Published in final edited form as:

Anat Rec (Hoboken). 2014 September ; 297(9): 1630–1636. doi:10.1002/ar.22969.

Protein arginylation, a global biological regulator that targets actin cytoskeleton and the muscle

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Abstract

Posttranslational addition of Arg to proteins, mediated by arginyltransferase ATE1 has been first observed in 1963 and remained poorly understood for decades since its original discovery. Recent work demonstrated the global nature of arginylation and its essential role in multiple physiological pathways during embryogenesis and adulthood and identified over a hundred of proteins arginylated in vivo. Among these proteins, the prominent role belongs to the actin cytoskeleton and the muscle, and follow up studies strongly suggests that arginylation constitutes a novel biological regulator of contractility. This review presents an overview of the studies of protein arginylation that led to the discovery of its major role in the muscle.

Discovery of protein arginylation and arginyl transfer enzyme (ATE1)

Protein arginylation was originally discovered in 1963, when it was found that ribosome-free extracts from cells and tissues exhibit prominent incorporation of specific radioactive amino acids. This phenomenon proved to be dependent on tRNA, but did not require any other components of the translation machinery. The amino acids were found to be transferred from aminoacyl tRNAs onto existing proteins, thus causing a posttranslational modification. Such ribosome-independent amino acid incorporation was first observed in prokaryotes using Leu and Phe [1–4], and subsequently was discovered in liver extracts using Arg [5, 6]. Attempts to observe incorporation of other amino acids in a similar way had failed, showing that this new mechanism is indeed specific to Leu/Phe in bacteria and Arg in mammals. All the accumulating evidences then indicated that this discovery represents a true posttranslational modification [6–8].

In summary, at the time of the discovery it was found that (i) this system is independent of regular conventional protein synthesis, and (ii) this system depends on a new enzyme that modifies the existing proteins by addition of Arg [1–3, 6, 9]. Thus, this system was originally named “soluble amino acid incorporation system”. However, International Enzyme Nomenclature Commission headed by late Dr. Waldo Cohn during the late 1970s decided that the official name for this protein should be Arginyl-tRNA Protein Transferase.

It should be noted that amino acid addition to proteins is not a unique phenomenon in eukaryotic systems, which also contain enzymes mediating posttranslational glycylation (addition of Gly [10]), glutamylation (addition of Glu [11]), and tyrosination (addition of

Tyr [12, 13]) (see also [14, 15] for recent reviews). But these other modifications, described primarily for tubulin, do not utilize aminoacyl-tRNA.

Studies in different systems resulted in biochemical identification of this enzyme in plants [16] and in guinea-pig hair follicles [17]. In 1990, this enzyme was cloned and characterized in yeast, receiving its current name ATE1 (for Arginyl Transfer Enzyme [18]). Since ATE1 transfers Arg from arginyl tRNA, the arginylation reaction depends on Arg-tRNA synthetase activity in cells [19]. Later studies identified Ate1 genes in multiple species, and it is now known that ATE1 is present in all eukaryotes [16, 18, 20, 21]. In mice and human (and possibly some of the lower species), Ate1 gene encodes at least four isoforms generated by alternative splicing [20–22]. While evidence indicates that these isoforms have different activity, tissue expression, and substrate specificity, the functional distinction between these isoforms in vivo remains unclear.

Biological pathways regulated by arginylation

Ate1 knockout in yeast does not affect their viability [18], a finding that triggered the long-term label for arginylation as a “non-essential” process. However, later studies in other biological systems demonstrated that this was, in fact, far from true. A series of later studies implicated arginylation in regulation of embryogenesis [23] aging [24, 25], stress and heat shock, [26–28], regenerative processes [29–32], and protein degradation in the skeletal muscle [33]. In mice, Ate1 knockout results in embryonic lethality, abnormal cardiac morphogenesis, and impaired angiogenesis. Detailed analysis of tissue-specific and complete Ate1 knockout mouse models reveals defects in neural crest-dependent craniofacial morphogenesis [34], the formation and contractility of the cardiac muscle [35], and abnormalities in gametogenesis [36]. Postnatal deletion of Ate1 affects multiple physiological systems, leading to weight loss, mental retardation, and infertility [37]. Ate1 deletion in *Arabidopsis thaliana* leads to delayed leaf senescence [38, 39], defective shoot and leaf development [40] and abnormal seed germination [41]. Genomic screens show that while Ate1 does not affect viability of *C. elegans* it leads to embryonic lethality in *Drosophila*, suggesting that the key physiological systems that depend on arginylation for survival have evolved somewhere between these species [42]. Thus, arginylation is essential in many eukaryotic systems and is implicated in a number of physiological pathways (Fig. 1).

