Mechanism of active substrate delivery by the AAA+ protease adaptor ClpS

by

Izarys Rivera-Rivera

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Signature of Author..... Signature redacted
August 28, 2015
Department of Biology

Certified by..... Signature redacted
Tania A. Baker
E.C. Whitehead Professor of Biology
Thesis Supervisor

Certified by..... Signature redacted
Robert T. Sauer
Salvador E. Luria Professor of Biology
Thesis Co-supervisor

Accepted by..... Signature redacted
Amy E. Keating
Professor of Biology
Co-chair, Biology Graduate Committee

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Abstract

AAA+ molecular machines power myriad cellular processes including protein degradation, microtubule severing, membrane fusion, and initiation of DNA replication. Protein quality control in all organisms involves deployment of ATP-dependent proteases, consisting of hexameric AAA+ rings that unfold and translocate specific substrates into an associated peptidase barrel. Adaptor proteins assist in recognition and degradation of certain substrates, but how enzyme-adaptor pairs ensure proper substrate selection is incompletely understood.

In this thesis I focus on the delivery mechanism employed by the bacterial adaptor protein ClpS. The ClpS adaptor collaborates with the AAA+ ClpAP protease to recognize and degrade N-end rule substrates. ClpS binds the substrate's N-degron and assembles into a high-affinity ClpS-substrate-ClpA complex, but how the N-degron is transferred from ClpS to the axial pore of the AAA+ ClpA unfoldase to initiate degradation is not known. Here, we demonstrate that the unstructured N-terminal extension (NTE) of ClpS enters the ClpA processing pore in the active ternary complex and that ClpA engagement of the ClpS NTE is crucial for ClpS-mediated substrate delivery.

In addition, I report evidence that ClpA engagement of the ClpS NTE drives structural rearrangements in ClpS important for N-end rule substrate delivery. Furthermore, our preliminary experiments suggest that ClpS is able to resist degradation by ClpAP due to a combination of a high local stability and a challenging translocation sequence at the junction of the NTE and folded core domain. I propose a model in which ClpA remodels ClpS by translocating the NTE, triggering delivery of the N-end rule substrate. Similar mechanisms may be employed by other AAA+ enzymes that collaborate with adaptor proteins to remodel/disassemble substrates without destroying them by degradation.

Thesis Supervisor: Tania A. Baker
Title: E.C. Whitehead Professor of Biology

Thesis Co-Supervisor: Robert T. Sauer
Title: Salvador E. Luria Professor of Biology

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CHAPTER ONE

Introduction

Importance of protein degradation

Intracellular protein degradation is fundamental for cellular survival. It allows cells to respond to environmental stresses as well as developmental cues (1-3). Misfolded or damaged proteins must be degraded in order to prevent toxic aggregation, and functional proteins are degraded for regulatory purposes. In both cases, degradation recycles amino acids. An important example of degradation as a regulatory tool in eukaryotes is the control of cell cycle progression by various cyclin-dependendent kinases (CDKs), which are activated upon binding to their partner cyclins. Cyclins undergo a continuous cycle of synthesis and degradation during cell division. CDKs are inactivatated by proteolysis of their partner cyclins, leading to transitions between stages of the cell cycle. Different cyclins are degraded at different stages to allow for cell cycle progression (4, 5). Failure to degrade cyclins at a specific time leads to the arrest of dividing cells, highlighting the importance of these precise proteolytic events.

Intracellular proteolysis occurs through various pathways. In the lysosomes of eukaryotic cells, for example, protein degradation is catalyzed by relatively non-specific ATP-independent proteases (6). In bacteria, archaea, and the cytoplasm and nucleus of eukaryotic cells, proteolysis of specific target proteins is carried out by peptidases that recognize only a limited number of peptide sequences or by ATP dependent proteases that are members of the AAA+ superfamily (3, 7, 8).

The AAA+ Superfamily

The AAA+ (ATPases associated with various cellular activities) superfamily is a ubiquitous family of proteins that utilize the energy of ATP binding and hydrolysis to

power mechanical work (1, 9-11). They work in a wide range of cellular events that ensure the healthy growth and maintenance of the cell under normal growth as well as under stress conditions. Examples of AAA+ enzymes include DNA polymerase clamp loaders, DNA helicases, the molecular motor dynein, and protein unfoldases. A defining feature of AAA+ family members is the presence of a structurally conserved AAA+ module (~250 residues), which consists of a large and small domain. This fold has multiple motifs that define the superfamily and are required for ATP binding and hydrolysis (*e.g.* Walker A motif and Walker B motif, sensor 1, sensor 2, and arginine fingers). The Walker A motif plays an important role in nucleotide binding and metal-ion coordination, which is necessary for ATP hydrolysis. In contrast, the Walker B motif contains residues that are more directly involved in ATP hydrolysis and that participate in metal ion coordination (1, 3). Unfoldases comprise a major class of AAA+ enzymes that assemble into hexameric rings and perform ATP-dependent remodeling on macromolecular substrates (12).

Protein unfoldases play critical roles in protein metabolism. Members of this subfamily use cycles of ATP binding and hydrolysis to drive conformational changes in the ring that partially or completely unfold protein substrates (13). One member of this family is the bacterial unfolding chaperone ClpB. ClpB extracts unfolded polypeptides from aggregates via substrate threading through its central channel. By using mixed aggregates consisiting of protein fusions of misfolded and native domains, Haslberger *et al.* proposed that even partial unfolding of a misfolded moiety by ClpB can be sufficient to solubilize aggregates (14).

In some cases, protein unfoldases translocate unfolded polypeptides into a partner protease for degradation (15). For instance, a major class of AAA+ protease substrates arises from the bacterial tmRNA system, which adds a degradation tag (degron) to the products of stalled ribosomal translation (16-18). These tagged proteins are recognized by the unfoldase ClpX, which translocates the polypeptide to the ClpP peptidase for degradation (19, 20).

