-arginylation.

There are several methods of N-terminomics that enrich N-terminal peptides [4]. COFRADIC and TAILS, for example, are well-known methods that have been widely practiced since their introduction [5, 6]. The N-terminomics methods consist of three main experimental steps: blocking of all primary amines,

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digestion of proteins into peptides using a protease such as trypsin, and removal of internal peptides generated during digestion. In principle, since only internal peptides would have exposed primary amines following digestion, any beads with amine-reactive functional groups might be used to remove internal peptides while leaving the original protein N-terminal peptides undisturbed.

Here, we present a workflow for Nt-arginylation analysis, in which N-terminal peptides are obtained through our own N-terminal peptide enrichment method, iNrich [7]. iNrich enriches N-terminal peptides from 25–5000 μg proteins, depending on extensive prefractionation. The key advantage of iNrich over the aforementioned methods is that iNrich achieves the maximum identification of N-terminal peptide by utilizing a single-staged reactor, which lessens sample loss throughout the enrichment procedure (Fig. 1). Due to the rarity of Nt-arginylation, the N-terminal peptides thus obtained are mainly acetylated or have D_3-acetylation introduced in the blocking step of free amines. To identify bona fide Nt-arginylation, we apply a protocol with several optimizations while analyzing LC-MS/MS spectra. In brief, mass spectra are first searched for common N-terminal modifications, including Nt-acetylation and Nt-D_3-acetylation, and then for Nt-arginylation using only those spectra that are not filtered for target-decoy validation. It is probable that a peptide spectrum annotated with this modification is misannotated due to the indistinguishable mass difference between arginylation (+156.1011 Da) and combinations of amino acid residues, glycine and valine (+156.0899 Da), for example (Fig. 2) [1]. Therefore, among the spectra annotated as Nt-arginylated, those spectra in which the preceding residue(s) in the original sequence is R, GV, or VG are discarded. The detailed procedure follows. We present the experimental methods in two ways according to the amount of sample. Follow Subheadings 3.2 and 3.3 when the amount is small (for 25–200 μg), and Subheadings 3.4 to 3.6 when it is large (for 200 μg–5 mg). Subheadings 3.1, 3.7, and 3.8 are common.

2 Materials

1. Lysis and thiol blocking.
2. Water, HPLC grade.
3. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 0.5 M solution.
4. Cell lysis buffer: 50 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS) pH 8, 6 M guanidine, 10 mM TCEP, 40 mM 2-chloroacetamide, 1× Halt Protease Inhibitor (*see* **Notes 1**).

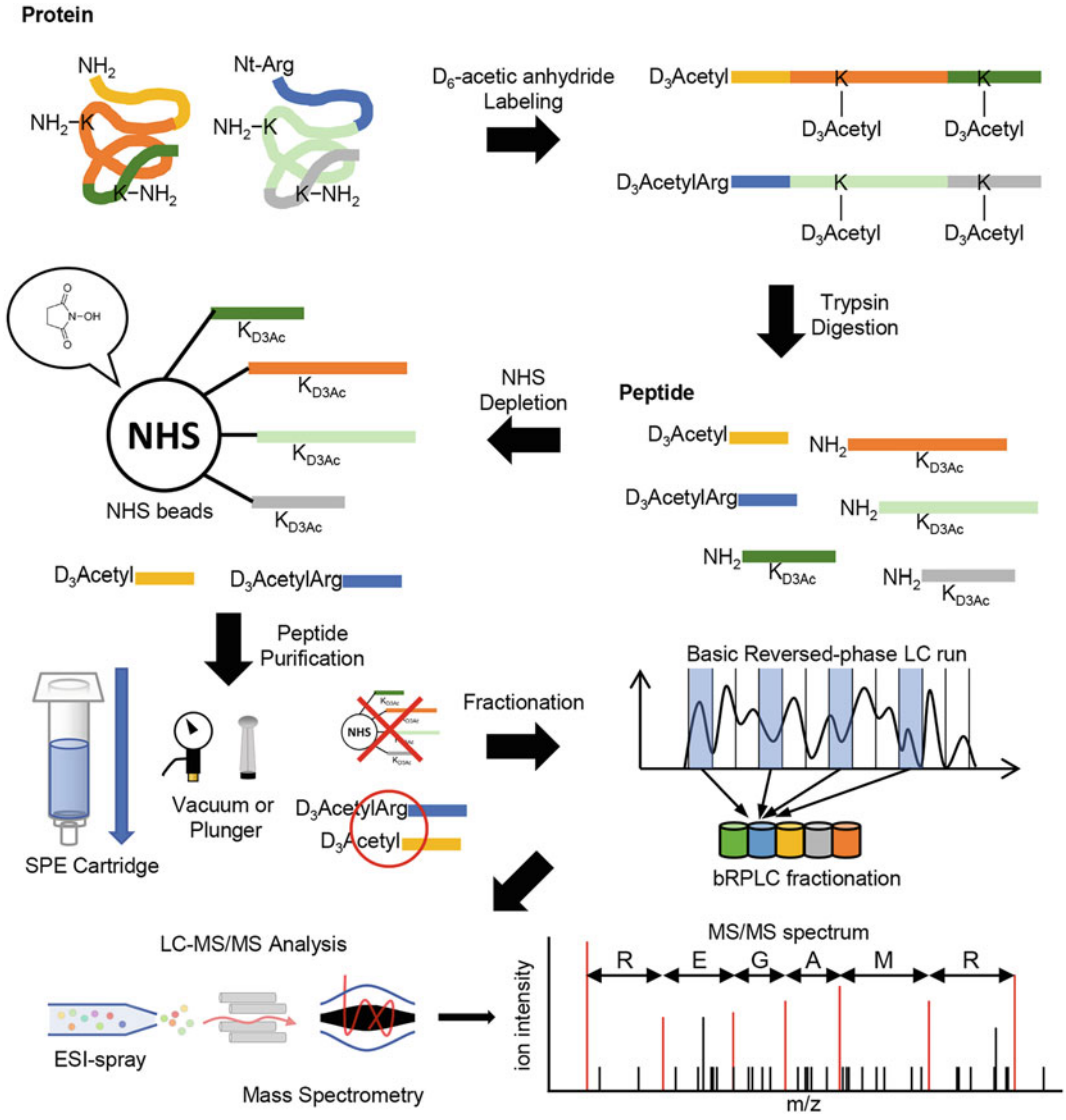

Fig. 1 Overview of N-terminal peptide enrichment procedure described in this chapter. Primary amines of proteins are labeled before digestion. Hence, only the internal peptides generated after digestion will have a primary amine, which in turn are captured by N-hydroxysuccinimide (NHS)-activated agarose beads by nucleophilic substitution. The beads are retained during solid-phase extraction (SPE)

5. Thermomixer (Eppendorf 5382000015) with well plate holder.
6. Ultrasonic obliterator with microtip.
7. Temperature controllable tabletop microcentrifuge (>12,000 × *g*).
8. Acetone, −20 °C.
9. Methanol, −20 °C.

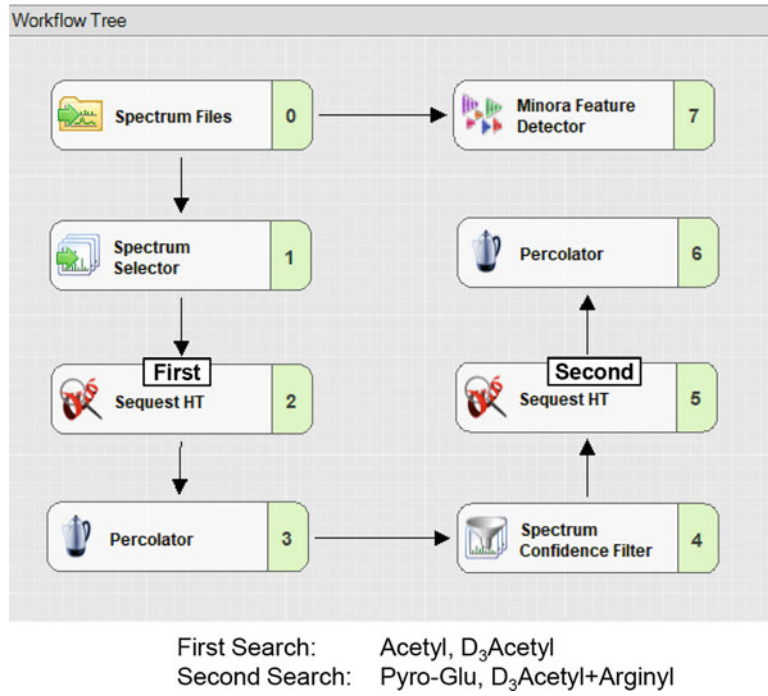

Fig. 2 Overview of nodes for processing workflow in proteome discoverer. The spectra matched in the first search are filtered out for further analysis. Target-decoy validation node (percolator) cannot validate the mass spectra properly when there are limited counts of spectra. To ensure proper validation, pyro-glu modification is included in the second search

10. Protein lysis buffer: 200 mM EPPS pH 8, 6 M guanidine hydrochloride (GuHCl).
11. Bicinchoninic acid (BCA) assay kit (23225, Thermo Fisher Scientific).
12. Bovine serum albumin standard protein, 2 mg/mL in the ampule.
13. Labeling of primary amines and digestion to peptides (for 25–200 μg).
14. Polypropylene NMR tube cap (5 mm size, Z153281, Sigma-Aldrich).
15. 1 mL pipette tip holder.
16. D_6-acetic anhydride (175641, Sigma).
17. Pyridine (270970, Sigma).
18. 1 mL disposable pipette tip holder.
19. HLB 1 cc solid-phase extraction (SPE) cartridge (30 mg sorbent, WAT094225, Waters).
20. Acetonitrile, HPLC grade.

21. Equilibrium buffer: 200 mM EPPS pH 8.
22. Microcentrifuge tube rotator (SLRM-2 M, SeouLin Bioscience).
23. Trypsin solution in 1 mM HCl in 0.1 mg/mL (*see* **Notes 2**).
24. Labeling of primary amines and digestion to peptides (for 1 mg).
25. 5 mL Eppendorf tube.
26. HLB 6 cc solid-phase extraction (SPE) cartridge (200 mg sorbent, WAT106202, Waters).
27. Negative selection of N-terminal peptides (for 25–200 μg).
28. Pierce™ NHS-activated agarose spin columns, 33 mg × 25 (26198, Thermo Fisher Scientific).
29. (Optional) Vacuum manifolds (Waters).
30. 50 mM EPPS pH 8, 0.15 M NaCl.
31. Washing buffer: 0.1% trifluoroacetic acid (TFA), LCMS grade.
32. Elution buffer: 0.1% formic acid (FA), 50% acetonitrile, LCMS grade.
33. Vacuum evaporator.
34. Negative selection of N-terminal peptides (for 1 mg).
35. Pierce™ NHS-activated agarose spin columns, 330 mg × 5 (26199, Thermo Fisher Scientific).
36. Basic reversed-phase liquid chromatography (bRPLC) fractionation (for 1 mg).
37. 1290 Infinity HPLC system (Agilent).
38. Fraction collector+ (Agilent).
39. XBridge Peptide BEH C18 column, 130 Å, 3.5 μm, 4.6 mm × 250 mm (186003570, Waters).
40. XBridge BEH C18 guard column, 3.5 μm (186007768, Waters).
41. 96-well microplates+.
42. bRPLC stock buffer: 200 mM ammonium formate pH 10 (*see* **Notes 3**).
43. bRPLC buffer A: 10 mM ammonium formate pH 10.
44. bRPLC buffer B: 10 mM ammonium formate pH 10, 90% acetonitrile.
45. LC-MS/MS analysis.
46. Q Exactive mass spectrometer (Thermo Fisher Scientific).
47. UltiMate 3000 RSLCnano system (Thermo Fisher Scientific).
48. EASY-Spray column, 50 cm × 75 μm ID, PepMap RSLC C18, 2 μm (ES803, Thermo Fisher Scientific).

49. Reconstitution buffer: 0.1% FA, 2% acetonitrile, LCMS grade.
50. Ultrasonic bath (Branson).
51. Mobile phase A: 0.1% FA, LCMS grade.
52. Mobile phase B: 0.1% FA, 80% acetonitrile, LCMS grade.
53. Data analysis.
54. Proteome Discoverer software v2.4 (Thermo Fisher Scientific).

3 Methods

3.1 Cell Lysis and Thiol Blocking

1. Add cell lysis buffer to harvested cell. We recommend 300 μL per 20.0×10^6 cells (based cell count at confluency in 150 mm dish).
2. Boil the cell mixture in thermomixer for 10 min at 95 °C, set r.p.m. to 600.
3. Cool boiled cell mixture in ice.
4. Obliterate cell mixture using ultrasonication for 10 s on, 10 s off cycles for six times in prechilled aluminum cold block or ice.
5. Centrifuge at 12000 × *g* at 4 °C for 10 min.
6. Transfer supernatant to 5 mL centrifuge tube.
7. Add ice-cold acetone with eightfold volumes of transferred supernatant and ice-cold methanol with onefold volume.
8. Store the tube at −80 °C for at least 2 h.
9. Place the tube in ice to equilibrate temperature (*see* **Note 4**).
10. Centrifuge at 12000 × *g* at 4 °C for 10 min.
11. Carefully discard the supernatant and add 2 mL of ice-cold methanol, and mix.
12. Centrifuge at 12000 × *g* at 4 °C for 10 min.
13. Carefully discard the supernatant and add 2 mL of ice-cold methanol, and mix.
14. Centrifuge at 12000 × *g* at 4 °C for 10 min, and discard the supernatant.
15. Air-dry the pellet. *Important*: Excessive drying of the pellet may seriously impair protein solubilization.
16. Resuspend sample in 500 μL of protein lysis buffer.
17. Quantify protein concentration with Pierce BCA Assay Kit.
18. Dilute the protein lysate until its concentration reaches 4 μg/μL with protein lysis buffer.
19. Store the sample at −20 °C until further processing.

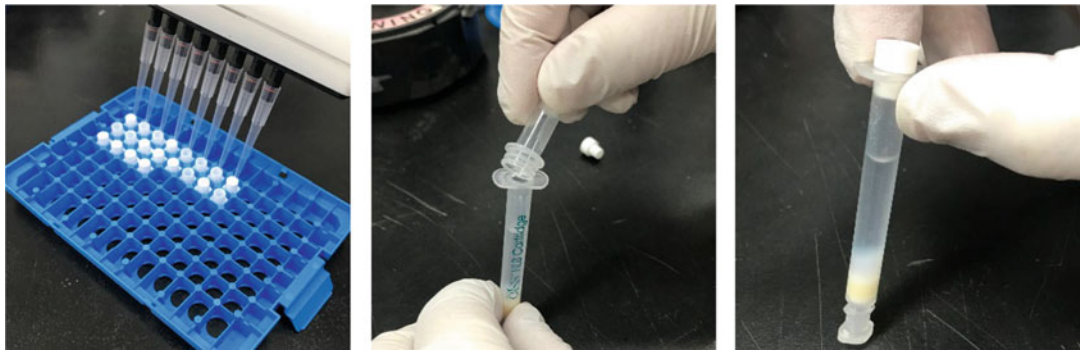

Fig. 3 Three images of iNrich method in practice. Image (left) shows NMR tube caps in 1 mL tip holder. Image (middle) describes how to pour NHS beads into an SPE cartridge. Image (right) shows a complete set of iNrich reactor plugged at both ends with a Luer plug (bottom) and an NMR tube cap (top)

3.2 Labeling of Primary Amines and Digestion to Peptides (for 25–200 µg)

1. Place polypropylene NMR tube cap into the rack (backside of typical 1 mL pipette tip holder) (Fig. 3).
2. Transfer 25–200 µg of proteins into an NMR tube cap.
3. Add 200 mM D_6-acetic anhydride and 200 mM pyridine. *Example*: 12.5 µL of the lysate (50 µg of protein), add 0.21 µL of pyridine (12.24 M) and add 0.25 µL of D_6-acetic anhydride.
4. Cover with any 96-well plate cover. Incubate at RT for 1 h.
5. Incubate at 30 °C at 600 r.p.m. for 1 h on a thermomixer.
6. Pre-activation of HLB 30 mg sorbent cartridge: Wash the cartridge three times with 1 mL of acetonitrile and equilibrate the cartridge three times with 1 mL of 0.2 M EPPS pH 8.
7. Plug the cartridge outlet with a Luer plug from waters or a plug supplied with NHS-activated spin columns.
8. Add a tenfold volume of 0.2 M EPPS pH 8 into the cartridge for dilution.
9. Connect NMR tube cap with the cartridge.
10. Incubate at RT for 1 h on a tube rotator at 50 r.p.m.
11. Add trypsin solution at an enzyme/substrate ratio of 1:50, and incubate at 37 °C for 2–4 h on a tube rotator set at 50 r.p.m. (*see* **Note 5**).

3.3 Negative Selection of N-terminal Peptides (for 25–200 µg)

1. Add 33 mg dry NHS-activated agarose beads from the spin column per 100 ug proteins directly into the SPE cartridge.
2. Incubate at RT for 2 h on a tube rotator at 50 r.p.m.
3. Place the cartridge on a vacuum manifold.
4. Remove the cap.
5. Transfer any residual solution in the cap into the cartridge.