Identification of arginylated proteins in vivo

Ever since the original discovery of arginylation in 1963, the biggest question in the field revolved around the identity of proteins arginylated in vivo, which would likely point to its biological role as well as to its specific intracellular functions. However, identification of arginylated proteins remained an elusive task, largely due to the fact that Arg added to proteins by the action of ATE1 would be too similar to that incorporated by conventional translation, and thus any distinction of posttranslationally arginylated proteins from the bulk of proteins produced via translation is extremely difficult.

Early studies attempted to tackle arginylated protein identification by using various protein and peptide substrates in an in vitro reaction which utilized enriched ribosome-free fractions

from cell extracts as the source of ATE1 activity. Other strategies involved developing methods for observing translation-independent Arg incorporation by inhibiting protein synthesis and detecting protein bands on the gel that contain radioactively labeled added Arg. Using these methods, it was found that Arg can be incorporated into membranes and chromatin [6, 9, 43], as well as dozens of gel bands in preparations from different tissues and/or different species [44], [24, 45–47], [48–50]. In vitro and in vivo ATE1 targets identified in earlier studies include ornithine decarboxylase [51–53], regulators of G-protein signaling (RGS) [54, 55], BSA, alpha-lactalbumin, and thyroglobulin, (arginylated on the N-terminus after the removal of signal peptides [19, 56]). Another class of ATE1 targets includes regulatory peptides and hormones, including neurotensin (identified in vivo, [57], as well as in-vitro-tested beta-melanocyte stimulating hormone [58], insulin [59], and angiotensin II [58]. A pattern that emerged in the course of these studies suggested only one commonality: ATE1 appeared to exhibit strong preference to proteins and peptides containing N-terminally exposed acidic residues, Asp or Glu.

A new era in identification of arginylated proteins opened after the development of high precision mass spectrometry methods, which enabled higher throughput screens that could reveal in vivo proteins and peptides containing posttranslationally added Arg [60, 61]. This analysis has enabled identification of multiple additional arginylated proteins in complex samples from different tissues and cell types, expanding the list of known arginylated proteins to hundreds and suggesting that this posttranslational modification can potentially play a truly global role. In addition, this analysis suggested that N-terminal arginylation in vivo may not be restricted to Asp and Glu. Multiple peptides containing posttranslationally added Arg on the N-terminus of virtually every other exposed amino acid residue were also discovered. To date, it is not clear whether this new type of arginylation is also mediated by ATE1 or if other arginyltransferases with different substrate specificity also exist in vivo. It is clear, however, that the studies of protein arginylation and its specificity are still in the beginning stages.

Functional consequences of arginylation on protein targets

Identification of ATE1 as the enzyme mediating arginylation (Balzi 1990) came in the wake of the discovery of the N-end rule pathway of protein degradation, which relates the identity of the N-terminal residue to the protein's half-life. According to these studies, test substrates engineered to include N-terminally exposed Arg are metabolically unstable in yeast [62, 63]. Furthermore, engineered proteins which contained N-terminal Asp and Glu undergo N-terminal arginylation in yeast, rendering them metabolically unstable [63–65].

Independently it was also found that N-terminal Arg attracts Ub conjugation machinery, which can lead to protein ubiquitination and degradation [66]. All this evidence converged into a prevailing view that arginylation in vivo constitutes a mechanism for degradation of pre-processed proteins or proteolytic fragments that bear Asp and Glu on their N-termini.

Recent functional studies expanded this view, suggesting that protein degradation may not be the only, or even the prevalent function of arginylation. First, these screens themselves, performed on protein fractions isolated from wild type (i.e., arginylation-positive) cell and tissue extracts, relied on abundance of arginylated proteins, and thus were naturally biased

against those proteins which are metabolically unstable after arginylation. Functional studies on some of these proteins further confirmed that arginylation may have nothing to do with regulation of their metabolic stability. Arginylation of calreticulin during the ER stress facilitates its role in stress granules rather than cause its removal from the cells [67–70]. Arginylation of beta amyloid protein was suggested to facilitate its alpha helical conformation, preventing its pathological misfolding and accumulation [71]. Arginylation of a proteolytic fragment of the cell adhesion protein talin mediates its novel role in cell-cell adhesion [72]. Arginylation of myosin in platelets affects its regulation by phosphorylation and its normal contractility [73]. N-terminal arginylation of beta actin facilitates cell migration [74] and actin polymerization in vivo [75], independently of proteasome-mediated degradation.