AAA+ proteases

In all kingdoms of life, large barrel-shaped assemblies named AAA+ proteases carry out regulated ATP-dependent proteolysis. Although their complexity varies among different kindgdoms, they all share a common architecture that consits of a hexameric AAA+ unfoldase ring and a multi-subunit compartmental peptidase that contains the active sites for degradation (Figure 1.1) (1, 15, 21, 22). Active sites for compartmentalized proteases vary among enzymes. For example the ClpP peptidase of the ClpAP and ClpXP proteases contains a His-Asp-Ser catalytic triad in its active site, whereas the proteolytic active site for the Lon protease is composed of a Lys-Ser dyad (23, 24). Several structural studies show that the AAA+ unfoldase ring and the peptidase bind such that their central pores are aligned (25-28). The unfoldase AAA+ ring recognizes the substrate through an intrinsically disordered degradation tag, or degron, in an otherwise folded protein that is unable to pass through the narrow central pore (Figure 1.2) (15). Degrons vary significantly among organisms and among AAA+ proteases. Examples of degrons utilized by different organims as well as by different AAA+ proteases will be described in the substrate recognition section. Translocation of the degron attached to the folded protein through the central AAA+ pore generates an

unfolding force as the enzyme pulls a large structure through a narrow channel (15). The translocation power stroke can be driven by ATP hydrolysis in a single subunit of the ring. The nucleotide-binding site is located between the large and small AAA+ domains, and ATP binding and hydrolysis can alter the orientation of these domains, causing rigid-body motions that propagate around the ring. These movements are transmitted to the substrate in part by conserved pore loops that protude into the central pore (29, 30). Finally, translocation of the denatured substrate into the degradation chamber results in its degradation (Figure 1.2). Additionally, pore loops also play important roles in substrate recognition as mutation of pore loop residues affects the recognition and processing of substrate proteins (31-34).

AAA+ degradation machines guarantee that truncated, damaged and unwanted proteins are eliminated from the cell, thereby ensuring homeostasis of the proteome. The next two sections will focus on the main AAA+ proteolytic machines in both eukaryotic and bacterial cells.

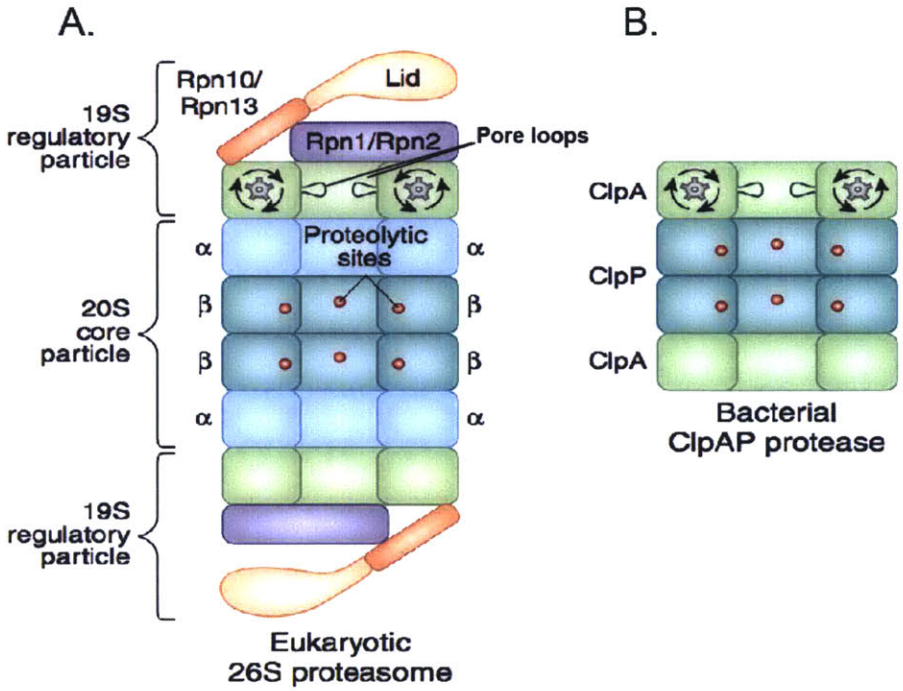

Figure 1.1. AAA+ proteases, varied complexity, common architecture. Cartoon of the eukaryotic 26S proteasome and the bacterial ClpAP protease. Both proteases share a common architecture in which the protease sites are buried in an internal chamber of the compartmentalized peptidase and AAA+ hexameric rings flank the peptidase (35, 36). (*A*) Cross section of the eukaryotic 26S proteasome. The 20S core particle is flanked by 19S regulatory particles. The proteolytic sites in the 20S core particle are located in the β-rings of the peptidase. The scaffold proteins Rpn1 and Rpn2, the ubiquitin receptors Rpn10 and Rpn13 and the loops lining the ATPase ring are shown. Only one set of loops lining the ATPase ring is shown. (*B*) Cross section of the bacterial ClpAP protease. The ClpP peptidase is flanked on both sides by ClpA ATPase rings. Only one set of loops lining the ATPase ring is shown. Figure taken from Schrader et al. 2009 (37).

The proteasome

The proteasome is the main proteolytic machine in archea and in the cytosol and nucleus of eukaryotic cells (38). It is responsible for degrading hundreds of regulatory proteins and for destroying damaged proteins (38). In eukaryotes, this multi-component protease is called the 26S proteasome and is composed of two subcomplexes, the 20S core particle and the 19S regulatory particle (Figure 1.1A) (39).

The 20S core particle is composed of four seven-membered rings of α and β subunits.

The inner two rings each consist of seven related β-subunits that are arranged to form an internal cavity (35). Three of the subunits in each ring contain a proteolytic site that faces the cavity. A ring of seven related α-subunits on each side flanks the β-rings, and substrates enter the proteolytic cavity formed by the β-rings through a pore at the top of the α-ring (Figure 1.1 A) (35). By itself, the pore of the 20S core particle is too narrow to allow folded proteins to enter through diffusion, and even unfolded peptides are inefficiently degraded (40).