6. Add 50 μL of 0.2 M EPPS pH 8 into the cap and scrape residual solutions with pipetting, and transfer to the cartridge.
7. Pre-activate a HLB 200 mg sorbent cartridge by washing the cartridge three times of 6 mL of acetonitrile.
8. Equilibrate the cartridge three times with 6 mL of 50 mM EPPS pH 8, 150 mM NaCl.
9. Hold the vacuum at the flow rate of 1 drop in 2–3 s.
10. Wash with 0.5 mL of 50 mM EPPS pH 8, 0.15 M NaCl.
11. Wash four times with 1 mL of 0.1% TFA in water.
12. Elute two times with 0.5 mL of 0.1% FA, 50% acetonitrile into a 1.5-mL Eppendorf tubes.
13. Dry the sample via vacuum centrifugation. And proceed to Methods 3.7 (LC-MS/MS Analysis).

3.4 Labeling of Primary Amines and Digestion to Peptides (for 200 μg–5 mg)

1. Transfer 1 mg of proteins into a 5 mL Eppendorf tube.
2. Add 200 mM D_6-acetic anhydride and 200 mM pyridine.
3. Incubate at 30 °C at 600 r.p.m. for 1 h on a thermomixer.
4. Add a tenfold volume of 0.2 M EPPS pH 8 into the cartridge for dilution.
5. Incubate at 30 °C at 600 r.p.m. for 1 h on a thermomixer.
6. Add trypsin solution at an enzyme/substrate ratio of 1:50, and incubate at 37 °C at 600 r.p.m. for 16 h on a thermomixer (*see* **Note 5**).
7. Negative selection of N-terminal peptides (for 25–200 μg).
8. Add 33 mg dry NHS-activated agarose beads from the spin column per 100 ug proteins directly into the SPE cartridge.
9. Incubate at RT for 2 h on a tube rotator at 50 r.p.m.
10. Place the cartridge on a vacuum manifold.
11. Remove the cap.
12. Transfer any residual solution in the cap into the cartridge.
13. Add 50 μL of 0.2 M EPPS pH 8 into the cap and scrape residual solutions with pipetting, and transfer to the cartridge.
14. Pre-activate a HLB 200 mg sorbent cartridge by washing the cartridge three times of 6 mL of acetonitrile.
15. Equilibrate the cartridge three times with 6 mL of 50 mM EPPS pH 8, 150 mM NaCl.
16. Hold the vacuum at the flow rate of 1 drop in 2–3 s.
17. Wash with 0.5 mL of 50 mM EPPS pH 8, 0.15 M NaCl.
18. Wash four times with 1 mL of 0.1% TFA in water.

19. Elute two times with 0.5 mL of 0.1% FA, 50% acetonitrile into a 1.5-mL Eppendorf tubes.
20. Dry the sample via vacuum centrifugation.

3.5 Negative Selection of N-Terminal Peptides (for 200 μg–5 mg)

1. Add 330 mg dry NHS-activated agarose beads from the spin column per 1 mg proteins into the 5 mL Eppendorf tube.
2. Incubate at RT at 1000 r.p.m. for 2 h on a thermomixer.
3. Place the HLB 200 mg sorbent cartridge on a vacuum manifold.
4. Pre-activate a cartridge by washing the cartridge three times of 6 mL of acetonitrile.
5. Equilibrate the cartridge three times with 6 mL of 50 mM EPPS pH 8, 150 mM NaCl.
6. Transfer the peptides and NHS beads in a 5 mL Eppendorf tube into the SPE column.
7. Bind the peptides to the HLB column with the use of a vacuum manifold and pump.
8. Add 2500 μL of 50 mM EPPS pH 8, 150 mM NaCl into the leftover 5 mL Eppendorf tube, and mix it thoroughly.
9. Transfer the residual solution of the 5 mL Eppendorf tube into the HLB column and flow it through.
10. Wash five times with 5 mL of 0.1% TFA in water.
11. Elute two times with 2.5 mL of 0.1% FA, 50% acetonitrile into 5 mL Eppendorf tubes.
12. Dry the sample via vacuum centrifugation.

3.6 bRPLC Fractionation (Only for 200 μg–5 mg)

The bRPLC fractionation is a well-established peptide separation method for LC-MS/MS analysis. Its procedure does not involve another desalting of the fractions, which improves overall sensitivity and reproducibility. Wang et al. provided an excellent explanation of the method [8]. We observed a fourfold increase in the overall identification of N-terminal peptides, as well as an improvement in the identification of N-terminally arginylated peptides [7].

1. Add 105 μL of 10 mM ammonium formate pH 10, 2% acetonitrile into the dried samples.
2. Mix using a vortex mixer and sonicate for 10 min in an ultrasonic bath.
3. Centrifuge the sample at 12000 × *g* at 4 °C for 10 min.
4. Transfer the supernatant to an LC autosampler vial.
5. Place two 96-well plates in the fractionator.
6. Inject 100 μL.

7. Use the following bRPLC parameters:
   - Flow rate: 0.5 mL/min.
   - Column temperature set point: 42.0 °C.
   - LC timetable: from 0% to 5% B in 10 min, from 5% to 40% B in 38.5 min, from 40% to 70% B in 14 min, hold at 70% B for an additional 10 min, from 70% to 5% B in 10 min, and hold at 5% B for 10 min.
   - Fractionator timetable: Time-based with 0.4 min time slices start in 10 min and stop in 77.2 min.
   - Fraction counts: 168.
8. Combine 168 fractions into 24 fractions by concatenating $n + 0$, $n + 24$, $n + 48$, $n + 72$, $n + 96$, $n + 120$, and $n + 144$ into 1.5 mL Eppendorf tube, where n is concatenated fraction number. The volume of a concatenated fractions is expected to 200 μL × 7 = 1.4 mL.
9. Dry the concatenated fractions in a vacuum concentrator for 1 h.
10. (Optional) Concatenate again between the adjacent concatenated fractions, for example, first and second concatenated fractions, to settle into 12 fractions.
11. Dry the concatenated fractions in a vacuum concentrator, and store at −20 °C until LC-MS/MS analysis.

3.7 LC-MS/MS Analysis

1. Reconstitute the samples in 5 μL of 0.1% FA, 2% acetonitrile solution, sonicate in an ultrasonic bath for 10 min, and spin down in a benchtop centrifuge at RT for 30 s.
2. Transfer the samples into autosampler vials.
3. Inject 2 μL of sample for LC-MS/MS analysis.
4. Notable MS parameters are as follows: MS2 parameter, fixed first mass of 100 m/z, and higher-energy collision dissociation (HCD), normalized collision energy of 32.

3.8 N-Terminal Arginylome Data Analysis

The chance to discover N-terminal arginylated peptides appear less likely in general [9], as a fragment spectrum would highly be likely to match with N-terminal acetylation or heavy N-terminal acetylation, which is labeled by D_6-acetic anhydride. To discover bona fide N-terminal arginylation peptides, a workflow for analyzing mass data described here comprises three major steps: searching the fragment spectra against N-terminal acetylation first, filtering out matched spectra in the first search, and searching the fragment spectra against N-terminal arginylation (Fig. 2). Proteome Discoverer™ 2.4 software was used in this protocol.

1. Prepare UniProt reference proteome database from the FTP site of UniProt, and upload it into Proteome Discoverer software [10].

2. Administration → Maintain Cleavage Reagents.
3. Click a checkbox for "Is Active" for Acetyl:2H (3) (+45.029395 Da). Add follow modifications and click checkboxes for "Is Active": ArgAcetylD3 (Delta Mass; +201.130506 Da, Delta Average Mass; +201.2409 Da, Substitution; H(11) 2H (3) C(11) N (4) O (2), Position; Any_N_Terminus, Amino Acid Site; Aspartic Acid, Glutamic Acid), ArgAcetylD3NQ (Delta Mass; +202.114522 Da, Delta Average Mass; +202.2257 Da, Substitution; H (10) 2H (3) C (11) N (3) O (3), Position; Any_N_Terminus, Amino Acid Site; Asparagine, Glutamine).
4. Modules for processing step workflow:
   (a) Spectrum Files.
   (b) Spectrum Selector.
   (c) Sequest HT, first:
      - Enzyme Name: Trypsin_R (Semi). Max. Missed Cleavage Sites: 2.
      - N-terminal modifications (peptide terminus): Acetyl/+42.011 Da (N-Terminus), Acetyl:2H (3)/+45.029 Da (N-Terminus).
      - Static modifications: Carbamidomethyl/+57.021 Da (C), Acetyl:2H (3)/+45.029 Da (K).
      - Dynamic modifications: Max. equal modifications per peptide; 3, Oxidation/+15.995 Da (M).
   (d) Percolator, first.
      - Target/Decoy Selection; Concatenated.
      - Validation based on; q-Value.
      - Target FDR (Strict); 0.01.
      - Target FDR (Relaxed); 0.05.
      - Spectrum Confidence Filter. Spectrum Confidence; Worse Than High.
   (e) Sequest HT, second.
      - Enzyme Name: Trypsin_R (Semi).
      - Max. Missed Cleavage Sites: 2.
      - N-terminal modifications (peptide terminus): Glu- > pyro-Glu/−18.011 Da (E).
      - ArgAcetylD3/+201.131 Da (D,E), ArgAcetylD3NQ/+202.115 Da (N,Q).
      - Static modifications: Carbamidomethyl/+57.021 Da (C), Acetyl:2H (3)/+45.029 Da (K).

- Dynamic modifications: Max. equal modifications per peptide; 3, Oxidation/+15.995 Da (M).

(f) Percolator, second.

- Target/Decoy Selection; Concatenated.
- Validation based on; q-Value.
- Target FDR (Strict); 0.01.
- Target FDR (Relaxed); 0.05.

(g) Minora Feature Detector. This module is for label-free quantitation (LFQ).

5. Modules for consensus step workflow. Use an exemplary workflow provided by manufacturer suitable for the experiment.
6. Export peptide group level data table.
7. Filter out any peptides that have features described below:

   −1 or −2 position or both residue is arginine (*see* **Note 6**).

   −2 and −1 position residues are comprised of valine and glycine or glycine and valine [1].

4 Notes

1. We recommend adding TCEP, 2-chloroacetamide, and 100× protease inhibitor to 50 mM EPPS pH 8, 6 M guanidine solution (protein lysis buffer), because these reagents are not stable for prolonged storage. Best prepared fresh or minimize possible freeze and thaw cycles.
2. Although trypsin is generally the best protease for N-terminal peptide enrichment, we found that N-terminal arginylated peptides identified using chymotrypsin were highly complementary to the N-terminally arginylated peptides from trypsin.
3. To make 200 mM ammonium formate, we used concentrated ammonium hydroxide with estimated concentration of 14.534 M based on the density of 0.9 g/mL, the formula weight of 35.05 g/mol, and a concentration of 56.6% w/w. For 500 mL stock bRPLC buffer, we first poured 400 mL of HPLC grade water, added 6.76 mL of ammonium hydroxide, added 0.7 mL of concentrated formic acid with estimated concentration of 25.179 M, and finally added HPLC grade water up to 500 mL.
4. The tube just taken out from the −80 °C refrigerator is extremely fragile and can crack or break during high g force centrifugation.
5. We observed that 2 h of digestion would be sufficient for amine-blocked protein samples. However, we found that

1 mg scale of proteins is much easier to aggregate in digestion conditions with low concentration of a chaotropic chemical, guanidine.

6. We suspect that peptides generated by tryptic cleavage might be misannotated as having N-terminal arginylation as a result of a combination of modifications. For example, in an article published by Xu et al., the combination of several possibilities of amino acid residues, posttranslational modification (PTM), and intrinsic limit of mass spectrometric resolution leads to an ambiguous identification due to a generous mass error window of search engine parameter.

Acknowledgments

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Chapter 26

Monitoring the Activation of Selective Autophagy via N-Terminal Arginylation

Chang Hoon Ji, Su Bin Kim, Min Ju Lee, and Yong Tae Kwon

Abstract

In addition to generating N-degron-carrying substrates destined for proteolysis, N-terminal arginylation can globally upregulate selective macroautophagy via activation of the autophagic N-recognin and archetypal autophagy cargo receptor p62/SQSTM1/sequestosome-1. To evaluate the macroautophagic turnover of cellular substrates, including protein aggregates (aggrephagy) and subcellular organelles (organellophagy) mediated by N-terminal arginylation in vivo, we report here a protocol for assaying the activation of the autophagic Arg/N-degron pathway and degradation of cellular cargoes via N-terminal arginylation. These methods, reagents, and conditions are applicable across a wide spectrum of different cell lines, primary cultures, and/or animal tissues, thereby providing a general means for identification and validation of putative cellular cargoes degraded by Nt-arginylation-activated selective autophagy.

Key words In vitro p62/SQSTM1 self-oligomerization, Detergent-insoluble/soluble fractionation, Punctate formation/co-localization, Autophagic flux, ATE1, Nt-arginylation

1 Introduction

At any given moment in time, up to 30% of all nascent peptide chains are misfolded [1]. Given that the structure of a protein equates to its functionality, such misfolding usually induces loss-of-function toxicity [2]. Additionally, this misfolding also exposes hydrophobic motifs, which otherwise are thermodynamically hidden, resulting in the aggregation of the above protein species as an effort to lower entropy and inducing gain-of-function toxicity [2]. As part of a collective decision-making process to combat such loss- or gain-of-function toxicity, cells employ protein quality control (pQc) to (1) refold/correct, (2) sequester, or (3) degrade pathological proteins [1–3]. Among these, proteolysis occurs via either the ubiquitin-proteasome system (UPS) or the autophagy-lysosome pathway (ALP).

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The proteinaceous substrates of the N-degron pathway were traditionally assumed to be degraded only by the proteasome, naturally due to the identification of UBR family E3 ligases as N-recognins via their UBR box and N domains [4]. However, our laboratory first identified that a subset of arginylated proteins, including cytosolic protein species (C-terminal fragments of CDC6 and BRCA1) and ER chaperones (BiP/GRP78, PDI, and CRT), carrying N-terminal arginylation-permissive residues were upregulated during proteotoxic ER stress, retrotranslocated to the cytosol and N-terminally arginylated by ATE1 [5–7]. The N-terminal arginine (Nt-Arg) residue of these proteins was found to bind the archetypal autophagy cargo receptor p62/SQSTM1/Sequestosome-1 via its ZZ domain, which was structurally analogous to the UBR box of UBR E3 ligase/N-recognins [5, 6, 8, 9]. This N-degron-mediated interaction induces a conformational change of p62, exposing its PB1 and LC3-interacting domains, which accelerates its self-oligomerization and targeting to autophagosomes in tandem with not only the Nt-arginylated substrate but also ubiquitinated cargoes interacting with the UBA domain of p62 [8, 9]. In addition, p62, whose ZZ domain is occupied with N-terminal Arg, acts as an autophagic inducer that facilitates autophagosome biogenesis [8–10]. Through this bimodal mechanism, activated p62 accelerates the autophagic proteolysis of cellular cargoes, including organelles and protein aggregates that are too large for the UPS to handle [9–12]. Consequently, these results substantiated the existence of an autophagic branch of the Arg/N-degron pathway that dynamically protects cells from proteotoxicity [3, 9, 11, 13].

Traditional approaches to studying protein degradation via the N-degron pathway have relied on confirming the arginylation and degradation of the putative N-degron substrate [4]. The materials, methods, and protocols described below are made possible from the fact that ATE1-mediated N-terminal arginylation of N-degron substrates activate the autophagic Arg/N-degron pathway via the conformational and thus biological activation of p62 [8–11]. Consequently, the first part of the procedure below focuses on determining whether p62 has been activated via an in vitro oligomerization assay involving nonreducing SDS-PAGE [8, 10, 11]. Second, analysis of punctate formation and co-localization of p62 with the target protein/cargo will be detailed below to confirm not only activation of p62 but also the increased delivery of substrates to autophagic membranes [8, 10, 11, 13]. This will be complemented by a lysosomal flux assay following detergent-insoluble/soluble fractionation to verify acidic hydrolysis of the target cargo, which is more often than not found as an insoluble and aggregated species under proteotoxic conditions [8, 10, 11]. As this protocol combines both in vitro and in vivo analysis of the activation of the autophagic Arg/N-degron pathway and the

subsequent degradation of would-be substrate cargoes, it has been successfully replicated in various conditions and sample types, allowing for the identification of a number of autophagic substrates degraded by the autophagic Arg/N-degron pathway [10, 11].

2 Materials

2.1 In Vitro p62/SQSTM1 Oligomerization Assay

1. p62-myc/his encoding plasmid.
2. Lipofectamine 2000.
3. Lysis buffer: 50 mM HEPES (pH 7.4), 0.15 M KCl, 0.1% NP-40, 10% glycerol. Stored at 4 °C.
4. Protease and phosphatase inhibitors: protease inhibitors cocktail (100×), 50 mM bestatin, 500 mM PNPP (p-nitrophenyl phosphate) phosphatase inhibitor cocktail (100×).
5. BCA assay kit: BCA Reagent A, BCA Reagent B, Albumin Standards from 0 to 2 μg.
6. Oligomerization inducing drugs (e.g., p62-activators), dipeptides (Arg-Ala, Ala-Arg) diluted in DMSO.
7. 4× nonreducing LDS buffer: 40% glycerol, 4% LDS (lithium dodecyl sulfate), 4% Ficoll 400, 0.8 M triethanolamine-CL (pH 7.6), 0.025% phenol red, 0.025% Coomassie, 2 mM EDTA disodium.
8. 4–20% gradient SDS-PAGE gels.
9. p62 antibody, MYC antibody.
10. Liquid LN_2, water bath.