In the case of beta actin, an unexpected link to arginylation-dependent degradation suggests that such degradation is likely responsible for removal of the closely homologous gamma actin if it becomes incorrectly arginylated in cells. Such selection occurs through an unconventional mechanism that relates mRNA coding sequence to protein stability and translation rates [76]. Thus, in the case of actins (and possibly other closely homologous but selectively arginylated protein isoforms), ubiquitin-dependent degradation appears to be a mechanism to ensure the selectivity of arginylation toward specific protein targets.

However, other lines of studies continue to argue toward the “conventional” degradation-dependent function of ATE1. Arginylation renders RGS4 and RGS5 metabolically unstable [54, 55]. Since these proteins negatively regulate cardiac morphogenesis, this mechanism may possibly be responsible for cardiac abnormalities seen in Ate1 knockout mouse embryos [23], which can potentially be explained by accumulation of RGS in the absence of arginylation. Arginylation has also been implicated in targeting oxidized proteins [59] and proposed to regulate oxygen sensing [54, 77]. It is likely that both degradation-dependent and degradation-independent effects play a role in protein regulation by arginylation in vivo.

Mid-Chain Arginylation of Intact Proteins

A recent discovery of a new type of arginylation, on midchain sites of intact proteins, has significantly expanded the potential biological scope of arginylation.

All the past screens and identification strategies for proteins arginylated in vivo relied on the postulate that proteins can only be arginylated at the N-terminus. This postulate originated from the assumption that a reaction of amino acid addition to a protein that utilizes charged tRNA must resemble the peptide elongation step on the ribosome (and thus must target the amino group of the N-terminal residue). Later on, the discovery and characterization of the N-end rule pathway reinforced the impression that arginylation, as a branch of this pathway, concerns exclusively the protein’s N-terminus. However, since arginylation by ATE1 was clearly prevalent toward Asp and Glu, this assumption meant that intact proteins can never be arginylated (since they all start with the N-terminal Met), and that only the action of specific aminopeptidases and proteases can expose the target site for arginylation. This, in turn, limited the potential pool of arginylation substrates to a subset of proteins that must be pre-processed prior to arginylation.

An apparent contradiction to this postulate emerged with the discovery that the biological peptide neurotensin can be isolated from an *in vivo* neuropeptide fraction with Arg added to a midchain Glu [57]. While there was no indication in this study whether this midchain reaction could be performed by ATE1 and whether it occurred on other peptides or proteins, it opened a possibility that arginylation *in vivo* may happen by different mechanisms that enable modification of intact proteins, in addition or instead of the conventionally postulated mechanism of Arg addition to the N-terminus.

A recent study performed a mass spectrometry screen without imposing the N-terminus restriction and assuming that Arg can be added to any Asp and Glu residue regardless of its position within the peptide (Wang et al, *in press*). Remarkably, screening under these conditions revealed a large number of proteins modified on midchain Asp and Glu in otherwise intact sequence. Follow-up studies showed that this reaction could be directly mediated by ATE1 in addition to the N-terminal reaction. Moreover, evidence suggested that midchain addition of Arg may be prevalent in the case of protein rather than peptide substrates. This discovery greatly expands the potential biological scope of arginylation, suggesting that in addition to protein fragments and pre-processed proteins it may also regulate intact proteins similarly to other global posttranslational modifications.

Regulation of arginylation

One of the big questions that remains relatively unexplored in protein arginylation field is the question of how this posttranslational modification is regulated *in vivo*. ATE1 expression exhibits a prominent peak at mid-development [20] and decreases during aging [24]. ATE1 levels can also vary significantly in different tissues [21]. Various proteins, drugs, and physiological compounds can significantly affect posttranslational Arg incorporation *in vivo*, however most of them are likely acting via non-specific mechanisms (see [42] for a recent review). It is feasible to imagine that proteins or non-protein inhibitors may exist *in vivo* that could potentially regulate the ATE1 enzyme itself or its molecular complex *in vivo*.