The 19S regulatory particle recognizes, unfolds and translocates substrates into the core particle for degradation. It contains ~18 proteins and two major subassemblies, the lid and the base. The base is a hetero-hexamer of AAA+ ATPases (Rpt1-Rpt6) that form a ring and peform the mechanical unfolding of substrates. The base also contains the Rpn1 and Rpn2 subunits, which bind to the ATPase ring and mediate binding of various ubiquitin receptors (Figure 1.1A) (41-43). The Rpt1-Rpt6 ring contains a long channel at the center, and the C-terminal ends of their AAA+ domains dock into the 20S core particle and trigger pore opening within the peptidase (35, 40). This pore opening facilitates the entrance of denatured proteins into the proteolytic chamber (40). Subunits of the lid include ubiquitin receptors (Rpn10 and Rpn13) and a de-ubiquitinase (Rpn11), among others (Figure 1.1A) (35, 43).

Proteins are targeted to the proteasome by a two-part degron consisting of a disordered region within the substrate and a reversibly-attached polyubiquitin tag (Ub_n) (43, 44). Ubiquitin is a small (76 residue) globular protein that performs myriad functions in eukaryotic cells (45). Covalent conjugation to other proteins, a process named

ubiquitylation or ubiquitination, is important for the degradation of numerous proteins by the 26S proteasome (38). A polyubiquitin tag is attached to substrates by ubiquitinases and this process can be reversed by de-ubiquitinases (46). The ubiquitination process will be described in detail in the substrate recognition section. The proteasome recognizes substrates at the ubiquitin tag via ubiquitin receptor subunits and initiates degradation at the disordered region (44, 47). Once the proteasome has engaged a substrate, it unravels the protein through the central cavity of the regulatory particle into the core particle (45). The intrinsic de-ubiquitinase Rpn11 removes and recycles the polyubiquitin tag as unfolding and degradation begins (48).

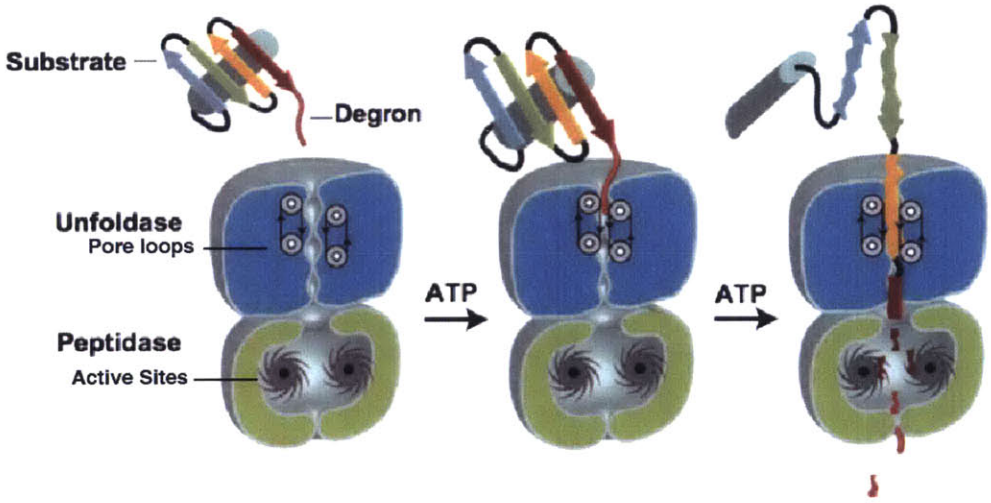

Figure 1.2. Cartoon of the general mechanim utilized by AAA+ proteases. A degron on a protein substrate is recognized by the AAA+ unfoldase. Afterwards, through cycles of ATP binding and hydrolysis, the substrate is unfolded and subsequently translocated into the peptidase for degradation. Figure from Sauer and Baker, 2011 (15).

Bacterial proteases

The study of bacterial proteases has provided insights into the general mechanisms

employed by AAA+ enzymes. Whereas the cytosol and nucleus of eukaryotic cells contain a single ATP-dependent protease, bacteria utilize several different proteases (49). *E. coli* has five distinct proteases with partially overlapping specificities: cytosolic ClpAP, ClpXP, HslUV, and Lon, and the membrane-anchored FtsH. Some of these enzymes contain both the AAA+ domain and the protease domain in a single polypeptide chain (*e.g.* Lon and FtsH), whereas others consist of separate AAA+ unfoldase and peptidase partners (*e.g.* ClpAP, ClpXP, and HslUV, where P and V are the peptidases). Each of these unfoldases contains at least one AAA+ module (1). ClpA is unique amongs this group of enzymes as it has two AAA+ modules in each of its 6 subunits (named D1 and D2) (50). Many bacterial species possess the ClpA-related ClpC unfoldase, which also has two AAA+ modules (51). Biochemical experiments suggest that the two ATPase rings of ClpA can fire and function independently as protein unfoldases (although the D2 ATPase ring is more important for unfolding of stable susbtrates) (50, 52).

All AAA+ proteases contain a family-specific accessory domain, usually located at the N-terminus of the AAA+ polypeptide (N-domain). HslU contains an intermediate domain (I domain) within the AAA module sequence (26). In contrast to the AAA+ domains, the N-domains of different families share no structural homology. These family-specific domains are typically not required for basic AAA+ protease function as variants of ClpA, ClpX and FtsH lacking these domains are still active in degradation of some substrates (53, 54). HslU is an exception, as deleting the I domain results in a ~50-fold reduction of its basal ATP hydrolysis rate (55). In most cases, N-domains play an important role in substrate recognition.

Because of the irreversible nature of protein degradation, it is critical that substrate recognition is closely regulated. In the next section, I will address some examples of the numerous strategies cells use to regulate substrate recognition.

Substrate Recognition

With millions of proteins crowding both prokaryotic and eukaryotic cells, it is important that substrate recognition by each protease is tightly controlled to prevent wasteful or toxic destruction. At the simplest level, proteolysis by AAA+ proteases is controlled via access to the active sites that are encapsulated inside the compartmentalized peptidase (15, 21, 22). For example, the ClpP peptidase is able to degrade small peptides in the absence of an AAA+ partner; however, an ATPase partner is needed to allow regulated degradation of protein substrates (56). The ATPase ring controls access to the active sites of the peptidase by directly binding to an exposed degron in the substrate to unfold and translocate the denatured polypeptide into the degradation chamber (Figure 1.2) (15).