2.2 Punctate Formation and Co-localization Analysis

1. 4% paraformaldehyde (PFA).
2. 10% Triton X-100 in PBS.
3. 2% BSA solution: Bovine serum albumin in PBS.
4. Coverslip (15 mm diameter, for 24-well plate), slide glass.
5. 10% PLL (poly-L-lysine) in distilled water.
6. Puncta formation-inducing drugs (e.g., autophagy inhibitors, p62-activators).
7. Antibodies: Primary antibodies for detecting target proteins (e.g., p62, LC3, and marker proteins of organelles or aggregates), fluorescence-conjugated secondary antibodies (e.g., Alexa flour 488 anti-rabbit IgG, Alexa flour 555 anti-mouse IgG).
8. Laser scanning confocal microscope.
9. LMS image software.

2.3 Detergent-Insoluble/Soluble Fractionation-Based Autophagy Flux

1. Autophagic inhibitor: Baf. A1 (bafilomycin A1), HCQ (hydroxychloroquine). To analyze autophagic flux, drugs should inhibit the late step of autophagy.
2. Lysis buffer: 100 mM NaCl, 0.5 mM EDTA, 20 mM Tris-HCl (pH 8.0), 0.5% Triton X-100. Stored at 4 °C.
3. Protease and phosphatase inhibitors: Protease inhibitors cocktail (100×), 50 mM bestatin, 500 mM PNPP (p-nitrophenyl phosphate) phosphatase inhibitor cocktail (100×).
4. 5× sample buffer.
5. Sonicator.
6. 1× PBS, autoclaved distilled water.
7. Antibodies for detecting target proteins, GAPDH, LC3, and p62.
8. Coomassie blue staining solution, destaining solution.

3 Methods

Carry out all procedures on ice, unless otherwise specified.

3.1 In Vitro p62/SQSTM1 Oligomerization Assay

1. Seed 8 × 10^6 cells/mL in a 100 mm dish and then transiently insert the plasmid encoding p62-myc/his using lipofectamine 2000. In the original paper, HEK293T cells were used. The yield of proteins is approximately 10 μg for a 100 mm dish.
2. Harvest cells and resuspend in lysis buffer supplemented with protease inhibitors cocktail, PNPP, and phosphatase inhibitor.
3. Repeat freezing (liquid N_2) and thawing (42 °C) the cell suspension 10–20 times until mixture turns yellowish.
4. Centrifuge the suspension at 13,000 rpm for 20 min and isolate the supernatant.
5. Measure the concentration of proteins in the supernatant by BCA.
6. Dilute the lysis buffer for 1/4 with distilled water (20 μL per the number of samples), and add protease inhibitor cocktail, PNPP, and phosphatase inhibitor cocktail.
7. 1 μg of protein was incubated with 50 mM of p62 oligomerization-inducing dipeptides or 1000 μM of chemicals. Add 100 μM of bestatin and incubate the samples at room temperature for the required time (*see* **Note 1**). A remaining amount of 1/4 diluted lysis buffer to finalize the volume of each sample up to 20 μL.
8. Add 4× nonreducing LDS buffer to samples and heat at 95 °C for 10 min.

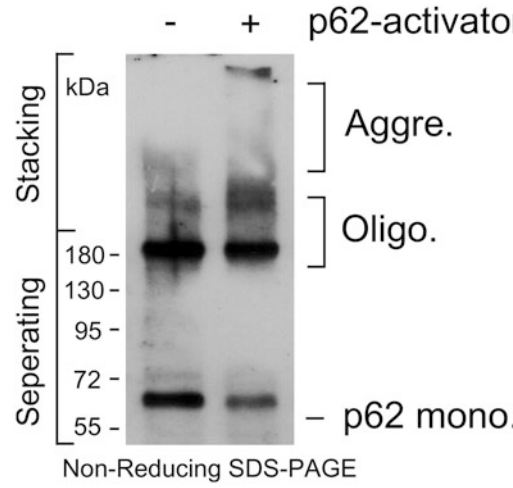

Fig. 1 In vitro p62 oligomerization assay in HEK293T cells incubated with the p62-ZZ ligands (p62-activator). 1000 mM of ligands were incubated for 2 h with lysates at room temperature (*see* [10] for the originally published result)

9. Resolve the samples using 4–20% gradient SDS-PAGE. Separate gels until the minimum size of proteins are approximately 50 kDa (monomer size of p62 is 62 kDa.).

   Caution: Do not remove stacking gels when samples are transferred to membranes.

10. Immunoblot the samples using p62 and MYC antibodies. A typical result is shown in Fig. 1.

3.2 Punctate Formation and Co-localization Analysis

1. Seed cells on coverslips coated with PLL**.** 5×10^4 cells/mL in a 24-well plate are recommended (*see* **Note 2**).
2. Incubate in the presence or absence of drugs that induce puncta formation.
3. Remove the culture medium by aspiration and wash the cells with 1× PBS twice. 500 μL of 1× PBS is recommended for a 24-well plate.
4. Fix the cells with 4% paraformaldehyde (PFA) and incubate for 15 min at room temperature (RT).
5. Rinse the coverslips with 1× PBS three times for 5 min/each.
6. Permeabilize the cells using 0.5% Triton X-100 solution for 15 min at RT.
7. Rinse the coverslips with 1× PBS three times for 5 min/each.
8. Block the cells with 2% BSA/PBS solution for 1 h at RT.
9. Incubate the cells in the primary antibody diluted in 2% BSA/PBS solution for the required time (*see* **Note 3**). To analyze the co-localization of two or three proteins, two primary

antibodies from different species or fluorescence-conjugated constructs should be used.

Caution: Incubation time is a critical parameter for staining. The required concentration of antibodies and incubation time should be tested before being used in the analysis.

Caution: If the antibodies require overnight incubation, the cells should be stored at 4°C.

10. Rinse the coverslips with 1× PBS three times for 10 min/each.
11. Incubate the cells in the fluorophore conjugates secondary antibody diluted in 1× PBS solution for 30–35 min at RT. The wavelength of the fluorophores should be considered (*see* **Note 4**).

    Caution: Incubate in dark by wrapping the plate in the following steps.
12. Rinse the coverslips with 1× PBS three times for 10 min/each.
13. Incubate the cells in the DAPI antibody diluted in 1× PBS solution for 20 min at RT (if you use DAPI-included mounting medium, skip to **step 14**).
14. Mount coverslips with the antifade-mounting medium on the glass slide, and then wait until the media dry completely.

    Caution: Minimize air bubbles in the mounting medium.
15. Obtain confocal images by laser scanning confocal microscope. Over 60× magnification is required to observe puncta in cells.
16. Count the number of total puncta or colocalized puncta. Analyze and modify the images by LSM image browser. Exemplary data are shown in Figs. 2, 3, and 4.

3.3 Detergent-Insoluble/Soluble Fractionation-Based Autophagy Flux

1. Seed approximately 1.2 × 10^6 cells/ml per well in 6-well plates, and then incubate the cells with culture media with condition of interest in the presence or absence of autophagic inhibitors. Treatment condition: Baf.A1 (200 nM, 6 h), HCQ (25 μM, 24 h).
2. Harvest cells by centrifuge at 13,000 rpm for 2 min.
3. Resuspend the cell pellet with 75 μL of protease inhibitors cocktail, phosphatase inhibitor cocktail-added lysis buffer, and incubate 30 min on ice. Vortex samples every 10 min.
4. Centrifuge at 13,000 rpm for 10 min.
5. Transfer the supernatant to new Eppendorf tubes (approximately 70 μL), and then add 30 μL of 5× sample buffer and resuspend to obtain the detergent-soluble fraction.
6. Wash centrifuged pellet with 1× PBS 3–5 times. Add 1× PBS, and vortex samples then centrifuge at 13,000 rpm for 2 min.

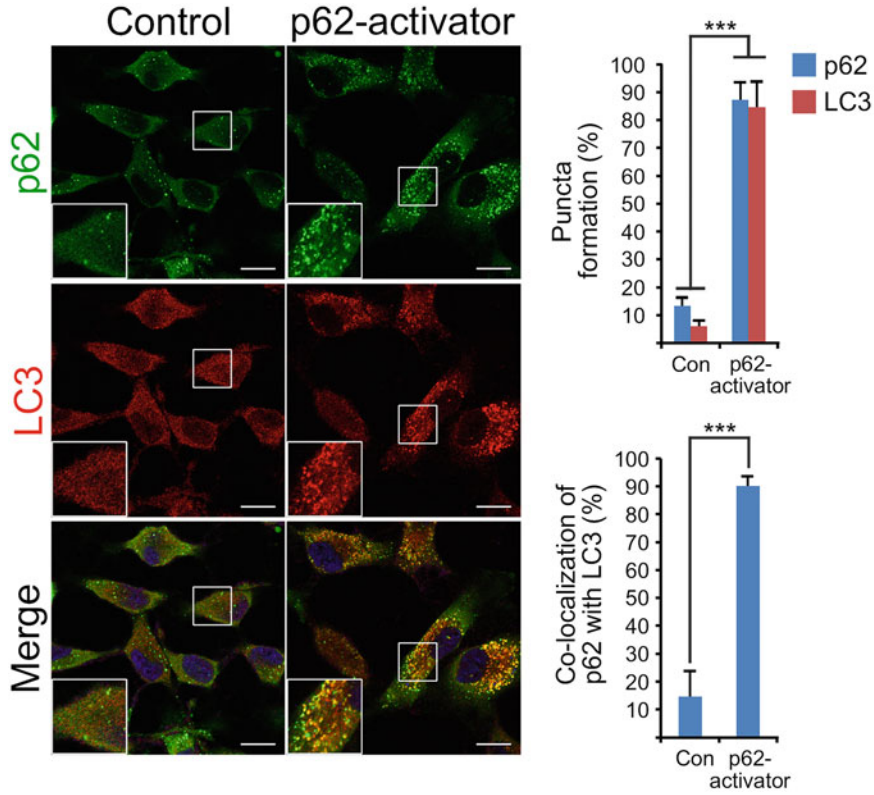

Fig. 2 Immunocytochemistry and co-localization analysis of HeLa cells treated with p62-activator (2.5 μM, 24 h). Scale bar, 10 μm. For analyzing puncta formation and co-localization, each counting 50 cells in three biologically independent experiments are used (*see* [10] for the originally published result)

7. Suspend pellet with 70 μL of autoclaved distilled water (use sonicator if necessary). Then, add 30 μL of 5× sample buffer, and resuspend to obtain the detergent-insoluble fraction.
8. Heat all samples at 100 °C for 10 min.
9. Load 5 μL of soluble samples and 10 μL of insoluble samples in each well of SDS-PAGE gels.
10. Immunoblot using antibodies of target proteins and loading control. Coomassie blue can be used as a loading control for the insoluble fraction. LC3 or p62 could be used as a positive control of autophagy flux (Fig. 5) [10].
11. Compare the protein level of target proteins. Autophagy flux (A.F.) indices are based on the ratio of substrate levels in the presence or absence of autophagic inhibitors (Fig. 6) [10]. A typical result is shown in Fig. 5.

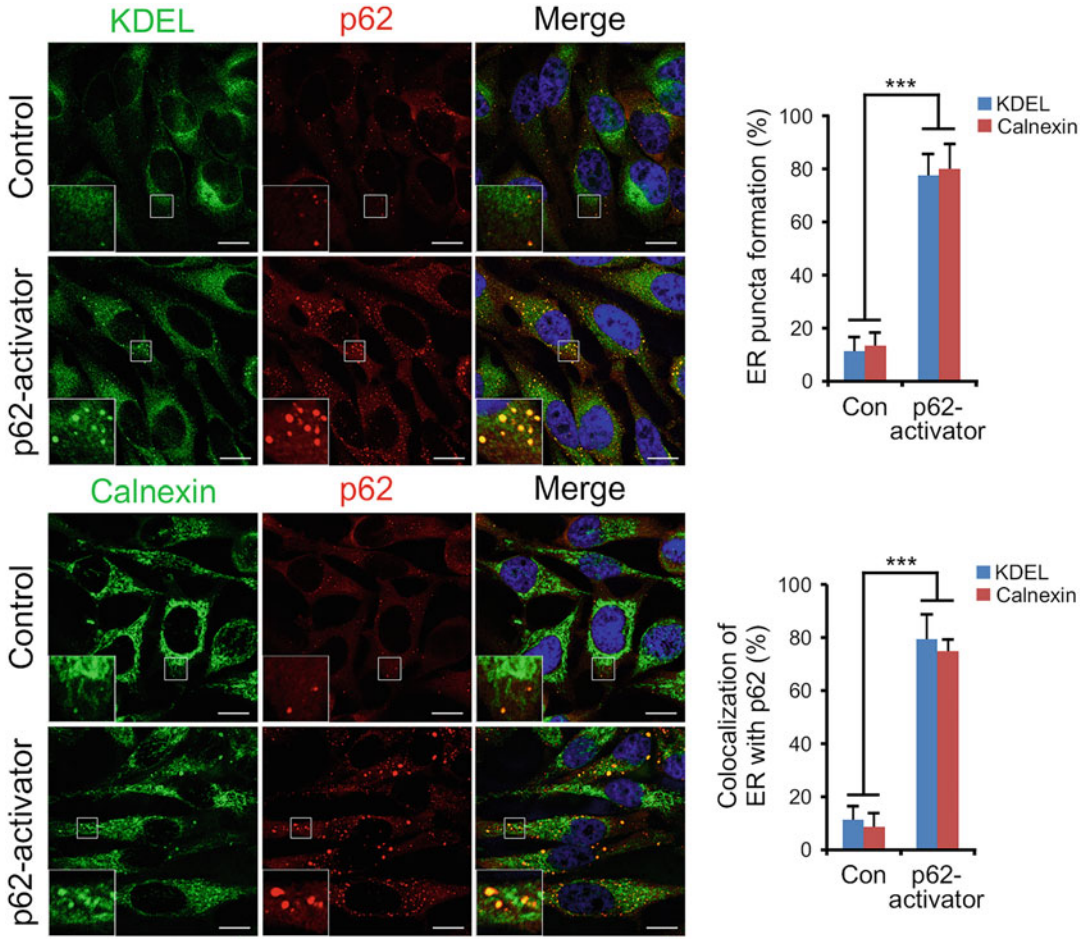

Fig. 3 Puncta formation and co-localization analysis for ER-phagy (organellophagy). Co-localization assays of p62 and ER compartments in HeLa cells treated with p62-activator (2.5 μM, 5 h). Scale bar, 10 μm. Quantification of punctate formation of ER compartments and co-localization of ER compartments with p62 (*see* [11] for the originally published result)

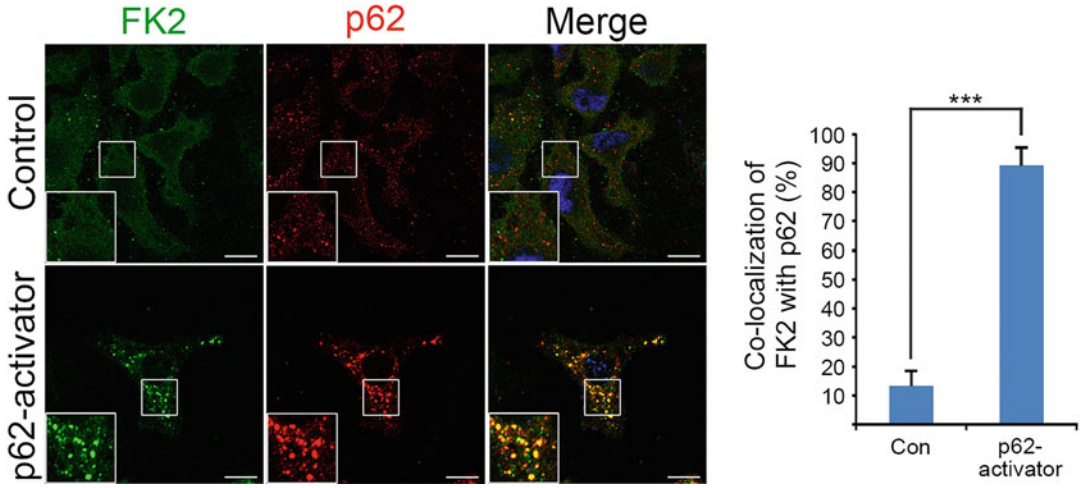

Fig. 4 Puncta formation and co-localization analysis for aggregates. Co-localization assays of p62 and FK2 in HeLa cells treated with p62-activator (1 μM, 24 h). Scale bar, 10 μm. Quantification of co-localization of aggregates with p62 (*see* [10] for the originally published result)