Another well characterized regulator of arginylation involves ubiquitin-dependent protein degradation. For those proteins which are rendered metabolically unstable after arginylation, ubiquitin can facilitate their rapid removal. As demonstrated in the case of beta and gamma actin [72], this mechanism can efficiently facilitate arginylation specificity and ensure the removal of closely homologous proteins that were not meant to be arginylated *in vivo*.

Finally, an attractive, but so far hypothetical activity that could regulate arginylation *in vivo* involves de-arginylation enzyme(s), which may be capable of removal of posttranslationally added Arg from protein substrates. The action of these enzymes, if they exist, is expected to resemble the action of previously characterized Aminopeptidase B (which can remove Arg from the protein and peptide N-terminus) and Carboxypeptidase B (which can in principle remove Arg from the side chain carboxyl groups). Finding whether these or other similar enzymes may be involved in regulation of arginylation *in vivo* constitutes an exciting direction of further studies.

Arginylation as a regulator of cardiac contractility and the heart muscle

One of the main originally described phenotypes in arginylation-deficient ATE1 knockout mouse was a defect of cardiac morphogenesis, resulting in severe malformations and hypoplasia of the heart [23]. Follow-up studies showed that in addition to these gross morphogenic defects the cardiac muscle was also severely malformed, with multiple abnormalities in the myofibril structure leading to defects in cardiac contractility and likely contributing to embryonic lethality in these mice. These results suggested that the heart in general and cardiac myofibrils in particular constitute a major physiological system regulated by arginylation.

To dissect the specific role of ATE1 in the heart, a conditional knockout mouse line was constructed, in which targeted deletion of ATE1 was confined to the heart muscle (driven by alpha myosin heavy chain promoter that activates in differentiated cardiomyocytes)[78]. In these mice, cardiac morphogenesis occurred normally, suggesting that these early morphogenic defects are not specific to cardiomyocytes and/or originate at stages preceding heart formation in the embryo. However, the myofibrils from ATE1-deficient hearts showed defects largely similar to those observed in the embryos of complete ATE1 knockout (Fig. 2), suggesting that the formation and contractility of myofibrils within the cardiomyocytes depends on arginylation in these cells. With age, these heart-specific ATE1 knockout mice developed severe dilated cardiomyopathy and thrombosis, leading to high rates of lethality due to heart failure after 6 months of age.

Mass spectrometry analysis of the isolated myofibrils identified a limited subset of proteins that are arginylated, most of them implicated directly in contractility and/or in the maintenance of the structural integrity of the myofibrils (Table 1). Their identity also raised a possibility that regulation of the muscle structure and contractility by arginylation may constitute a general mechanism that also affects other types of muscle and regulates their properties, interactions, and possibly metabolic stability.

Uncovering the exact role of arginylation on each of these sites constitutes an exciting direction of further studies.

Acknowledgments

This work was supported by NIH grant R01GM104003 to A.K.

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Figure 1.
Major biological pathways regulated by arginylation.

Figure 2. Knockout of arginyltransferase ATE1 leads to major defects in the heart muscle. Electron micrographs of wild type (WT) and ATE1 knockout (KO) muscle show diffuse Z-bands, irregular sarcomere lengths, and overall disintegration of the cardiac myofibrils, leading to dilated cardiomyopathy and heart failure. See [78] for further details.

Table 1

Arginylated proteins in cardiac myofibrils.

Name	Accession	Arginylated Residue
alpha actinin 2	NP_150371.4	L510
fibrillin 1	NP_032019.2	E640 ^{**} , Q671, L1266, E1794 [*]
laminin gamma 1	NP_034813.2	E803 [*]
myosin heavy polypeptide 6	NP_034986.1	L747 [^] , K999 [^] , L1001 [^] , ^{**} , V1027 [^] , L1486 [^] , Q1534, L1578 [*] , N1647
myosin light polypeptide 3	NP_034989.1	A20, T81 ^{**} , M117
titin N2B	NP_082280.2	L7960, V15013, C24818 [*]
tropomyosin alpha-1	NP_077745.2	L50, K77
cardiac troponin T2	NP_001123650.1	A184

[^] also found arginylated in myosin heavy polypeptide 7 (Myh7; NP_542766.1; L745, K997, L999, V1025, L1484, and L1576)

^{*} found as monomethylated Arg as described in [79]

^{**} found as dimethylated Arg as described in [79]