Proteins slated for degradation must bear a specific signal that is distinct from stable proteins. Degrons vary greatly in complexity and structure. In bacteria, for example, proteins targeted for degradation are often recognized through short peptide sequences encoded in their primary structure. These peptide sequences are often located near or at the N or C terminus of substrates, presumably to make them more accessible for recognition by the AAA+ protease (Figure 1.2). For example, the ClpXP protease attempts to degrade any protein with an accessible Ala-Ala at the C terminus (15). Another strategy for proteolysis regulation is control of degron accessibility, whereby

exposure of degrons requires an additional step. Examples include unfolding of a substrate's secondary structure, a primary cleavage event by another peptidase, subunit dissociation, or a conformational change within the substrate (57, 58).

Another common mechanism used to regulate protein degradation is the covalent addition of polypeptide sequences that mark substrates for destruction. Addition of degradation markers can occur cotranslationally or posttranslationally. In the next sections examples of posttranslational and cotranslational degron attachment will be described.

Ubiquitination

In eukaryotes, the post-translational addition of the ubiquitin protein is the major method for targeting proteins to degradation (43). Ubiquitin is enzymatically cross-linked to substrate proteins for recognition and degradation by the 26S proteasome (46). At least four ubiquitin units must be attached to substrates for efficient proteasome recognition (47). Additionally, susbtrates must contain an unstructured region for initiation of unfolding and degradation (44). A series of three enzymatic activities are responsible for fusing the ubiquitin tag onto substrate proteins (Figure 1.3). First, a ubiquitin-activating enzyme (E1) forms a thiol-ester bond with the carboxy-terminal glycine of ubiquitin in an ATP-dependent process. E1 then transfers the ubiquitin to the active-site cysteine of E2, an ubiquitin-carrier enzyme, by a *trans*-thiolation reaction. Lastly, an E3 ubiquitin ligase recognizes a specific substrate protein and catalyzes the transfer of ubiquitin from E2 to the substrate by the formation of an isopeptide linkage between a substrate lysine and one ubiquitin, followed by attachment of additional ubiquitins to form a

polyubiquitin chain. There are two E3 families, the HECT familiy and the ring finger family. The ring finger family is believed to mediate the direct transfer of ubiquitin from the E2 to the substrate, whereas the HECT-family forms a thiol-ester intermidiate with ubiquitin as part of the process of substrate ubiquitination (Figure 1.3) (46). Regulation of substrate recognition by the appropriate E3 is the central control point in proteasomal degradation. For example, in humans there are over 600 ubiquitin ligases (46). Additional mechanisms of E3 regulation include inhibition by substrate mimics that lack a modifiable lysine. Finally, the intrinsic de-ubiquitinase of the proteasome, Rpn11, removes the bound ubiquitins by cleaving the isopeptide bond (46).

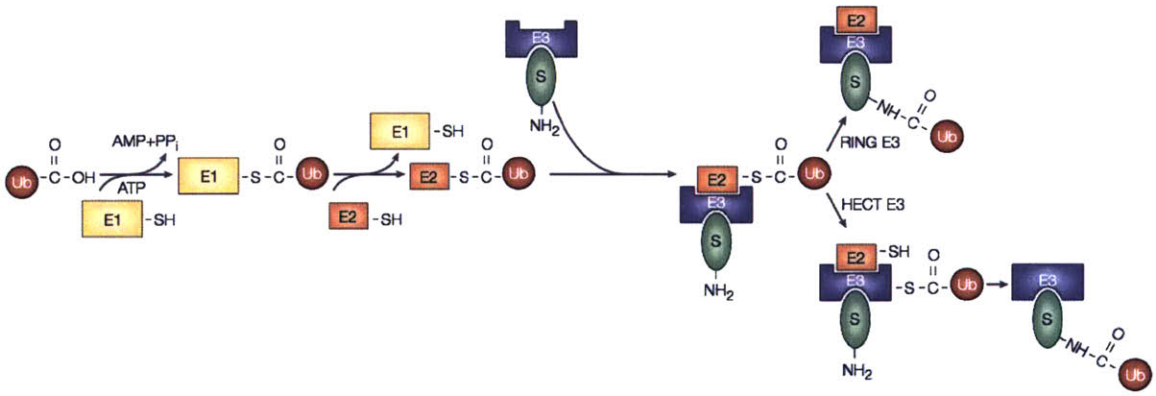

Figure 1.3. Reaction scheme for Ubiquitination. Free ubiquitin (Ub) is activated by the formation of a thiol-esther linkage between E1 and the C terminus of ubiquitin, in an ATP-dependent manner. Afterwards, ubiquitin is transferred to an ubiquitin carrier protein (E2). E2 then associates with one of the numerous E3s, which might or might not have a substrate already bound. For HECT domain E3s, ubiquitin is transferred to the active site cysteine of the HECT domain followed by transfer to the substrate (S). For RING E3s, the ubiquitin is transferred directly to the substrate. Figure from Weissman, 2001 (59).

Pupylation

For many years, posttranslational modifications that target proteins for degradation were considered an exclusive feature of eukaryotic cells. However, the discovery of

pupylation, modification of lysine residues with a prokaryotic ubiquitin-like protein (Pup), revealed that some bacteria use a tagging pathway conceptually similar to ubiquitination to target proteins for degradation. This pathway is essential for mycobacteria to survive nitrogen starvation (60). Despite both being recognition "flags" to target substrates to the proteasome, Pup and ubiquitin differ in sequence, structure, method of activation and conjugation, and mechanism for substrate delivery. For example, Pup is an instrinsically-disordered protein, whereas ubiquitin adopts a β-grasp fold (61, 62).