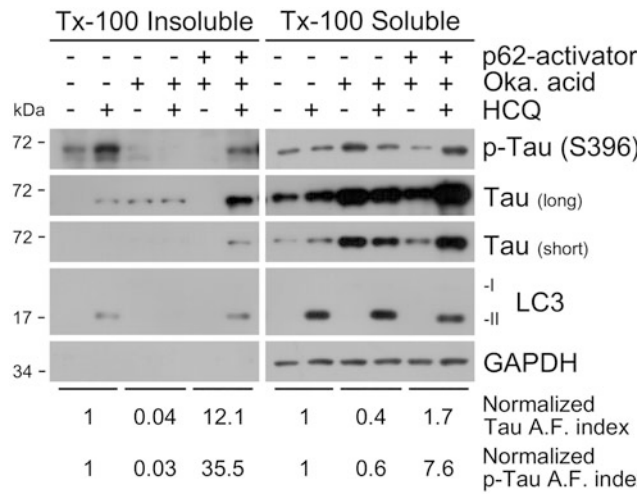

Fig. 5 Triton X-100-fractionation assay in SH-SY5Y-tauP301L cells treated with a combination of HCQ (10 μM, 24 h), okadaic acid (15 nM, 24 h), or p62 activator (0.1 μM, 24 h) (*see* [10] for the originally published result)

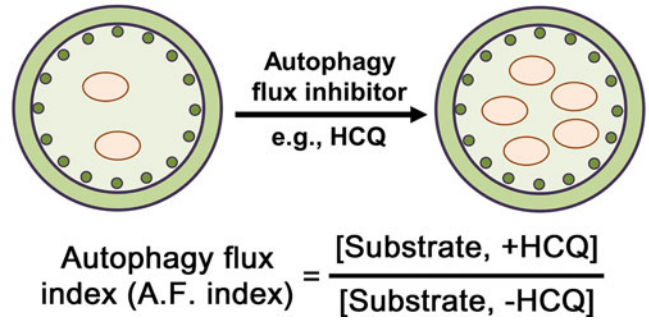

Fig 6 A schematic formula for autophagy flux index [10]

4 Notes

1. Required incubation time for the formation of aggregates should be between 1 and 8 h.
2. Insert coverslips in each well of the plate, and then incubate 30 min with 10% PLL in autoclaved distilled water to polarize coverslips. Wash coverslips three times with autoclaved distilled water.
3. 300–500 μL of the solution is required to cover the coverslip. Optionally, transfer coverslips onto inverted Eppendorf caps to save antibodies. 50–100 μL of primary antibody solution is enough for covering each coverslip.
4. Wavelength of fluorophores should be considered to analyze the co-localization of proteins. It is recommended to select two or three different fluorophore probes that have the least spectral overlap.

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Chapter 27

Analyzing the Interaction of Arginylated Proteins and Nt-Arg-Mimicking Chemical Compounds to N-Recognins

Chang Hoon Ji, Min Ju Lee, Su Bin Kim, and Yong Tae Kwon

Abstract

Characterizing and measuring the interactome of N-degrons and N-recognins are critical to the identification and verification of putative N-terminally arginylated native proteins and small-molecule chemicals that structurally and physiologically mimic the N-terminal arginine residue. This chapter focuses on in vitro and in vivo assays to confirm the putative interaction, and measure the binding affinity, between Nt-Arg-carrying natural (or Nt-Arg-mimicking synthetic) ligands and proteasomal or autophagic N-recognins carrying the UBR box or the ZZ domain. These methods, reagents, and conditions are applicable across a wide spectrum of different cell lines, primary cultures, and/or animal tissues, allowing for the qualitative analysis and quantitative measurement of the interaction of arginylated proteins and N-terminal arginine-mimicking chemical compounds to their respective N-recognins.

Key words Pull-down assay, Microscale thermophoresis (MST), Nt-arginine-mimicking chemical compound, ATE1, Nt-arginylation

1 Introduction

Since the discovery of protein arginylation in 1963 and the N-end rule pathway (now N-degron pathway) in 1986, a large number of various proteins as arginylation-permissive substrates have been identified and characterized [1, 2]. These endogenous substrates range from viral proteins and nuclear transcription regulators to ER chaperones and kinases, accentuating the importance of protein arginylation and the Arg/N-degron pathway in proteostasis and cellular function [1–3]. Moreover, accumulating evidence have highlighted the non-degradative role of protein arginylation, facilitating other cellular functions ranging from alpha helical conformation, stress granule scaffolding, cell-cell adhesion, other post-translational modifications, and self-polymerization [4, 5].

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Traditional methods to study protein arginylation and the Arg/N-degron pathway have relied on identifying whether putative substrates undergo ATE1-mediated N-terminal arginylation [1, 3]. Relatively less interest was focused on expanding the set of verified N-recognins that recognize N-terminally arginylated substrates, not least because the UBR box and the N domain of UBR E3 ligases were considered the only N-recognins for some time [2, 3]. However, our laboratory as well as other groups have reported the identification and characterization of non-UBR N-recognins and responsible domains or motifs that recognize the N-terminal arginine residue, most notably the ZZ domain [2, 6, 7]. Given that protein arginylation is not exclusively limited to inducing the proteolytic turnover of the corresponding substrate, expanding the set of verified N-recognins has become increasingly important to characterize the role that protein arginylation may have on not only the endogenous substrate but on cellular functions as well [2–5].

In this chapter, we report a multipartite protocol for qualitatively determining or quantitatively measuring the interaction of an N-terminally arginylated protein to an N-recognin, specifically via its UBR box, ZZ domain, or other Nt-Arg-recognizing domains and motifs. This protocol can be useful in identifying a putative N-recognin via testing its interaction with known N-terminally arginylated proteins/peptides, and vice versa with putative Nt-Arg-carrying substrates with known N-recognins. Likewise, the same principle can be readily applied to small-molecule chemical compounds that mimic the binding mode of N-degrons to N-recognins. The protocol below initially describes an in vitro X-peptide/small-molecule pull-down assay to qualitatively analyze the physical binding of a target N-degron protein, peptide, or molecule to a target N-recognin [2, 5, 6, 8]. The second part of the protocol will complement the qualitative analysis provided above by describing the method based on microscale thermophoresis (MST) to directly measure the binding affinities between putative N-degron peptides, proteins, or small molecules and N-recognins [9, 10]. The incorporation of a qualitative analysis and quantitative measurement has resulted in successful identification and characterization of putative N-degrons and N-recognins, as well as the development and structure activity relationship (SAR)-dependent optimization of N-degron-mimicking small-molecule chemical compounds.

2 Materials

2.1 Pull-Down Assay

2.1.1 Peptide Conjugation

1. Beads (Streptavidin Agarose Resins) solution: Beads are 50% slurry.
2. Phosphate-buffered saline (PBS): Prepare a 10× solution with bi-distilled water containing 10.6 mM KH_2PO_4, 30 mM

Na_2HPO_4, $2H_2O$, and 1.54 M NaCl, and sterilize with a 0.2 μM filter. The 1× solution obtained following dilution with bi-distilled water will have a pH of around 7.4.

3. Peptides: Chemically modified peptides should be approximately 11-mer amino acid in length.

2.1.2 Preparation of Cell Lysate

1. Eukaryotic cells.
2. Phosphate-buffered saline (PBS): Prepare a 10× solution with bi-distilled water containing 10.6 mM KH_2PO_4, 30 mM Na_2HPO_4, $2H_2O$, and 1.54 M NaCl and sterilize with a 0.2 μM filter. The 1× solution obtained following dilution with bi-distilled water will have a pH of around 7.4.
3. Trypsin EDTA.
4. Buffer A (hypotonic buffer): Prepare a solution with bi-distilled water containing 10 mM KCl, 1.5 mM $MgCl_2$, and 10 mM HEPES (pH 7.9). The solution will have a pH of around 7.9.
5. Antiprotease cocktail (100×): Mix protease inhibitors cocktail and phenylmethylsulfonyl fluoride (PMSF).
6. Liquid LN_2, water bath.
7. BCA assay kit: BCA Reagent A, BCA Reagent B, Albumin Standards from 0 to 2 μg.

2.1.3 Pull-Down Assay

1. Buffer B (binding buffer): Prepare a solution with bi-distilled water containing 0.05% Tween 20, 10% glycerol, 0.2 M KCl, and 20 mM HEPES (pH 7.9). The solution will have a pH of around 7.9.
2. Biotinylated peptide of interest.
3. 2× sample buffer.

2.2 MST (MicroScale Thermophoresis)

1. For illustration, the protein domain to be used is the ZZ domain of p62 (MVHPNVICDGCNGPVVGTRYKCSVCP DYDLCSVCEGKGLHRGHTKLAFPSPFGHLSEGFSH). The stock concentration is 10 mg/mL.
2. Protein buffer: The solution is mixed with 200 mM NaCl, 50 mM Tris (pH 7.5), and 1 mM TCEP in bi-distilled water.
3. Protein labeling kit (*see* **Note 1**): The kit includes Dye RED-NHS 2nd Generation (10 μg), labeling buffer NHS.

   RED-NHS 2nd (the buffer is mixed with 130 mM $NaHCO_3$ and 50 mM NaCl and will have a pH from 8.2 to 8.3 at room temperature (RT)), A-column, B-column, and adapter for 15 ml centrifuge tube. The Monolith Protein

Labeling Kit Generation is optimized for labeling and purification of proteins with a molecular weight higher than 5 kDa. The dye carries a reactive NHS-ester group that reacts with primary amines (lysine residues) to form a covalent bond. RED dyes are suited for Monolith NT.115 series and Monolith NT. Automated instruments with a RED detector (Nano and Pico).

4. Monolith NT.115 Capillaries.
5. Nanotemper.

3 Methods

3.1 Pull-Down Assay

3.1.1 Peptide Conjugation

1. Transfer 1 mL beads (Streptavidin Agarose Resins, 50% slurry) solution to a new 15 mL conical tube using cleaved tip.
2. Centrifuge at 2000 rpm at 4 °C for 1 min. Discard supernatant.
3. Add 5 mL PBS (pH 7.4) and resuspend carefully.
4. Centrifuge at 2000 rpm at 4 °C for 1 min (*see* **Note 2**).
5. Repeat **step 3** and **step 4** five times.
6. After the last wash, spin down at 3000 rpm at 4 °C for 3 min, remove the PBS, and store the beads at 4 °C.
7. Solubilize peptide of interest (e.g., dissolve 10 mg peptide in 500 μL DMSO to obtain a final concentration of 20 μg/μL).
8. Prepare mixture for peptide conjugation. Add 20 μL solubilized peptide, 800 μL beads, and 5 mL PBS.
9. Incubate on rotor at 4 °C overnight.
10. Centrifuge at 2000 rpm at 4 °C for 3 min and discard supernatant.
11. Repeat **step 3** and **step 4** five times and go to **step 12** (*see* **Note 3**).
12. After the last wash, remove the PBS and add 700–800 μL new PBS (*see* **Note 4**).
13. Store at 4 °C (*see* **Note 5**).

3.1.2 Preparation of Cell Lysate

1. Seed eukaryotic cells at 8×10^6 in 100 mm cell culture dish, and incubate overnight at 37 °C in 5% CO_2.
2. Cool cells by placing dish on ice and wash cells with 1× PBS. Incubate cells at 2 min with 200 μL trypsin EDTA, add 1 mL PBS, and harvest cells.
3. Centrifuge at 13,000 rpm at 4 °C for 2 min and remove supernatant. Suspend cells with 100× antiprotease cocktail-added Buffer A. The volume of Buffer A is usually 100–200 μL for a 100 mm dish.

4. Repeat freezing (liquid LN2) and thawing (42 °C) the cell suspensions 5–20 times until the buffer gets yellowish color.
5. Spin down at 13,000 rpm at 4 °C for 15 min and get supernatant.
6. Measure the concentration of proteins in the supernatant by BCA.

3.1.3 Pull-Down Assay

1. Prepare 130–140 μg protein lysates (concentration of lysate: 1 μg/μL) including protein input (this method is based on 100 μg of protein input).
2. Transfer 30–40 μL protein lysates into a new tube for input. The percentage of input is 30–40% of total.
3. Put 500 μL Buffer A adding 100× antiprotease cocktail in the 100 μL lysates, and invert several times.
4. Centrifuge at 5000 rpm at 4 °C for 1 min, and discard supernatant.
5. Repeat **step 3** and **step 4** five times for washing.
6. Add 30–50 μL biotinylated peptide and ~200 μL Buffer B (binding buffer). The volume of final mixture is up to 300 μL.
7. Incubate on 4 °C rotor for 2–4 h.
8. Drain supernatant and wash by Buffer B five times, similar to **step 5**.
9. Centrifuge at 5000 rpm at 4 °C for 2 min.
10. Completely remove the supernatant.
11. Put 35 μL 2× sample buffer and boil at 100 °C for 10 min. At this time, boil the input at 100 °C for 7 min.
12. Load the input and the pull-down sample on SDS-polyacrylamide gel (*see* **Note 6**).
13. Immunoblot the sample using anti-protein of interest (Figs. 1 and 2).

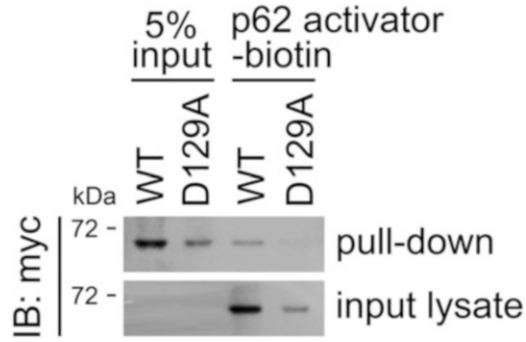

Fig. 1 Pull-down assay using biotinylated small-molecule p62 activator in HEK293T cells transiently expressing wild-type or D129A p62-myc constructs (*see* [5] for the originally published result)

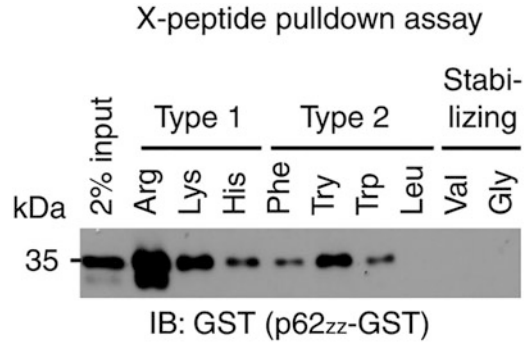

Fig. 2 X-peptide pull-down assay using 93-residue $p62_{ZZ}$-GST containing intact ZZ domain (*see* [6] for the originally published result)

3.2 MST (MicroScale Thermophoresis)

3.2.1 Peptide Conjugation

1. From a −80 °C deep freezer, place the protein and buffer on ice in advance, set the microcentrifuge centrifuge at 4 °C, and then perform cooling.
2. After confirming that the protein is melted (about 30 min), perform centrifuge at 13,000 rpm at 4 °C for 10 min.
3. Pick up only the supernatant.
4. Dilute to 10 μM.

3.2.2 Protein Buffer Exchange

1. Add 3 mL autoclaved distilled water (A/C D.W) to labeling buffer NHS. At this time, after pipetting until the powder is dissolved, confirm that it is completely dissolved and close the lid.
2. Invert the A-column three times to resuspend slurry.
3. Twist off the bottom and remove cap.
4. Place A-column in a fresh microcentrifuge tube.
5. Centrifuge at 1500 rpm at 4 °C for 1 min.
6. Discard flow-through from microcentrifuge tube, and place A-column back in the microcentrifuge tube. Add 300 μL labeling buffer NHS to equilibrate the A-column. Avoid contacting the inner walls of the column, and load the buffer directly in the center of the resin bed.
7. Centrifuge at 1500 rpm at 4 °C for 1 min and discard flow-through.
8. Repeat **step 7** and **step 8** three times and go **step 10**.
9. Place A-column in a fresh microcentrifuge tube.
10. Pipette 100 μL of the 10 μM protein sample in the center of the column resin.
11. Centrifuge at 1500 rpm at 4 °C for 2 min. Then, the protein is in the collected flow-through.

3.2.3 Protein Staining

1. Reconstitute with 25 μL DMSO right before use by pipetting up and down.
2. In a microcentrifuge tube, mix 7 μL of Dye RED-NHS 2nd Generation. Freshly prepared in DMSO with 7 μL labeling buffer NHS.
3. Add 10 μL of the 300 μM dye solution to 90 μL of the 10 μM protein sample in a microcentrifuge tube.
4. Mix carefully by pipetting up and down several times.
5. Incubate for 30 min at RT in the dark.
6. In the meantime: Perform to equilibrate the B-column.
7. Remove top cap from B-column and pour off the storage solution. Then, remove the bottom cap. Save both caps and set aside.
8. Replace the cap from the 15 mL centrifuge tube with the adapter.
9. Then, place the column on top of the adapter and in the tube.
10. Fill column with assay or equilibration buffer of choice, and allow buffer to enter the packed resin bed completely by gravity flow.
11. Repeat **step 10** and **step 11** three times.
12. About 8–10 mL of buffer should be used in total for all steps.
13. Discard the last flow-through so the tube is ready for the next step.
14. After the labeling reaction incubation, transfer the 100 μL of dye-protein solution to B-column equilibrated. Avoid contacting the inner walls of the column, and load the sample directly in the center of the resin bed. Let sample enter the resin bed completely.
15. Add 550 μL of assay or equilibration buffer of choice, and allow buffer to enter the resin bed.
16. To elute the protein, place the fresh microcentrifuge tube under the column. Add 450 μL of assay or equilibration buffer onto column and collect the flow-through that contains the labeled protein. Avoid contacting the inner walls of the column, and load the buffer directly in the center of the resin bed.

3.2.4 MST

When performing an MST experiment, a microscopic temperature gradient is induced by an infrared laser, and the movement of fluorescent molecules away from the heated area is monitored. The movement varies depending on whether a ligand is bound to the molecule. The difference in motion is used to calculate binding affinity.

1. Control program.
   (1) Start New Session.
   (2) Click wavelength BLUE or RED.
   (3) Pretest: Measure fluorescence power. Check adsorption and protein aggregation.
2. Measure binding affinity.
   (1) Make a generous 30 μL:
   10 mM ligand 3 μL + assay buffer 27 μL).
   (2) Make dilution solution:
   (DMSO 20 μL + assay buffer 180 μL).
   In order to put the same DMSO in No. 2–16.
   (3) No. 1: 20 μL aliquot of **(1).**
   (4) No. 2: No. 1 10 μL + **(2)** 10 μL, pipetting.
   (5) No. 3: No. 2 10 μL + **(2)** 10 μL, pipetting.
   (6) No. 4: No. 3 10 μL + **(2)** 10 μL, pipetting.
   (7) No. 5: No. 4 10 μL + **(2)** 10 μL, pipetting.
   (8) No. 6: No. 5 10 μL + **(2)** 10 μL, pipetting.
   (9) No. 7: No. 6 10 μL + **(2)** 10 μL, pipetting.
   (10) No. 8: No. 7 10 μL + **(2)** 10 μL, pipetting.
   (11) No. 9: No. 8 10 μL + **(2)** 10 μL, pipetting.
   (12) No. 10: No. 9 10 μL + **(2)** 10 μL, pipetting.
   (13) No. 11: No. 10 10 μL + **(2)** 10 μL, pipetting.
   (14) No. 12: No. 11 10 μL + **(2)** 10 μL, pipetting.
   (15) No. 13: No. 12 10 μL + **(2)** 10 μL, pipetting.
   (16) No. 14: No. 13 10 μL + **(2)** 10 μL, pipetting.
   (17) No. 15: No. 14 10 μL + **(2)** 10 μL, pipetting.
   (18) No. 16: No. 15 10 μL + **(2)** 10 μL, pipetting.

   Example data is shown in Fig. 3.

4 Notes

1. Bring all components to room temperature (RT) before use. When stored appropriately, the kit components should be stable for approximately 12 months. Do not exceed recommended centrifuge time or speed.
2. Streptavidin Agarose Resins are stored in ethanol so that beads need to wash several times.

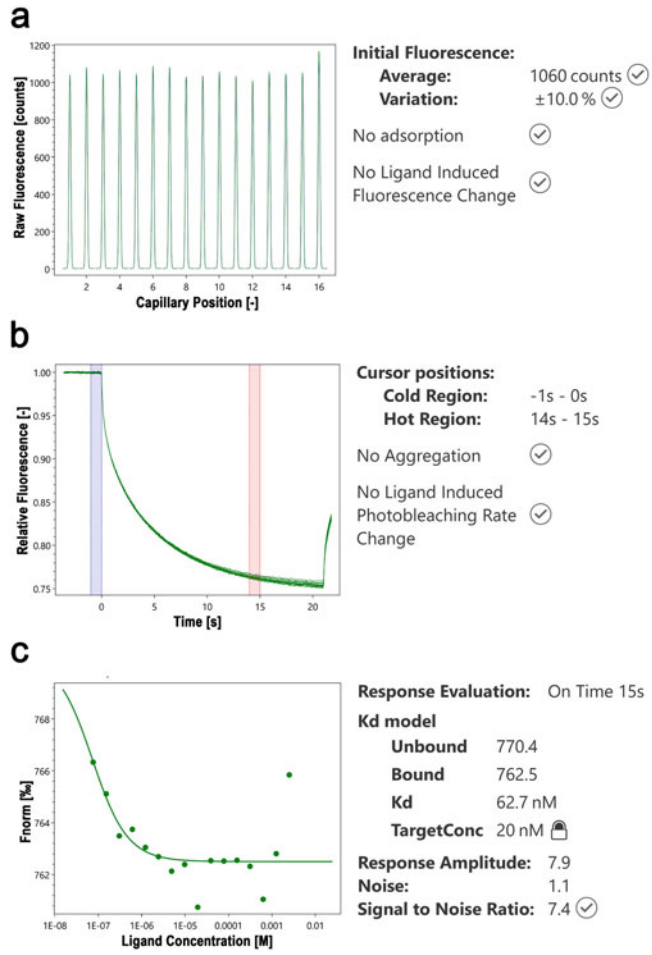

Fig. 3 MST result. (**a**) Capillary scans of a dilution series of the fluorescently labeled molecule. (**b**) MST traces. The thermophoretic movement of a fluorescent molecule changes upon binding to a nonfluorescent ligand, resulting in different traces. (**c**) Dose response. For analysis, the change in thermophoresis is expressed as the change in the normalized fluorescence (*Fnorm*), which is defined as F_{hot}/F_{cold} (*F*-values correspond to average fluorescence values between defined areas marked by the red and blue cursors, respectively). Titration of the nonfluorescent ligand results in a gradual change in thermophoresis, which is plotted as *Fnorm* to yield a binding curve, which can be fitted to derive binding constants

3. After add PBS and invert the tube, mix completely up to bottom of beads by tapping the tube.
4. The final biotinylated peptide is adjusted to 1 mL.
5. The biotinylated solution is stored during a month at 4 °C.
6. The loading volume of input is about 5–10% of loading volume of the sample.

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Chapter 28

Synthesis of Stably Charged Arg-tRNA^{Arg} for Structural Analysis

Yuka Yamaki, Howard Gamper, and Ya-Ming Hou

Abstract

Posttranslational protein arginylation catalyzed by arginyl transferases is a mechanism to regulate multiple physiological processes. This protein arginylation reaction uses a charged Arg-tRNA^{Arg} as the donor of arginine (Arg). The inherent instability of the ester linkage of the arginyl group to the tRNA, which is sensitive to hydrolysis at the physiological pH, makes it difficult to obtain structural information on how the arginyl transfer reaction is catalyzed. Here, we describe a methodology to synthesize stably charged Arg-tRNA^{Arg} that would facilitate structural analysis. In the stably charged Arg-tRNA^{Arg}, the ester linkage is replaced with an amide linkage, which is resistant to hydrolysis even at alkaline pH.

Key words Arginylation, tRNA, Structural Analysis, Arg-tRNA^{Arg}

1 Introduction

Posttranslational protein arginylation is conserved in all eukaryotes, targeting many cellular proteins and playing an essential role in cellular activities [1]. This reaction is catalyzed by protein arginyl transferases, using the activated arginyl group of Arg-tRNA^{Arg} as the arginylation donor. Synthesis of Arg-tRNA^{Arg} appears to use all available tRNA^{Arg} species as the donor, without specificity regarding the sequence of isoacceptors or isodecoders [2]. Even the acceptor helix alone of a tRNA^{Arg}, lacking the rest of the L-shaped tertiary structure, is competent [2], indicating a substrate recognition mechanism by protein arginyl transferases that emphasizes on the chemical moiety of the activated arginyl group. This chemical moiety is formed by esterification of the activated carbonyl group of the amino acid Arg with the 3′-OH group of the terminal adenosine of the A76 nucleotide of the acceptor tRNA^{Arg}. The resulting ester linkage of the arginyl group to the tRNA stores the high energy derived from ATP hydrolysis to synthesize the activated intermediate of the aminoacylation reaction Arg-AMP

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(arginyl adenylate), where the carbonyl group of Arg is activated. As such, the ester linkage in Arg-$tRNA^{Arg}$ is highly labile, easily hydrolyzed even at pH 5–6, making structural analysis highly difficult for probing the mechanism of protein arginylation.

Here, we describe a method to synthesize stably charged Arg-$tRNA^{Arg}$ that would facilitate structural analysis. Note that "charging" of $tRNA^{Arg}$ with Arg is often written as "aminoacylation" of $tRNA^{Arg}$ with Arg. In this synthesis of stably charged Arg-$tRNA^{Arg}$, we replace the ester linkage in the normal Arg-$tRNA^{Arg}$ with an amide linkage, which is stable and resistant to hydrolysis even at alkaline pHs. The replacement is achieved by using the CCA-adding enzyme (abbreviated as the CCA enzyme) to exchange the terminal adenosine of the A76 nucleotide in the acceptor $tRNA^{Arg}$ with a 3′-amino-3′-deoxy analog of ATP (abbreviated as nATP) to synthesize 3′-amino-3′-deoxy in the terminal adenosine [3]. The resulting 3′-amino substituted terminal ribose still permits aminoacylation by natural aminoacyl-tRNA synthetases but provides a stable amide linkage for aminoacyl attachment [4]. We have termed this 3′-amino substitution of tRNA as a 3′--tailing procedure that establishes a molecular tail for stable attachment of amino acids to tRNA [3]. In this chapter, we use *E. coli* $tRNA^{Arg}$(ICG) as an example to describe the synthesis of stably charged Arg-$tRNA^{Arg}$. The triplet ICG of the tRNA is the anticodon, and the inosine (denoted as I) in the anticodon is the product of a posttranscriptional deamination reaction of the normally adenosine residue in the wobble position, which is present in both bacteria and eukaryotes. The advantage of using *E. coli* $tRNA^{Arg}$(ICG) as a model is because it has been well characterized and can be produced in large quantities from an *E. coli* culture [3, 5–8].

2 Materials

All solutions and steps below use autoclaved double-distilled water (ddH_2O). Preferred suppliers and catalog numbers are listed for all the reagents, but, if unavailable, similar reagents at similar grade could in principle be used instead.

1. nATP: From BioLog (Life Science Institute, #A 114), Bremen, Germany.
2. Streptavidin Sepharose High Performance: GE Healthcare, #17-5113-01.
3. RNaseOut solution: Invitrogen, #10777019.
4. Pyrophosphatase: NEB, #M0361L.
5. Ultrafree-MC microcentrifuge filter: 0.22 μm, Millipore, #M9535.

6. Capture oligonucleotides: Ordered from IDT (Integrated DNA Technology, Coralville, Iowa, USA) or equivalent.
7. L-[4,5-^{3}H] arginine ([^{3}H]-Arg)) from American Radiolabeled Chemicals.
8. Oligo Clean and Concentrator kit: ZYMO Research, #D4061.
9. Counting cocktail: Econo-Safe Economical Biodegradable counting cocktail, Research Products International, #111175-CS.
10. Tri-Carb 4910 TR liquid scintillation counter: Perkin Elmer.

The buffers used are shown below.

1. The tRNA deacylation buffer: 0.1 M Tris–HCl (pH 9.0).
2. The TE buffer: 10 mM Tris–HCl (pH 8.0) and 1 mM EDTA.
3. The 1× CCA buffer: 50 mM glycine, pH 9.0, 10 mM $MgCl_2$, and 1 mM DTT.
4. The TB buffer: 10 mM Tris–HCl (pH 7.5).
5. The 1× hybridization buffer: 10 mM Tris–HCl (pH 7.5), 0.9 M NaCl, and 0.1 mM EDTA.
6. The 1× aminoacylation buffer (1× AA buffer): 20 mM KCl, 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 80 μg/mL BSA, and 4 mM DTT.

3 Methods

3.1 Bacterial Cell Transformation and Growth

1. Transform *E. coli* cells with an overexpression plasmid derived from pKK223-3 that encodes the gene for *E. coli* $tRNA^{Arg}$(ICG) (*see* **Note 1**).
2. Pick a single bacterial colony, transfer into ~5 mL of LB (Luria-Bertani) broth with the antibiotic marker ampicillin (Amp) at 100 μg/mL, and grow overnight at 37 °C on a shaker. Inoculate the overnight culture into 2 L of fresh medium (1:100 dilution) with the same Amp concentration, grow to OD_{600} of 0.3–0.4 at 37 °C, and induce with IPTG at 0.3 mM at 37 °C to turn on expression of the tRNA gene. Harvest the induced cells after 15–18 hours (h) at 37 °C, wash cells with 0.9% NaCl, and store cells at –80 °C until use.

3.2 Isolation of the Total tRNA Pool and Deacylation

1. Isolate the total tRNA pool from cells according to the chapter "Preparation of an enriched $tRNA^{Arg}$ fraction for arginylation by expression in *E. coli*" by Avcilar-Kucukgoze et al. of this book.
2. Resuspend the total tRNA pool in 500 μL of the deacylation buffer. Incubate for 3 h at 37 °C for deacylation.

3. Ethanol precipitate the deacylated total tRNA pool with 1/10 volume (vol) of 3 M NaOAc (sodium acetate) (pH 5.0) and 3 vol of ethanol (100%) (*see* **Note 2**).
4. Resuspend the tRNA pellet in 500 μL TE for the 3′-amino tailing reaction below.

3.3 3′-Amino Tailing of the tRNA Pool

1. Start with 120 nmoles of the deacylated tRNA pool, and divide it into three tubes of 40 nmoles each. Mix in each tube the deacylated tRNA pool in a final vol of 800 μL in the 1× CCA buffer. Add to each tube 1.75 mM nATP, 1 mM inorganic pyrophosphate (PPi), 20 μM *E. coli* CCA enzyme (*see* **Note 3**), and 3.3 μL of the RNaseOut solution. Incubate all three reactions at 37 °C for 1 h. Each reaction is catalyzed by the CCA enzyme to exchange the terminal A76 of tRNA with nATP.
2. To each reaction, add 10.6 μL of 100 U/μL of pyrophosphatase to remove the PPi released from nATP after the exchange reaction. Incubate for 15 min at room temperature (RT).
3. Add 81.1 μL of 3 M NaOAc (pH 5.0) to each tube, and perform extraction with phenol-chloroform-isoamyl alcohol (24:5:1) in 100 mM NaOAc (pH 5.0) (*see* **Note 4**). To the supernatant, add an equal vol of isopropanol, incubate at 10 min at RT, and spin at 13,500 rpm for 15 min, 4 °C, to collect the 3′-tailed total tRNA (*see* **Note 5**). Wash the tRNA pellet with 70% ethanol, dry, and dissolve it in 200 μL ddH_2O each.
4. Ethanol precipitate the 3′-tailed tRNA from all three reactions, and dissolve the sum of all tRNA reactions in 800 μL of the TB buffer. Split the 800 μL tRNA solution into four aliquots of 200 μL each for affinity purification below.

3.4 Affinity Purification of $tRNA^{Arg}$(ICG) from the 3′-Tailed Total tRNA Pool

1. Prepare the resin. Pour 600 μL of the slurry of Streptavidin Sepharose High Performance into the upper cup of an Ultrafree-MC microcentrifuge filter and centrifuge for 10 s at 7500 rpm. Discard the flow-through and resuspend the slurry in 300 μL of the TB buffer by gentle vortex. Repeat washing the resin three times.
2. Design a 3′-biotinylated DNA oligonucleotide that is complementary to the sequence of *E. coli* $tRNA^{Arg}$(ICG) from U12 to C40 (*see* **Note 6**). Order the 3′-biotinylated DNA oligonucleotide from IDT. This is the oligonucleotide that will capture the tRNA of interest.
3. Binding the 3′-biotinylated DNA oligonucleotide to the washed resin. Add 400 μL of 26.2 μM of 3′-biotinylated DNA capture oligonucleotide in the TB buffer and mix with the resin.

4. Incubate for 10 min at RT. Centrifuge for 10 sec at 7500 rpm to remove unbound capture oligonucleotide. Wash the resin four times with 400 μL of TB by centrifugation. Monitor A_{260} of the wash solution to remove unbound capture oligonucleotide until the reading is stabilized. The washed resin is approximately 200 μL.
5. Add 200 μL of 2× hybridization buffer to each aliquot of 200 μL of 3′-tailed total tRNA from **step 5** of Subheading 3.3, resulting in a total of 400 μL each containing 10–100 nmoles of 3′-tailed total tRNA.
6. The 3′-tailed total tRNA is then incubated with the washed resin to selectively bind the tRNA of interest – i.e., *E. coli* tRNA^{Arg}(ICG). Add the 400 μL of each tRNA solution in the 1× hybridization buffer from **step 5** to the washed resin from **step 4** of this Methods section, resulting in a total of 600 μL of each tRNA-resin solution. Transfer the 600 μL of the tRNA-resin solution to a 1.5 mL Eppendorf tube. Resuspend each tRNA-resin solution by gentle vortex.
7. Incubate each tRNA-resin solution for 10 min at 65 °C in a heat block to denature the structure of the tRNA. After the heat block is slowly cooled to RT, which permits hybridization of the tRNA to the capture oligonucleotide on the resin, the resin can be stored at 4 °C until the next step.
8. Divide the 600 μL tRNA-resin solution into 2 aliquots of 300 μL each. Transfer each aliquot to a new Ultrafree-MC cup. Centrifuge the two cups each in a Ultrafree-MC cartridge to remove the unbound tRNA from the resin.
9. Wash each resin with 400 μL of the TB buffer at RT until A_{260} of the wash solution is below 0.2. This may require 5–8 washes.
10. Elute the bound tRNA by heating each resin at 65 °C in 400 μL of the TB buffer in the upper cup of an Ultrafree-MC cartridge. Recover the eluted tRNA by a quick spin. Repeat the heat elution step using fresh 400 μL of the TB buffer.
11. Combine the two aliquots of eluted tRNA into a new Corex tube.
12. Repeat **steps 6**–**10** of this Methods section. Combine all fractions of the eluted tRNA, precipitate the tRNA with ethanol, and resuspend it in one solution of 50 μL in water. Make a 1:200 dilution to measure A_{260} and determine the concentration of the eluted tRNA. Because the eluted tRNA might contain the capture oligonucleotide, Subheading 3.5 describes the removal of the capture oligonucleotide.