A Pup ligase, PafA, catalyzes isopeptide bond formation between Pup and substrates. Pupylation is counterbalanced by the depupylation enzyme Dop, which mediates cleavage of the isopeptide bond for the release of Pup from the substrate. After modification, binding of Pup to the N-terminal domain of the PAN AAA+ ring targets substrates to the proteasome. Subsequent engagement of Pup by the AAA+ pore initiates unfolding that leads to the degradation of substrate. Pup is degraded along with the substrate during *in vitro* degradation assays (61).

ssrA tags

A well-characterized example of cotranslational peptide addition in bacteria is the ssrA tagging system, involved in protein quality control in all eubacteria. SsrA tagging occurs when translation stalls. Ribosomal stalling (*e.g.* when an mRNA lacks a proper stop codon) triggers recruitment of the tmRNA tagging and ribosome rescue system. The tmRNA molecule encoded by the *SsrA* gene, which possesses both tRNA- and mRNA-like properties and is charged with an alanine, enters the A site of the ribosome, and alanine is appended to the nascent polypeptide. Afterwards the template switches from

the 3' end of the original mRNA template to the open reading frame of the tmRNA molecule which endcodes the ssrA degron. The sequence and length of the ssrA tag varies (9-36 residues) among baterial species. In *E.coli* the ssrA tag consists of the 11-residue AANDENYALAA-COO^- sequence (18, 63). SsrA-tagged substrates are principally degraded by the ClpXP protease, sometimes with the assistance of the SspB adaptor. Adaptor proteins and their mechanism will be described in the adaptor proteins section. In some bacteria with smaller genomes encoding only two proteases, the work of degrading ssrA-tagged substrates is taken up by the Lon protease (64). The ssrA tagging system assures that there is no build-up of aberrant proteins produced during failed translation and facilitates ribosome recycling.

N-degron Pathway

N-degrons are the smallest degradation tags known. This highly conserved degradation pathway relates the stability of a protein to the identity of its N-terminal amino acid (65, 66). Different organisms recognize different amino acids as either stabilizing or destabilizing. N-degron substrates are typically generated by endoproteolytic cleavage (67). For example, degradation of the protein cohesin, which holds together sister chromatids during DNA replication, occurs through the N-degron pathway. Before anaphase, the protease separase cleaves cohesin, revealing an N-degron on the C-terminal fragment. This fragment is then degraded through the ubiquitin-proteasome pathway (68).

A variety of amino acids are recognized through this pathway as either primary destabilizing amino acids, which are directly recognized, or as secondary or tertiary

destabilizing amino acids, which require further modifications to be recognized (Table 1.1). For example, in bacteria Trp, Tyr, Phe, and Leu are primary destabilizing amino acids, and Arg and Lys act as secondary destabilizing amino acids to which Leu or Phe is appended by specific amino-transferases (Table 1.1) (69).

In eukaryotes, the range of destabilizing amino acids is broader. In addition to the primary residues recognized in bacteria, Ile, His, and Lys also serve as primary destabilizing amino acids (66). Moreover, the eukaryotic N-degron pathway includes tertiary destabilizing amino acids that must be modified twice for recognition (Table 1.1). For example, in *S. cerevisiae* N-terminal Asn is converted to the secondary residue Asp by N-terminal amidohydrolase-catalyzed deamination and further modified by an argininyl transferase (ATE1) that attaches an N-terminal Arg, which is a primary destabilizing amino acid and can be directly recognized (70).

The mechanism of N-degron recognition differs from prokaryotes to eukaryotes. In *E. coli*, the degradation of substrates bearing N-degrons is carried out by the AAA+ protease ClpAP (71). Although ClpAP can recognize and degrade N-degron substrates when they are present at a high concentration, degradation is greatly enhanced by the ClpAP-specific adaptor ClpS (72, 73). In eukaryotes, by contrast, a family of E3 ubiquitin ligases recognizes and covalently modifies N-degron substrates by polyubiquitin addition. The E3 regions that recognize N-degrons are of two classes: type 1/UBR box and type 2/ClpS-like. The UBR box region is responsible for recognizing Lys, Arg, and His N-degrons, whereas the ClpS-like region recognizes hydrophobic side chains (Table 1.1) (74-76).

Eukaryotes			
Tertiary	**Secondary**	**Primary**	**Recognized by**
N[a] (modified by deamidation)	D (modified by Arg-ylation)	RD	Class I or UBR box
Q (modified by deamidation)	E (modified by Arg-ylation)	RE	
C (modified by oxidation)	C*[b] (modified by Arg-ylation)	RC*	
		R	
		K	
		H	
		L	Class II or ClpS like
		F	
		Y	
		W	
		I	
Bacteria			
Tertiary	**Secondary**	**Primary**	**Recognized by**
None	R (modified by Leu or Phe-ylation)	F/LR	ClpS (class II)
	K (modified by Leu or Phe-ylation)	F/LK	
	(M)[c] (modified by Leu-ylation)	FM	
		L	
		F	
		Y	
		W	

[a]Typical N-end-rule residues, divided in classes, represented in the one letter amino acid code.
[b]C* denotes oxidized cysteine.
[c]To date, only one substrate with this modification has been reported (74).

Table 1.1. N-degron classes in eukaryotes and bacteria. Table from Sauer and Baker, 2011 (15).

Adaptor proteins

An additional level of substrate specificity can be achieved by the use of small acessory proteins, termed adaptors. Adaptors are widely employed proteins that may regulate proteolysis by either preventing or facilitating the degradation of specific substrates (12, 15, 77). A common mechanism used by adaptors to deliver substrates is to bind to a region of the substrate while simultaneously binding to the N-domain of an AAA+ unfoldase and thereby enhance degradation of the substrate by tethering the degron to the AAA+ protease. This tethering mechanism increases the effective local

concentration of the substrate near the processing pore of the protease. Adaptor-mediated tethering facilitates efficient degradation at low concentrations of substrates, conditions in which substrates alone may not bind efficiently (12, 77). This thesis focuses on the novel active mechanism employed by the *E. coli* adaptor ClpS to deliver N-degron substrates to the ClpAP protease. In the next sections a brief description of various bacterial adaptor proteins is presented to emphasize their importance for cellular homeostasis and the mechanism they utilize for substrate recognition and delivery to their associated AAA+ proteases.