3.5 Removal of the Capture Oligonucleotide

1. Transfer a fresh aliquot of 30 μL slurry of Streptavidin beads to a fresh 1.5 mL Eppendorf tube. Centrifuge for 30 s at 7000 rpm and remove the supernatant. Wash the beads with 500 μL of the TB buffer with 0.1 M NaCl and centrifuge for 30 s at 7000 rpm. Remove the supernatant and wash the beads with the TB buffer containing 0.1 M NaCl twice.
2. Add 300 μL of 0.1 M NaCl to the eluted tRNA from **step 12** of Subheading 3.4 and mix in with the washed beads from **step 1**. Incubate in a vortex shaker at 1200 rpm for 10 min at RT. At this salt concentration, the tRNA is separated from the capture oligonucleotide, which remains bound to the resin. Centrifuge the mixture for 2 min at 7000 rpm at 4 °C to separate the supernatant from the resin-bound capture oligonucleotide.
3. Collect the supernatant, which contains the eluted tRNA, and transfer it to a 1.5 mL Eppendorf tube.
4. To the eluted tRNA, add 1/10 vol 3 M NaOAc (pH 5.0) and precipitate it with ethanol. Resuspend the eluted tRNA in 100 μL TE. Make a 1:100 dilution of the eluted tRNA, and measure A_{260} to determine the concentration.

3.6 Stable Charging of $tRNA^{Arg}(ICG)$

1. Order L-[4,5-^{3}H] arginine ([^{3}H]-Arg)). The information of the [^{3}H]-Arg used for this chapter is as follows: 40 Ci/mmol, 1 mCi/mL in 0.01 N HCl, and 1 mCi/vial. This information varies from batch to batch of the radiochemical.
2. Perform an analytical charging reaction. Mix 20 μM $tRNA^{Arg}$(ICG) in 1× AA buffer with 330 μM Arg, 5 mM ATP, 3.0 μL of the stock of [^{3}H]-Arg, and 10 μM purified *E. coli* arginyl-tRNA synthetase (ArgRS) (*see* **Note 7**) in a total vol of 15 μL. Remove 1 μL of the reaction mixture into 99 μL ddH_2O as the "specific activity" sample. Incubate the aminoacylation reaction for 30 min at 37 °C.
3. Terminate the reaction by extraction with phenol-chloroform-isoamyl alcohol (24:5:1) in 100 mM NaOAc (pH 5.0). Ethanol precipitate $tRNA^{Arg}$(ICG), which is then washed and dried.
4. Most of the $tRNA^{Arg}$(ICG) molecules should be stably charged with Arg. For those molecules that are not 3′-amino tailed, they are charged with Arg by ArgRS during the charging reaction via the normal ester linkage, which is sensitive to alkali. Such ester-linked Arg-$tRNA^{Arg}$(ICG) can be removed by a deacylation reaction. Dissolve the Arg-charged $tRNA^{Arg}$(ICG) from **step 2** in 50 μL of 0.1 M glycine (pH 9.0). Incubate it for 30 min at 37 °C to deacylate all ester-linked Arg-$tRNA^{Arg}$(ICG). The remaining species should be only stably charged Arg-$tRNA^{Arg}$(ICG).
5. Purify the stably charged tRNA from **step 4** using an Oligo Clean and Concentrator kit. Elute the stably charged tRNA in

15 μL ddH_2O. Read A_{260} to determine the concentration. This is the "stably charged" sample.

6. Determine radioactivity. To a 0.6 mL Eppendorf tube, add 0.4 mL of a counting cocktail. Prepare two such tubes. To one tube, add 5 μL of the "specific activity" sample, while to the other tube, add 5 μL of the "stably charged" sample. Mix each tube thoroughly. Measure the [^{3}H]-Arg radioactivity in a Tri-Carb 4910 TR liquid scintillation counter.

3.7 Calculation of the Stable Charging Efficiency

1. Calculate the total counts of [^{3}H]-Arg in the "specificity activity" sample. The concentration of Arg in the sample is 3.3 μM (1:100 dilution of 330 μM), and the vol used for counting is 5 μL. Thus,

$$(\text{DPM of the "specific activity" sample})/(3.3\mu\text{M} \times 5\mu\text{L}) = \text{specific activity (dpm/pmoles)}. \quad (1)$$

2. Calculate the "stably charged" Arg-tRNA^{Arg}(ICG), which is counted by 5 μL. Thus,

$$(\text{DPM of the "stably charged" sample})/[\text{specificity activity (dpm/pmoles, from Eq.1)} \times 5\mu\text{L}] = \text{amount of stably charged Arg}-\text{tRNA}^{Arg}\ (\text{ICG}) \text{ in } \mu\text{M}. \quad (2)$$

3. Calculate the charging efficiency:

$$[(\text{The amount of stably charged Arg-tRNA}^{Arg}(\text{ICG}), \text{ from Eq. 2})/(\text{total tRNA concentration in the aminoacylation reaction})] \times 100\% = \text{stable charging efficiency (\%) in the total amount of input tRNA}^{Arg}(\text{ICG}) \quad (3)$$

4. The total yield of affinity-purified 3′-tailed *E. coli* tRNA^{Arg}(ICG) at this point is 22 nmoles.

3.8 Perform a Preparative Charging Reaction

1. Prepare a solution of 22 nmoles of affinity-purified 3′-tailed *E. coli* tRNA^{Arg}(ICG) in 180 μL of ddH_2O. Heat denature the tRNA for 3 min at 85 °C, add 30 μL of 10× AA buffer, and slowly cool the tRNA to RT.
2. Add to the heat-cooled tRNA at the final concentration of 6.25 mM ATP, 0.8 mM Arg, 0.1 mg/mL BSA, 4 mM DTT, and 10 mM $MgCl_2$ in a final vol of 300 μL. Incubate for 30 min at 37 °C for aminoacylation of the tRNA with Arg.
3. Phenol extraction of the charging reaction. Precipitate the tRNA with an equal vol of isopropanol, followed by washing

the tRNA pellet with 70% ethanol and drying the pellet. Dissolve the tRNA pellet in 100 μL 0.3 M NaOAc (pH 5.0).

4. Ethanol precipitate the tRNA and dissolve it in 100 μL RNase-free ddH_2O.
5. Deacylate the tRNA by adding 10 μL of 1 M glycine (pH 9.0) and incubating the tRNA for 30 min at 37 °C. Ethanol precipitate the tRNA and dry the pellet.
6. Calculate the amount of stably charged Arg-tRNA^{Arg}(ICG). Multiply the amount of 3′-amino-tailed and affinity-purified tRNA (nmoles) with the "efficiency" of stable charging (%) (from Eq. 3). This yields the amount of 3′-amino-tailed and affinity-purified tRNA (nmoles). The final yield is the value multiplied by 0.9 to account for the potential loss during phenol extraction and ethanol precipitation.
7. The final yield of stably charged Arg-tRNA^{Arg}(ICG) from this protocol is 6.5 nmoles.

4 Notes

1. The *E. coli* plasmid for overexpression of *E. coli* tRNA^{Arg}(ICG) is available upon request [6].
2. Typical ethanol precipitation of tRNA is with 0.3 M NaOAc (pH 5.0), 3 vol ethanol (100%) for 30 min at –20 °C, followed by centrifuge at 13,500 rpm for 30 min at 4 °C, by washing the pellet with ethanol (70%) at 13,500 rpm for 15 min at 4 °C and by drying the pellet in a SpeedVac.
3. We purify *E. coli* CCA enzyme to homogeneity from an overexpression clones [9, 10]. This clone is available upon request.
4. Typical phenol extraction of tRNA is with 0.3 M NaOAc (pH 5.0) and equal vol of phenol-chloroform-isoamyl alcohol (24:5:1) in 100 mM NaOAc (pH 5.0). Vortex for 30 sec and centrifuge the solution for 2 min at 13,500 rpm at RT. Transfer the aqueous phase to a new tube.
5. Typical isopropanol precipitation of tRNA is with 0.3 M NaOAc (pH 5.0) and equal vol isopropanol (100%) for 10 min at RT, followed by centrifugation, a wash with 70% ethanol, and by drying the pellet as described for ethanol precipitation.
6. We design the capture oligonucleotide in 20–30 residues in length. It should be complementary to the anticodon of the target tRNA and extend into the D stem-loop region.
7. We purify *E. coli* ArgRS from an overexpression clone. This clone is available upon request [6].

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Chapter 29

A High-Throughput Colorimetric Microplate Assay for Determination of Plasma Arginase Activity

Natalie J. Smith, Mahnaz Maddahfar, Bavani Gunasegaran, Helen M. McGuire, and Barbara Fazekas de St Groth

Abstract

Arginase, an enzyme involved in the urea cycle, is gaining attention as a critical player in numerous chronic pathologies. Additionally, increased activity of this enzyme has been shown to correlate with poor prognosis in a range of cancers. Colorimetric assays that measure the conversion of arginine to ornithine have long been used to determine the activity of arginase. However, this analysis is hindered by a lack of standardization across protocols. Here, we describe in detail a novel revision of the Chinard's colorimetric assay used to determine arginase activity. Dilution series of patient plasma are plotted to form a logistic function, from which activity can be interpolated by comparison to an ornithine standard curve. Inclusion of patient dilution series rather than a single point increases the robustness of the assay. This high-throughput microplate assay analyzes 10 samples per plate to produce highly reproducible results.

Key words Arginase, Arginine, Colorimetric assay, Enzyme activity, Microplate

1 Introduction

Arginase catalyzes the final step in the urea cycle, hydrolyzing arginine to form ornithine and urea [1]. It is expressed in two isoforms: Arginase-1 is found in the cytosol of hepatocytes, macrophages, myeloid cells, and innate lymphoid cells, all of which also secrete it. Hepatocytes and tumor cells have also been shown to package arginase-1 into extracellular vesicles (EVs) with global metabolomic effects [2, 3]. Arginase-2 is expressed constitutively in the mitochondria of most cells and cannot be secreted [4, 5] (Fig. 1). Both isoforms of arginase have similar mechanisms of action and produce the same metabolites, with 100% homology observed in areas critical to enzyme function [6]. Studies of arginase expression and activity are of increasing relevance, as the importance of this enzyme becomes more thoroughly documented across pathologies. Arginase has emerged as crucial regulator of

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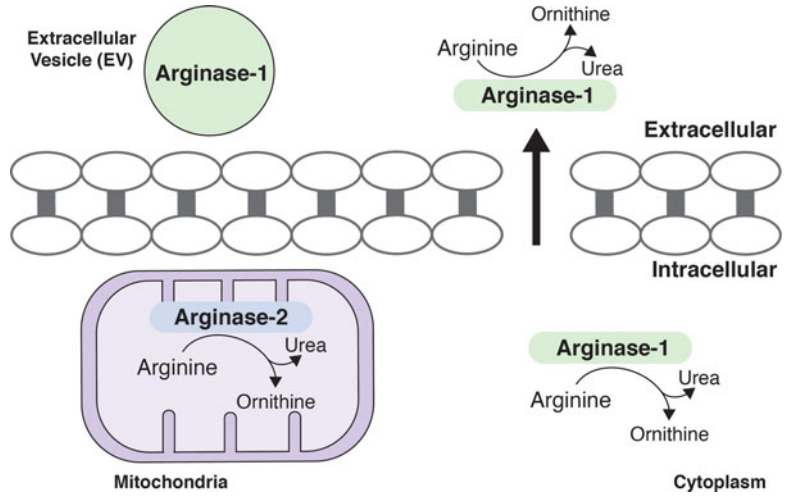

Fig. 1 Isoforms of arginase are differentially expressed in cells. Arginase catabolizes the amino acid arginine to form ornithine and urea. The two isoforms of arginase (ARG1/2) are found in different cellular locations. Arginase-1 (ARG1) is in the cytoplasm but can be secreted, either directly into the extracellular fluid or packaged into extracellular vesicles (EVs). ARG2 is the mitochondrial form of the enzyme, constitutively expressed in most cells

immunity in many chronic diseases, including cancer, autoimmunity, cardiovascular disease, and renal disease [7–11]. Increased arginase activity reduces the availability of arginine, an amino acid with strong immunomodulatory properties. Additionally, the presence of excess ornithine has been linked to structural defects in the cardiovascular, renal, and nervous systems [12, 13]. Accordingly, the measurement of arginase activity provides an important insight into the progression of a range of chronic pathologies.

Normal blood plasma contains only trace activity of arginase-1. However elevated activity has been detected in breast, ovarian, cervical, esophageal, prostate, gallbladder, liver, colorectal, and gastric cancers [2, 14–21]. However, studies of arginase-1 are complicated by the lack of standardized measurement criteria, hindering comparisons across studies. Colorimetric assays have long been used to determine arginase activity by measuring the conversion of arginine to ornithine, either in plasma or in tissue lysates [2, 21–25]. This method, first described by Chinard [26], is based on the principle that ninhydrin will react selectively with ornithine to generate a red colored product in acidic conditions. Chinard's method was subsequently adapted for use in a microplate [22, 25]. Measurement of this reaction, like those involved in radioimmunoassay and ELISA, generates a sigmoid dose-response curve that can be best approximated by fitting a logistic function fully described by parameters defining plateau, baseline, slope, and x-intercept [27]. The theoretical basis of biological assays

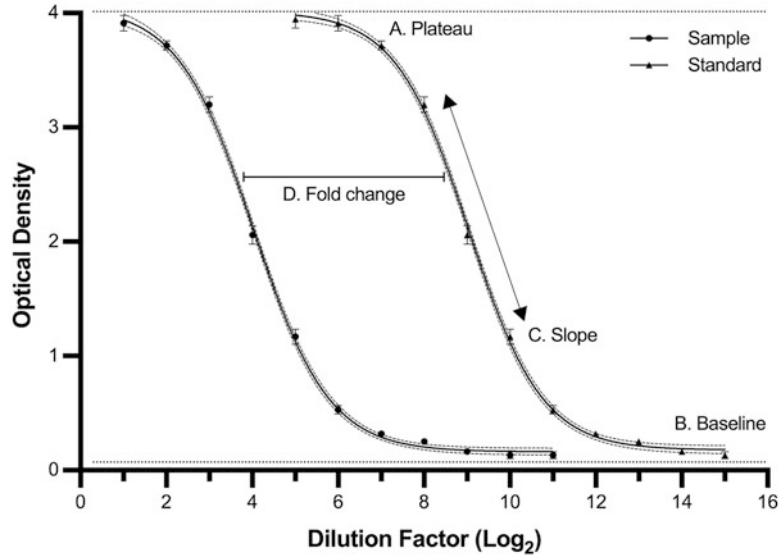

Fig. 2 Features of a sigmoidal curve. Sigmoidal curves of optical density plotted against dilution factor (log_2) annotated with (**a**) plateau, (**b**) baseline, (**c**) slope, and (**d**) fold change between the curves. 90% confidence intervals indicated by dashed line

generating sigmoid dose-response curves is that the dose-response curve for a test sample differs from the standard curve only by the x-intercept and that the difference between the two x-intercepts indicates the fold change in concentrations of the control and sample (Fig. 2). In the case of the arginase assay, the logistic functions fitted to control and sample data should have comparable plateau, baseline, and slope.

One commonly used method to calculate test values for assays with a logistic dose-response curve is to fit the standard logistic curve using untransformed data and then estimate the relative sample values from either a single test point or a number of replicate points at a single dilution. This approach assumes that sample data are all well approximated by a logistic curve with the same plateau, baseline, and slope as the control curve. The difficulty with this method is the choice of sample concentration: too high and the measurement may be reduced by nonspecific factors in the plasma; too low and the signal may be too close to the assay background. In addition, estimation of relative difference in concentration for the standard and test samples is most accurate where the slope of the standard curve is greatest, at its point of inflection [28]. Thus, even accurate assay measurements of sample activity that are near the standard curve plateau or baseline will generate activity estimates with wide margins of error.

For these reasons, we present a novel revision of the Chinard method that utilizes a dilution series rather than a single dilution as

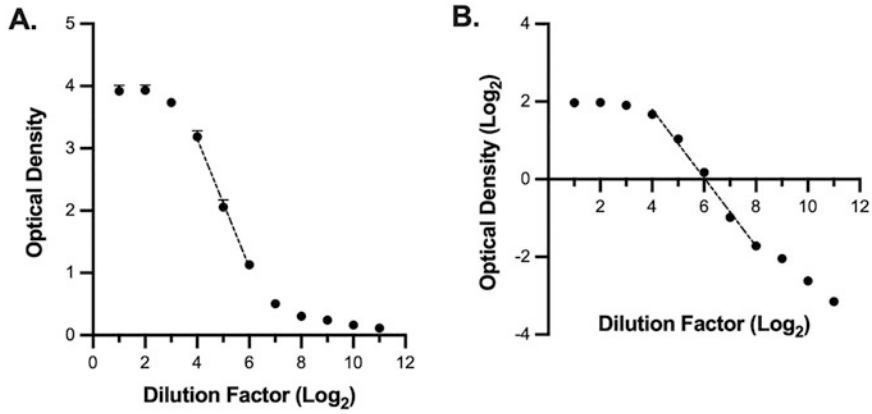

Fig. 3 Log transformation of optical density (y-values). (**a**) untransformed ornithine-HCl standard curve ($R^2 = 0.99$) and (**b**) log-transformed ornithine-HCl standard curve ($R^2 = 0.99$). The "linear" portion of the curve is indicated by a dotted line and includes more datapoints after log transformation of the optical density results, giving a more accurate estimation of fold change when compared to sample data

the basis of activity estimation for each sample. Ideally, the plateau, baseline, and slope of the fitted logistic curve would be estimated by combining the control and sample data, but there are no readily available computer programs that achieve this. We have used an alternative interpolation method, piecewise linear regression, following log transformation of the y-values (Fig. 3). This generates a slope measurement common to both the control and sample curves, while introducing an estimation error related to fitting a straight line to the "linear" portion of the logistic curve. This assay can be applied to a range of pathologies to generate highly reproducible measurements of arginase activity.

2 Materials

Carbonate buffer and stop solution stock may be prepared in advance. Solutions of arginine-HCl, ornithine-HCl, and $MnCl_2$ may be prepared the day of the experiment. Ninhydrin should be dissolved in the stop solution stock immediately prior to use in the assay.

2.1 Plasma Isolation by Density Gradient Centrifugation

1. Fresh whole blood collected in an anticoagulant-coated tube.
2. Centrifuge.

2.2 Colorimetric Microplate Assay

1. Phosphate-buffered saline (PBS).
2. 100 mM carbonate buffer: 0.760 g $NaHCO_3$, 4.34 g of Na_2CO_3, 500 mL milli-Q water, pH 10.5.
3. Stop solution stock: 6.5 mL 85% phosphoric acid (final concentration 1.1%), 455 mL 100% acetic acid (final concentration 91%), 38.5 mL milli-Q water.

4. 60 mM $MnCl_2$: 11.9 mg $MnCl_2$, 1 mL Milli-Q water (*see* **Note 1**).
5. 0.5 M arginine-hydrochloride (HCl): 52.6 mg arginine-HCl, 500 μL carbonate buffer.
6. 25 mM arginine-HCl: 1 mL 0.5 M arginine-HCl, 19 mL carbonate buffer (*see* **Note 2**).
7. Complete stop solution: 20 mL stop solution stock, 150 mg ninhydrin (final concentration 7.5%). Ninhydrin should be dissolved in stop solution stock immediately prior to use (*see* **Note 3**).
8. 100 mM ornithine-hydrochloride: 16.86 mg ornithine-HCl, 1 mL carbonate buffer.
9. 1 mM ornithine- hydrochloride: 10 μL 100 mM ornithine-HCl, 990 μL carbonate buffer.
10. 96-well, clear, flat-bottomed microplate.
11. Milli-Q water.
12. Heat-resistant plastic plate sealer.
13. 37 °C incubator.
14. Oven or pressure cooker set to 95 °C.

2.3 Analysis

1. Microplate reader (Tecan i-control infinite M1000 Pro): set at 515 nm.
2. GraphPad Prism.

3 Methods

3.1 Plasma Isolation by Density Gradient Centrifugation

1. Centrifuge blood vacutainers at 800 g for 15 min at room temperature with normal acceleration and the brake on.
2. Transfer plasma from the vacutainer in a 15 mL falcon tube using a sterile transfer pipette. Repeat the centrifugation, with 1600 g for 10 min (*see* **Note 4**).
3. Aliquot the double-spun plasma into labeled cryovials without disturbing the pellet. Store samples at −80 °C.
4. Subsequent Ficoll-Hypaque density gradient separation may be performed to isolate peripheral blood mononuclear cells (PBMCs) (*see* **Note 5**).

3.2 Standard Curve

1. Label 8 1.5 mL Eppendorf tubes with numbers 1–8. Aliquot 500 μL of carbonate buffer to tubes 2–8 and 1000 μL of 1 mM ornithine-HCl to tube 1.

Table 1
Concentration of ornithine-HCl at each point in standard curve

Dilution number	Concentration of ornithine-HCl (μM)
1	1000
2	500
3	250
4	125
5	62.5
6	31.3
7	15.6
8	Blank

2. Perform serial dilution by pipetting 500 μL of 1 mM ornithine-HCl from tube 1 to tube 2 Vortex the tube well and discard pipette tip.
3. Repeat six more times to form a seven-point twofold dilution series with ornithine concentrations starting at 1000 μM as in Table 1 (*see* **Note 6**). Leave tube 8 blank with carbonate buffer only.

3.3 Serial Dilution of Plasma

1. Remove plasma samples from storage at −80 °C and defrost on ice. Ten plasma samples can be analyzed on each microplate, requiring a total of 170 μL of plasma from each sample (*see* **Note 7**), with example plate layout (Fig. 4).
2. Aliquot 25 μL of PBS to all wells in columns 2–4, 6–8, and 10–12, excluding the standard curve wells (A1–8, B1–8).
3. Aliquot 85 μL of plasma sample 1 into wells C1 and D1, repeat with samples 2–10 by aliquoting into the remaining wells in columns 1, 5, and 9 as in Fig. 2.
4. Perform serial dilution by adding 60 μL of plasma to 25 μL of PBS.
5. Mix well and change pipette tips. Repeat three more times to form a four-point series with factor dilution $\sqrt{2}$ and dilution numbers 1, 1.5, 2, and 2.5 (*see* **Note 8**).
6. Discard last 60 μL. The final volume of plasma in each well should be 25 μL.

3.4 Colorimetric Assay

1. Aliquot 5 μL of 60 mM $MnCl_2$ solution into all wells containing plasma (final concentration 10 mM $MnCl_2$), leaving the standard curve wells empty. Mix well (see **Note 9**).
2. Incubate the microplate at 37 °C for 5 min to activate arginase.

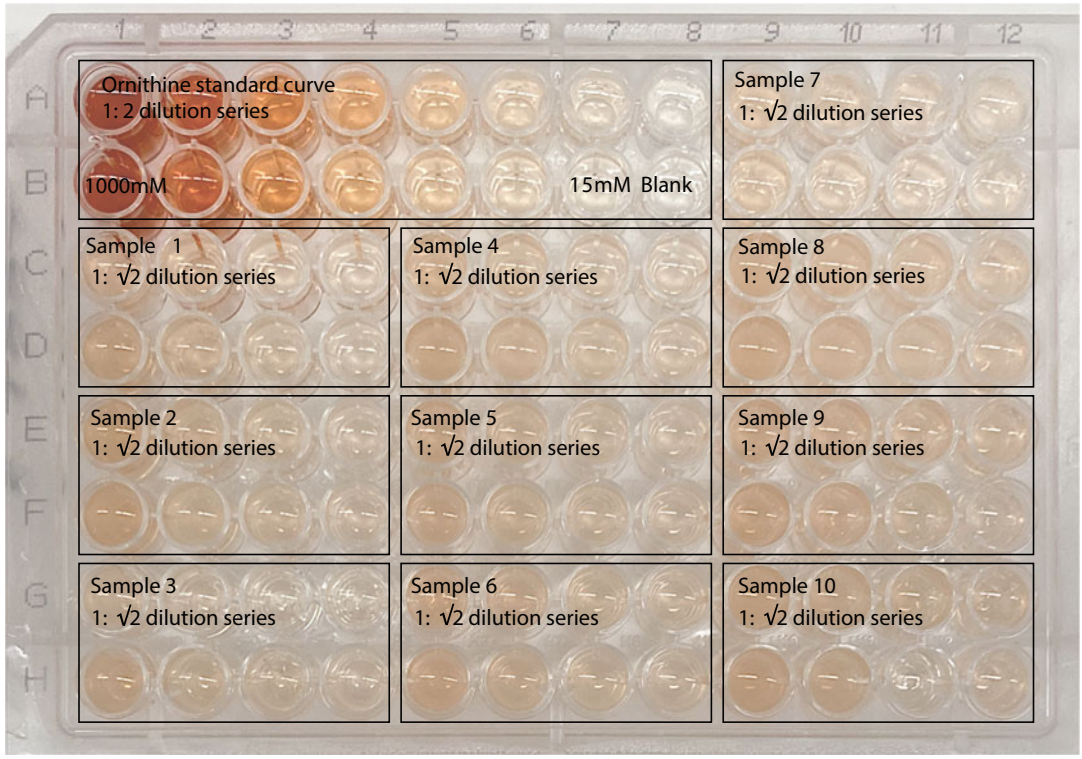

Fig. 4 Microplate layout. Plate layout for colorimetric assay, including ten patient serum dilution series and one ornithine-HCl standard curve. Standard and patient serum placement labeled on microplate

3. Remove from incubator and aliquot 50 μL of 25 mM arginine-HCl into all wells containing plasma.
4. Incubate the microplate at 37 °C for 40 min to hydrolyze arginine.
5. Meanwhile, prepare the complete stop solution (*see* **Note 10**).
6. Remove the microplate from the incubator. Vortex standard curve Eppendorf tubes well, and then transfer 80 μL from each Eppendorf tube to the microplate, changing tips between each tube (*see* **Note 11**).
7. Aliquot 190 μL of complete stop solution to all wells of the microplate.
8. Seal the plate with a heat-proof plastic plate sealer.
9. Heat the samples at 95 °C for 15 min. A color change reaction will occur between ninhydrin and ornithine, and a red-orange-colored product will form.
10. Let the plate cool to room temperature for 10 min (*see* **Note 12**).
11. Read optical density (OD) at 515 nm in a plate reader.

3.5 Analysis

1. Transform all OD readings by $\log_2$ and import log-transformed data into prism (*see* **Note 13**).
2. Plot $\log_2$(OD) of the standard curve against dilution number (*see* **Note 13**).
3. Determine the linear portion of the curve and exclude nonlinear points to optimize R^2.
4. Using the slope from the optimized linear standard curve, plot $\log_2$(OD) of each patient serum dilution series against dilution factor (*see* **Note 14**). Exclude nonlinear points to optimize R^2 of each patient plasma dilution series.
5. Determine the $\log_2$(fold change) between the standard and each sample by interpolating a value from both curves and subtracting the sample from the standard. Calculate 2 to the power of this value (2^x) to generate the fold change between the standard and each sample (Fig. 5).
6. To calculate the arginase activity of each sample, divide the neat activity of 80 U/L by the fold change (*see* **Note 15**).

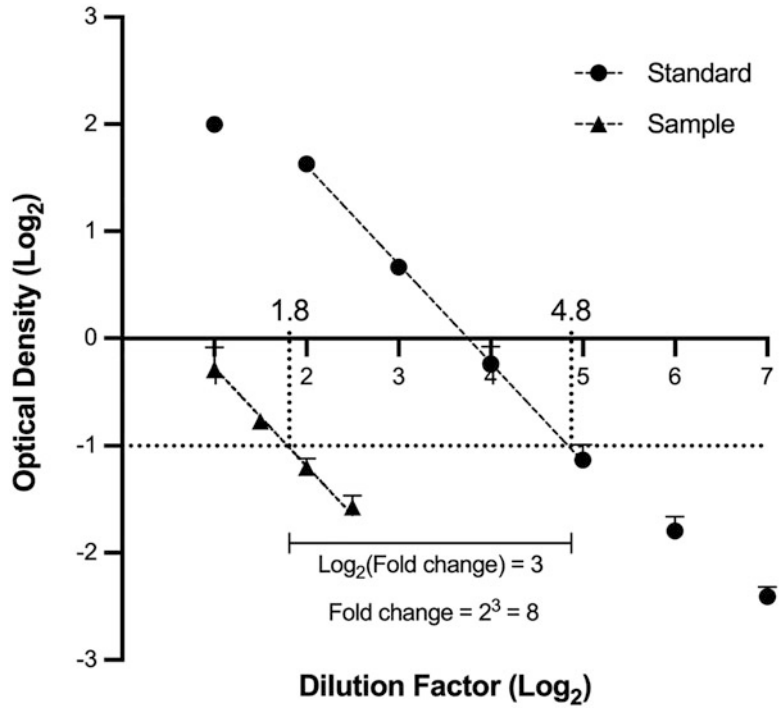

Fig. 5 Calculation of fold change between the standard and sample curves. Standard curve is plotted, and the linear portion determined to optimize R^2 ($R^2 = 0.99$). Patient plasma dilution series was plotted with the slope of the standard curve ($R^2 = 0.96$). An optical density value (-1) is interpolated from each curve (annotated on the graph). The interpolated dilution factor of the sample (1.8) is subtracted from the standard (4.8) to give the log fold change (3). The fold change is 2 to the power of this value (2^3). The neat activity (80 U/L) is divided by the fold change (8) to give the activity of the sample (8.9 U/L)

4 Notes

1. Arginases require divalent cations for activity; Mn^{+} ions are the physiological activator of this enzyme [29].
2. Arginine-hydrochloride is the salt form of the amino acid. Using arginine (not arginine-HCl) will cause the formation of a purple color called Ruhemann's purple as ninhydrin reacts nonselectively with arginine [30]. If a purple color forms even when using arginine-HCl, the stop solution may not be acidic enough or the concentration of the arginine-HCl solution may be too high.
3. Ninhydrin is light-sensitive and should be stored in the dark in a well-sealed container. Ninhydrin should be dissolved in the stop solution stock immediately prior to use. As this solution is not stable, the use of old complete stop solution will introduce variation and error to your assay. 20 mL is enough for 1 microplate. If the assay requires multiple microplates, a greater volume may be prepared, as long as it is used immediately.
4. When blood is drawn into tubes coated with an anticoagulant, the acellular fraction is plasma. In the absence of an anticoagulant, a clot will form and can be separated from the serum. The assay described here was optimized with plasma, not serum. However, it is likely that it will also be suitable for determination of arginase activity in serum.
5. Further, blood processing by Ficoll-Hypaque separation may be performed, if matched analysis with peripheral blood mononuclear cells (PBMCs) is warranted. In this case, add a volume of PBS+5%FCS equal to the original volume of blood to the plasma-depleted blood. Carefully underlay 10 mL of Ficoll-Paque plus, and then spin at 500 g for 30 min with the brake turned off. Remove the interface layer of PBMCs with a sterile transfer pipette and wash twice in PBS+5% FCS. Freeze at a concentration of 2×10^{6} cells/mL in 20% FCS and 10% DMSO in RPMI-1640.
6. The dilution factor is the number in the dilution series. The standard curve has been optimized to include dilution factors ranging from 1 to 7. OD readings are log-transformed to generate a dose-response curve with the longest "linear" segment. The range of the standard ornithine curve was set between 15.6 and 1000 μM to include all the values within the "linear" range while excluding repeated plateau and baseline values (Fig. 6).
7. Repeated freeze-thaw cycles are not recommended as this will introduce error to the assay and may lead to a higher activity calculation, potentially by rupturing extracellular vesicles that

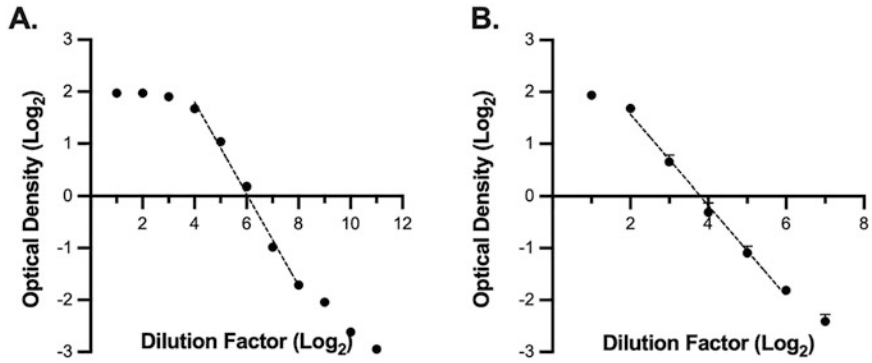

Fig. 6 Optimization of ornithine-HCl standard curve. (**a**) Original eleven-point standard curve with ornithine concentration ranging from 20,000 to 19 μM ($R^2 = 0.99$). (**b**) Optimized seven-point standard curve with ornithine concentration range in detailed in Table 1 ($R^2 = 0.99$). Linear portion of each curve indicated by dotted line

contain arginase-1 [2]. It is not recommended to perform analysis on plasma samples that have undergone more than two freeze-thaw cycles. If plasma is hemolyzed (pink in color), it should not be included in the assay as red blood cells contain arginase-1 and the calculated plasma activity will be too high [31].