SspB

E. coli SspB is a well-characterized adaptor of the AAA+ ClpXP protease (78-81). SspB is a dimeric adaptor that aids in the degradation of ssrA-tagged substrates. Each monomer of the adaptor contains a substrate-binding groove and a short ClpX-binding (XB) motif. The substrate-binding groove binds the N-terminal portion of the 11-residue ssrA-tag while leaving the two C-terminal residues available for the protease to bind and engage the substrate, allowing the substrate to be bound simultaneously by SspB and the ClpX pore. The XB motif binds specifically to a site on the ClpX N-domain (Figure 1.4) (79-81). Thus, SspB "tethers" the substrate to the protease and increases the local concentration of the ssrA-tag near the ClpX pore.

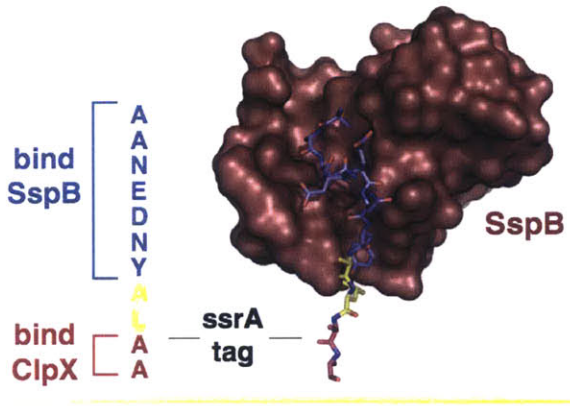

Figure 1.4. Recognition of the ssrA degron by SspB adaptor. The C-terminal alanines and α-carboxylate of the ssrA tag are recognized by the ClpX pore, wThehereas the N-terminal portion of the ssrA tag binds a grove in the body of the SspB adaptor, which has a tail that binds the N-terminal domain of ClpX. Figure from Sauer 2011 (15).

RssB

Bacteria have evolved a broad range of stress response mechanisms. For example, gram-negative bacteria respond to stress by the synthesis and/or activation of alternative RNA polymerase σ factors that direct transcription of regulons whose gene products counteract stress (82). *E. coli* cells enter stationary phase upon oxygen and nutrient limitation, heat stress, and osmotic stress, and the stress response is mediated by the master stress regulator, σ^S. In stationary phase, σ^S promotes the expression of ~100 stress response genes (82). Thus, σ^S is crucial for cellular homeostasis under stress conditions. Under favorable growth conditions, however, σ^S activity must be regulated for cells to resume exponential growth and to limit wasteful synthesis of unnecessary stress proteins. Regulation of σ^S activity occurs by tightly controlling its cellular concentration. σ^S is degraded by the ClpXP protease, and this degradation is enhanced by the adaptor RssB (82). RssB phosphorylation increases its affinity for σ^S and it delivers the substrate to ClpXP apparently by a tethering mechanism similar to that of SspB (83).

UmuD

Bacterial UmuD is a component of DNA polymerase V, an error-prone translesion polymerase. UmuD forms a heterodimer with UmuD', a truncated form of UmuD generated during DNA damage that lacks the first 24 N-terminal residues. Because of its low fidelity, the concentration of pol V must be tightly controlled and rise to a significant concentration only when DNA damage is severe. UmuD functions as a ClpXP adaptor to promote UmuD' degradation. UmuD contains a ClpX N-domain binding site not present in the truncated UmuD'. The UmuD-ClpX interaction positions UmuD' for degradation (84). This mechanism is another example of substrate tethering near the AAA+ processing pore.

MecA

MecA is a monomeric adaptor and obligatory activator of the ClpCP protease (85, 86). Unlike other AAA+ unfoldases, ClpC can only form functional hexamers in the presence of MecA. In *Bacillus subtilis*, the MecA-ClpCP complex is responsible for the degradation of the competence transcription factor, ComK. The adaptor's N-terminal domain recognizes ComK and its C-terminal domain interacts with the ClpC pore and acts as a degradation signal so that the adaptor is degraded with ComK (85, 86). Because MecA is degraded during substrate delivery, the mechanism is reminiscent of the Pupylation system in which Pup is directly recognized and degraded by the proteasome.

ClpS

The small *E. coli* ClpS protein is the only known adaptor of the ClpAP protease. The

ClpS gene was discovered as a short open reading frame upstream of the *ClpA* gene (87). Its structure consists of a core domain ($ClpS^{core}$), formed by three α-helices packed against three antiparallel β-strands. The $ClpS^{core}$ contains the substrate binding site as well as the ClpA binding site (73, 74, 88, 89). In addition to this tightly folded core, ClpS has a long and flexible N-terminal region (NTE) that lacks a stable secondary structure (Figure 1.5) (73). ClpS is involved in the N-degron pathway by directly binding to both the destabilizing N-terminal amino acid of a substrate and the ClpA N-domain to deliver the substrate to ClpAP for degradation (71-73, 90). ClpS enhances the rate of degradation of N-degron substrates and inhibits degradation of other substrates (71, 87, 91). For example, unlike ClpAP, ClpAPS cannot degrade ssrA-tagged substrates and does not display autodegradation of the ClpA unfoldase (91).

The mechanism of ClpS substrate delivery is more complex than simple tethering, as evidenced by mutations that preserve enzyme-ClpS-substrate complex formation but eliminate degradation (73, 91). An active hand-off mechanism that requires engagement of the ClpS NTE appears to be required to transfer the ClpS-bound N-degron substrate and allow engagement of the substrate N-terminal region by the ClpA pore (92). Evidence supporting the current view of the ClpS-ClpAP and N-degron early delivery steps are the subject of Chapter 2 and will be further discussed below.

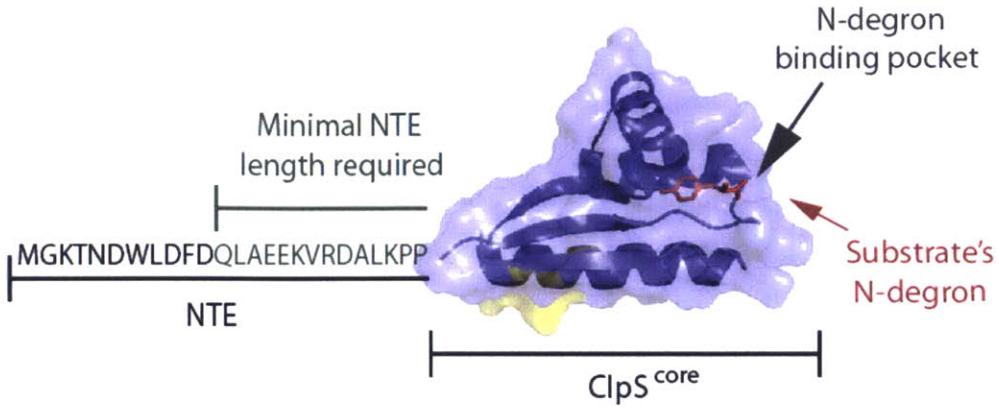

Figure 1.5. The adaptor protein ClpS. The ClpS adaptor has a long, flexible N-terminal extension (NTE; residues 1-25) and a folded core domain ($ClpS^{core}$; residues 26-106). The $ClpS^{core}$ binds N-degrons and the ClpA N-domain. A substrate's Tyr in the binding pocket is shown in red and ClpA N-domain interacting residues are shown in yellow (Protein Data ID code 3O1F). Succesful substrate delivery requires that the ClpS NTE be at least 14-aa long (shown in green).

ClpAPS and the N-degron pathway in *E.coli*

In *E. coli*, the residues Phe, Leu, Trp, or Tyr serve as primary N-end degrons (69). The ClpS adaptor binds these substrates via the N-degron residues and delivers them to the AAA+ ClpAP protease for degradation (71, 74, 89). As mentioned earlier, ClpAP, one of five degradation machines in *E. coli*, consists of the $ClpP_{14}$ serine protease and the $ClpA_6$ unfoldase. Each ClpA subunit contains a family specific N-domain and two AAA+ modules (D1 and D2) that form distinct rings in the hexamer (52). The D1 module is believed to play an important role in oligomerization of the unfoldase, whereas the D2 module is responsible for the majority of the ATP hydrolysis, as ClpAP can retain significant degradation activity when ATP hydrolysis mutations are present in the D1

ring (50). However, the activity of both ATPase domains may be especially important for unfolding substrates with high stability.

Degradation of substrates by ClpAP requires initial binding of the degron to the ClpA pore loops. Following engagement of the substrate, ClpA uses the energy of ATP binding and hydrolysis to drive conformational changes within the translocation channel that pull subtrates, this results in unfolding when a folded substrate is pulled against the narrow axial pore. Subsequently, substrates are translocated into the proteolytic chamber in ClpP (15). Although ClpAP can recognize and degrade N-degron substrates without adaptors, the K_M for degradation is dramatically lowered by the ClpAP-specific adaptor ClpS (from 29 μM without ClpS to 0.6 μM with ClpS for YLFVQ-titin) (72).

For efficient recognition by ClpS, N-degron substrates must have a free α-amino group, an unstructured region of at least four residues between the N-degron and the folded portion of the substrate, and preferentially neutral or positively charged residues adjacent to the N-degron (56, 74, 93, 94). Crystal structures of ClpS reveal that the substrate's destabilizing N-degron side chain is buried in a deep preexisting hydrophobic cleft on the surface of ClpS (73, 74, 95). Substrate binding is enhanced substantially (~ 100X) when ClpS binds $ClpA_6$. Reciprocally, N-degron substrates increase ClpS affinity for $ClpA_6$ (~10X) (73). Thus, the ClpAPS and N-degron substrate form a stable complex that must be broken for the reaction to continue and substrate to enter the ClpA unfolding pore. Importantly, substrate delivery cannot occur when the NTE of ClpS is deleted or lacks its first 12 amino acids; furthermore, numerous lines of evidence strongly indicate that the ClpA pore engages the NTE much like it engages

substrates, but that ClpS fails to be degraded by ClpAP (73, 91).

The ClpS adaptor: a model for substrate delivery

My thesis work centers on the molecular mechanism employed by the ClpS adaptor to deliver N-degron substrates to ClpAP. Chapter 2 highlights the role of the ClpS N-terminal extension (NTE) in the delivery mechanism and identifies features of the interactions between ClpA and ClpS that are critical for releasing the substrate from ClpAPS to enable the downstream steps of unfolding and degradation (92). Chapter 3 focuses on ClpA-dependent structural rearrengements that ClpS undergoes upon delivery of N-degron substrates as well as the molecular determinants of ClpS that render the adaptor resistant to degradation by ClpAP.

My results suggest that ClpS works together with ClpA in using an active substrate delivery mechanism rather than passive tethering. We propose a model in which, after formation of the high-affinity tertiary complex, ClpA-dependent translocation of the ClpS NTE begins to deform the $ClpS^{core}$ by pulling on the middle β-strand (β1-strand) of the three-stranded β-sheet. Partial or complete extraction of the β1-strand of ClpS facilitates substrate transfer by positioning the N-degron–binding pocket close to the ClpA pore and by weakening interactions between the substrate and the ClpS-binding pocket. Subsequently, ClpS resists further unfolding and is released from the ternary complex, allowing the adaptor to refold and translocation and degradation of the N-degron substrate to commence (Figure 1.6). Thus, we posit that the ClpS NTE acts as a degradation tag, and ClpS's stability promotes adaptor recycling. This thesis details a novel mechanism in which adaptors act as substrate mimics to collaborate with their

partner AAA+ protease.