8. A smaller dilution factor is used for the patient plasma ($\sqrt{2}$) than for the standard curve (2), because the amount of arginase activity in most plasma samples is too low to generate ornithine concentrations of more than 440 μM. For patient samples, a twofold dilution series thus generates only 1–2 measurements within the "linear" range of the standard curve. To generate additional measurements and allow more accurate calculation of the dose-response curve for each patient sample, an intermediate dilution of $\sqrt{2}$ was interpolated between each of the twofold dilutions. Reducing the dilution factor assists in identification of the "linear" portion of the dose-response curve, as distinct from baseline values (Fig. 7).
9. It is crucial to ensure that the amount of $MnCl_2$ is consistent across every sample well, as the differential activation of arginase will confound subsequent calculations of arginase activity. If not confident with reproducible pipetting of small volumes, it is possible to carry out the assay in two microplates. In this case, $MnCl_2$ should be first added to all wells (excluding standard curve) of an empty plate labeled "test plate." This will allow you to see the small volume and check that $MnCl_2$ has been added to all wells. Serial dilutions of patient plasma should be carried out in a second U-bottom microplate labeled "dilution plate," and then transferred across to the test plate containing $MnCl_2$. The assay can be continued from here as per the protocol.

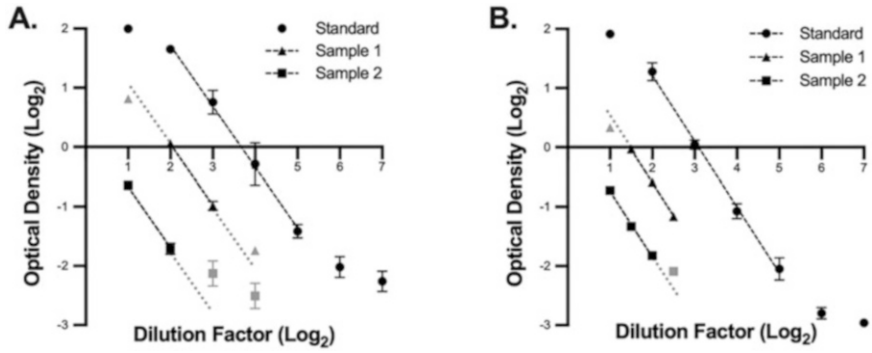

Fig. 7 Optimization of patient plasma dilution factor. (**a**) Standard curve and two plasma dilution series with factor 2 and (**b**) factor sqrt(2). Points in the plasma dilution series that were excluded from analysis are indicated in gray. In fig. (**b**), R^2 prior to exclusion of points was 0.97 and 0.92 for sample 1 and 2, respectively. Following exclusion of points, R^2 increased to 0.99 and 0.98

10. Ninhydrin crystals may be difficult to dissolve in the stop solution stock; if this is the case, vortex for 2 min in a 50 mL falcon tube covered in foil until no crystals are visible. All steps involving the stop solution should be performed inside of a chemical safety hood as this solution highly acidic and will burn the skin upon contact.
11. The ornithine-HCl standard curve should be quickly pipetted into the microplate, as the hydrolysis of arginine will continue until the stop solution is added. If not confident with quick pipetting, add the stop solution to the wells containing plasma before aliquoting the standard curve to the plate and then adding the stop solution to the standard curve. Keep this consistent between plate runs to ensure results are comparable. The reaction between ninhydrin and ornithine occurs under hot, acidic conditions and will not occur in full until the plate is heated to 95 °C.
12. Stable OD readings will be obtained for approximately 1 h after the microplate is removed from the pressure cooker. Ensure consistency in the time the plate is left to cool. A red-orange precipitate may form in the wells if cooled for too long. If this is the case, agitate the plate in the plate reader to resuspend the precipitate.
13. OD readings are log-transformed so as to generate a dose-response curve with the longest "linear" segment.
14. The neat activity is the activity of arginase that would generate the amount of ornithine present at the first point of the standard curve, i.e., 1000 μM. Activity is calculated as the amount of ornithine produced per minute per liter at 37 °C, according to the formula below.

$$Arginase\ Activity = \frac{Concentration\ of\ Ornithine\ (\mu M) * Ornithine\ Volume\ (\mu L)}{Reaction\ Time\ (minutes) * Sample\ Volume\ (\mu L)}$$

$$Arginase\ Activity = \frac{1000 * 80}{40 * 25} \qquad Arginase\ Activity = 80$$

15. This assay was optimized using 92 plasma samples from patients with lung cancer. A range of arginase activities between 3.0 U/L and 26.6 U/L with mean 11.07 U/L was found in this cohort of lung cancer patients. Similar ranges in plasma arginase activity have been observed in breast, ovarian, cervical, prostate, gallbladder, and colorectal cancers [2, 15, 17–21]. Plasma arginase activity has been observed to be lower in healthy individuals, with a documented mean of approximately 5 U/L. This finding has not been validated using our described method [2, 18, 19].

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Chapter 30

Development of New Tools for the Studies of Protein Arginylation

Anna S. Kashina

Abstract

Studies of posttranslational modifications present many unique challenges, stemming from their role as the major drivers of biological complexity. Perhaps the most immediate challenge to researchers working on virtually any posttranslational modification is the shortage of reliable easy-to-use tools that can enable massive identification and characterization of posttranslationally modified proteins, as well as their functional modulation in vitro and in vivo. In the case of protein arginylation, which utilizes charged Arg-tRNA that is also used by the ribosomes, detection and labeling of arginylated proteins is especially difficult, because of the necessity of distinguishing these proteins from the products of conventional translation. As of now, this difficulty remains the major obstacle to new researchers entering the field. This chapter discusses some of the strategies for developing antibodies for arginylation detection, as well as some general considerations for development of other tools for studies of arginylation.

Key words Arginylation detection, Arginylation antibodies, Tools for the studies of posttranslational modifications

Studies of posttranslational modifications present many unique challenges, stemming from their role as the major drivers of biological complexity. Perhaps the most immediate challenge to researchers working on virtually any posttranslational modification is the shortage of reliable easy-to-use tools that can enable massive identification and characterization of posttranslationally modified proteins, as well as their functional modulation in vitro and in vivo. In the case of protein arginylation, which utilizes charged Arg-tRNA that is also used by the ribosomes, detection and labeling of arginylated proteins is especially difficult, because of the necessity of distinguishing these proteins from the products of conventional translation.

As of now, mass spectrometry remains the only reliable method of identification of arginylated proteins in vitro and in vivo. This method is very powerful, but it also has its drawbacks. It is very costly and labor-intensive and requires the use of very precise

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high-end instruments, which are not available in every facility. It also involves very complex multistep procedures for data filtering and validation. In the end, the stringency of the analysis likely results in discarding some of the truly arginylated proteins that cannot be reliably separated from naturally or artificially occurring mass ambiguities.

The main method of arginylation detection utilized throughout the years involves the use of radioactively labeled Arg (^{3}H or ^{14}C), which can be detected by liquid scintillation or autoradiography. This method is useful for arginylation detection in vitro, in ribosome-free systems, but has met with only limited success when applied in the presence of active ribosomes, which also facilitate labeled Arg incorporation into proteins during translation. In addition, both ^{3}H and ^{14}C, although highly stable, are weak emitters, and for proteins with low abundance, detection of these isotopes is either not possible or, in the case of autoradiography, can take multiple weeks of film exposure.

A promising strategy for arginylation detection involves development of antibodies specific to arginylated proteins. In principle, this strategy is feasible based on the success with similar approaches in the studies of other posttranslational modifications (e.g., phosphor-specific antibodies). However, arginylation meets with the unique challenge because of the high similarity of the added Arg to those found in the protein backbone. This concluding chapter discusses some of our successful and ongoing strategies in developing antibodies for arginylation detection, as well as some general considerations for development of other tools for studies of arginylation.

1 "Pan-Arginylation" Antibodies

In the past, we have been partially successful with developing "pan-arginylation" antibodies, designed to recognize any protein with N-terminal Arg, followed by either Asp of Glu, the preferred target sites for arginylation [1]. To do this, we used the following procedure (*see* Fig. 1 for the overview):

1. *Antigen design*. As the antigens, we used peptides containing N-terminally arginylated sequences (Arg-Asp and Arg-Glu) as a part of highly immunogenic 5-amino-acid stretch (consisting of bulky charged residues), followed by a stretch of amino acid with predicted low immunogenicity (enriched in small uncharged residues). In this approach, the N-terminal 5-aa stretch is chosen, because this length represents the minimum size of an antigenic epitope reactive with an antibody. The context of the highly immunogenic epitope followed by the low-immunogenic stretch of amino acids ensures that the

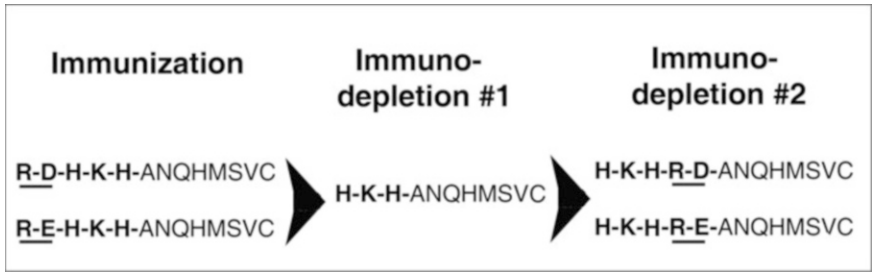

Fig. 1 Peptide design used for the development of "pan-arginylation" antibodies. (Panel adapted from Wong et al. [1])

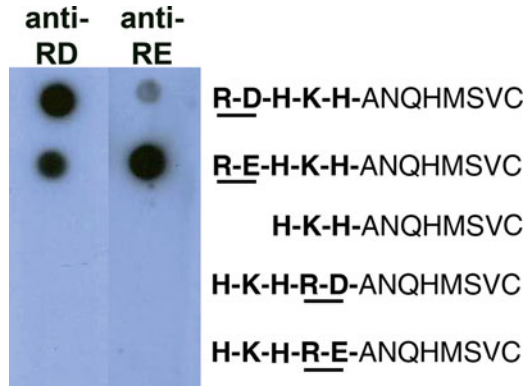

Fig. 2 "Pan-arginylation" antibodies recognize peptides bearing Arg-Asp (anti-RD) or Arg-Glu (anti-RE) sequences at the N-terminus and do not cross-react with similar non-arginylated peptides. Reactivity of the antibodies in dot blots (left) with the peptides listed on the right. Image based on data originally published in [1]

majority of the immune response will be confined to the N-terminal epitope. The peptide sequences used in our study are shown in Fig. 1.

2. *Immunodepletion*. To ensure that the resulting antibodies are specific to the N-terminally exposed Arg, we performed a two-step immunodepletion of the antiserum with (i) the peptide with the sequence similar to the low-immunogenic part of the peptide used for immunization (step 1) and (ii) the peptide similar to the antigen but with the Arg-Asp/Arg-Glu sequence moved into an internal position within the epitope (step 2). The resulting antibodies showed high specificity for the antigen but did not react with any peptides of the similar sequence that were used for immunodepletion (Fig. 2).

It should be noted that while we were successful in generating the antibodies with this design, the results we obtained with these antibodies were not as expected. First, likely due to their broad nature and their specificity to a single amino acid residue (Arg),

they did not show good reactivity on Western blots and stained the cells diffusely in immunofluorescence. This can be explained by the fact that, as we now know, hundreds of proteins are arginylated in vivo, and staining all these proteins at once would likely lead to diffuse staining in the cells. It is also possible that sample preparation for Western and/or cell fixation and processing for immunofluorescence can partially or completely mask the arginylated epitopes on the majority of protein targets.

Our antibodies were successfully applied in pulldowns, revealing hundreds of potentially arginylated proteins in each immunoprecipitate [1]. Among those, mass spectrometry analysis identified arginylated sites on 43 proteins – the first global screen for proteins arginylated in vivo. Surprisingly, however, very few of these proteins were arginylated on N-terminal Asp or Glu, suggesting that in vivo other sites can also be targeted for arginylation via ATE1-binding recognition factors or another, as yet unidentified, mechanism.

2 Antibodies to Ate1

Probing of arginylation activity in biological systems inevitably involves analysis of the level of the ATE1 enzyme. In the past, no reliable ATE1 antibodies have been available on the market, and even the older Affymetrix arrays contained no Ate1-specific probes. Thus, until recently, very little information has been gathered about ATE1 expression, tissue distribution, and potential changes in its intracellular levels in different physiological and disease conditions. Past studies preceding the development of modern tools characterized ATE1 levels by detecting overall arginylation activity in tissues and cell fractions (see, e.g., Chap. 6 of the current book). Recently our group has developed rat monoclonal anti-ATE1 [2], which is highly specific, has broad reactivity with ATE1 in different species, and has been commercially licensed by EMD Millipore (Catalog number MABS436).

3 Peptide Antibodies to Individual Arginylated Proteins

A promising approach to the studies of protein arginylation and its in vivo effects lies in raising antibodies to individual arginylated proteins. Overall, such antibodies represent the ultimate tool for functional dissection of the role of arginylation on specific proteins and protein sites. However, due to high cost and significant effort required to produce each antibody, this approach can be used only for proteins of high importance that have been shown by other methods to be functionally regulated by arginylation.

For N-terminally arginylated proteins, one possible strategy of producing such antibodies involves generating peptide antibodies by a design similar to that described above in Subheading 1 of this chapter. In this approach, the immune peptide should be selected to include the N-terminal sequence including the posttranslationally added Arg and the residues immediately adjacent to it for immunization; the same sequence without Arg should then be used for immunodepletion. Even with this setting, the success of each particular antibody would depend on the protein. Recently, EMD Millipore has developed the first successful antibody to N-terminally arginylated beta actin (Catalog# ABT264).

The recent discovery of midchain arginylation of intact proteins on the carboxy- side chains of Asp and Glu [3] enabled a new strategy of development of anti-arginylation antibodies, which seems highly promising even though it has not yet been tested in practice. In this strategy, the antigen should be designed as a synthetic peptide with side chain Arg addition to the internal Asp/Glu. The counterscreening and immunodepletion in this case should be performed with the use of the same peptide without the side chain Arg group. Overall, this strategy would resemble the approach widely used for development of phosphor-specific antibodies.

While the peptide synthesis during antigen preparation for such branched peptides is much more difficult than with N-terminally arginylated peptides, the resulting arginylated peptides should in principle be much more immunogenic, since the branched structure involving bulky positively charged group of the Arg side chain differs significantly from the regular protein structure in this position. Discovery of key side-chain-arginylated proteins should lead to the emergence of this approach in future studies of the functional role of protein arginylation.

4 Other Approaches to Arginylation Detection

While the tools described above and elsewhere in this book are often very powerful and have driven over 50 years of arginylation studies, their limitations and difficulty of use remain a significant barrier to entry for researchers interested in arginylation. Major breakthroughs in this area of study require the development of accessible tools for the entire scientific community, to enable easy arginylation detection and possibilities of its functional studies in vivo.

One of the prospective approaches involves the use of biotinylated Arg in arginylation reaction, which should enable easy pull-downs and labeling of arginylated proteins. This seems possible, since biotinylated Arg is used for in vitro translation and thus is known to be compatible with Arg-tRNA charging by Arg-tRNA-

synthetase. However, it is still unknown if ATE1 can utilize such biotinylated moiety for Arg transfer. It also does not solve the issue of specificity if the ribosomes are present in the system. Thus, the main use of such a tool would be to replace the radioactive label with the biotin tag, mostly for in vitro ribosome-free studies.

Another promising approach involves developing chemical analogs of tRNA, which cannot be utilized by the ribosome but can potentially be recognized by ATE1. Such an approach has been successfully employed with L/F transferase, the bacterial enzyme analogous to ATE1 that transfers Leu and Phe onto the N-terminal Arg [4]. This enzyme can utilize aminoacyl adenosine in place of tRNA as the carrier of donor amino acids (see [5, 6] and Chap. 21 in this book), providing a highly specific way of separating the modifications by L/F transferase from regular translation. In principle, if a similar chemical tRNA mimic could be found that is compatible with arginyl transfer reaction, it could become a powerful tool in arginylation studies. Excitingly, a new study recently showed that Arg-conjugated tRNA fragments (tRFs) can serve as Arg donors as efficiently as full length tRNA [7].

While pilot studies suggest that ATE1 is not able to utilize similar compounds for Arg transfer, it seems possible, and highly promising, to attempt developing small tRNA mimics that could mediate arginylation but not protein translation on the ribosome. Such mimics could become the ultimate tool for arginylation detection in vivo, enabling its real-time tracking from the addition of the Arg group to the functioning and metabolic targeting of the protein after arginylation. With the recent breakthrough of solving ATE1 structure [8, 9], these studies are entering a new era of discovery.

Protein arginylation stimulated imagination of generations of researchers and led to many breakthrough findings over the years. Now, more than 60 years after its discovery, we are witnessing a major expansion of this exciting and novel field.

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