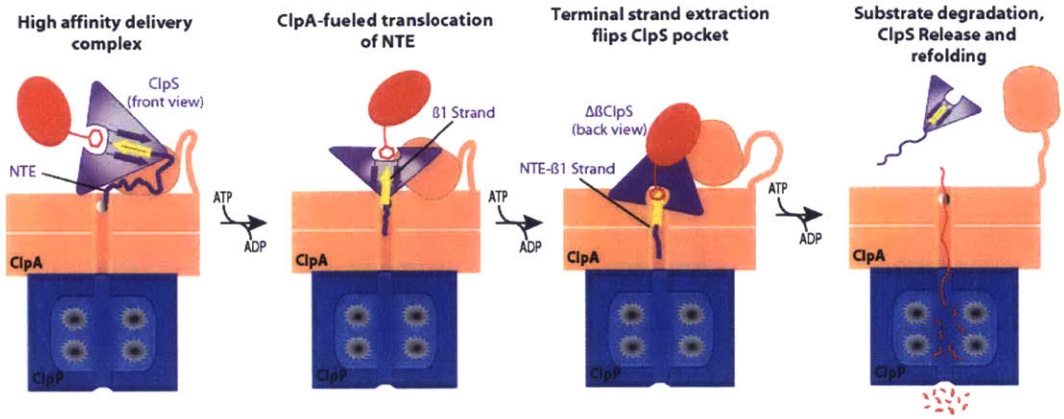

Figure 1.6. Model for ClpA-dependent N-degron substrate transfer. After formation of the high-affinity delivery complex ClpA dependent translocation of the ClpS NTE begins to deform the ClpScore by pulling on the middle β1-strand of the three-stranded β-sheet. Extraction of the β1-strand of ClpS facilitates substrate transfer by inverting the adaptor, thereby positioning the N-degron-binding pocket close to the ClpA pore, and by weakening interactions between the substrate and the ClpS-binding pocket. Subsequently, ΔβClpS resists further unfolding and thus is released from the ternary complex, allowing for refolding of the adaptor and translocation and degradation of the N-degron substrate to commence.

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Chapter Two

Remodeling of a delivery complex allows ClpS-mediated degradation of N-degron substrates

This chapter is a modified version of the paper that was previously published as Rivera-Rivera I, Román-Hernández G, Sauer RT, Baker TA. Remodeling of a delivery complex allows ClpS-mediated degradation of N-degron substrates. Proc Natl Acad Sci U S A. 2014 Sep 16;111(37):E3853-9. I Rivera and G Román performed the experiments. I Rivera, Sauer RT and Baker TA prepared the manuscript.

Abstract

The ClpS adaptor collaborates with the AAA+ ClpAP protease to recognize and degrade N-degron substrates. ClpS binds the substrate N-degron and assembles into a high-affinity ClpS-substrate-ClpA complex, but how the N-degron is transferred from ClpS to the axial pore of the AAA+ ClpA unfoldase to initiate degradation is not known. Here, we demonstrate that the unstructured N-terminal extension (NTE) of ClpS enters the ClpA processing pore in the active ternary complex. We establish that ClpS promotes delivery only in *cis,* as demonstrated by mixing ClpS variants with distinct substrate specificity and either active or inactive NTE truncations. Importantly, we find that ClpA engagement of the ClpS NTE is crucial for ClpS-mediated substrate delivery by using ClpS variants carrying "blocking" elements that prevent the NTE from entering the pore. These results support models in which enzymatic activity of ClpA actively remodels ClpS to promote substrate transfer, and highlights how ATPase/motor activities of AAA+ proteases can be critical for substrate selection as well as protein degradation.

Introduction

AAA+ molecular machines power cellular processes as diverse as protein degradation, microtubule severing, membrane fusion, and initiation of DNA replication, with the common theme that macromolecules are actively remodeled (1-3). Furthermore, protein-quality control in all organisms involves deployment of ATP-dependent proteases, consisting of hexameric AAA+ rings that unfold and translocate specific substrates into an associated peptidase barrel (3, 4). Adaptor proteins assist in recognition and degradation of certain substrates (5-8), but how enzyme-adaptor pairs ensure proper substrate selection is poorly understood.

In prokaryotes and eukaryotes, the N-end rule pathway governs degradation of proteins with specific N-terminal amino acids (9, 10). In *E. coli* the primary destabilizing N-degron amino acids are Phe, Tyr, Trp, and Leu (11, 12). ClpS, a widespread bacterial adaptor, recognizes and delivers N-degron substrates to the ClpAP or ClpCP AAA+ proteases (6, 11, 13). These enzymes consist of the AAA+ ClpA or ClpC unfoldases coaxially stacked with the ClpP peptidase (14-16). In eukaryotes, a family of E3 ligases shares homology with the substrate-binding region of ClpS (17, 18). These ligases recognize N-degron substrates and promote ubiquitination, which then targets the modified protein to the 26S proteasome (17, 18).

Multiple crystal structures reveal the regions of ClpS that bind to the N-degron as well as a patch that binds the N-terminal domain of ClpA (19-22). This bivalent binding to the substrate and the enzyme tethers N-degron substrates to ClpAP. However, tethering

alone is insufficient for ClpS to promote substrate delivery as deletion of 13 amino acids of the ClpS unstructured N-terminal extension (NTE, residues 1-25 in *E. coli* ClpS; Fig. 2.1A) prevents N-degron substrate degradation but does not block formation of a high-affinity delivery ternary complex (HADC) consisting of substrate, the ClpS adaptor, and the ClpAP protease (Fig. 2.1B) (19). Importantly, the identity of the NTE sequence is not critical for ClpS function (23). An active delivery model has been proposed in which the translocation pore of ClpA engages the ClpS NTE with subsequent translocation that remodels the delivery complex to achieve substrate engagement (Fig. 2.1C) (19).

Here, we investigate how the ClpS NTE functions during delivery of N-degron substrates. We show that the NTE can only promote delivery of substrates that are bound to the same ClpS molecule. Furthermore, we demonstrate that the NTE enters the ClpA translocation pore and provide strong evidence that ClpA pulls on the ClpS NTE to trigger substrate delivery.

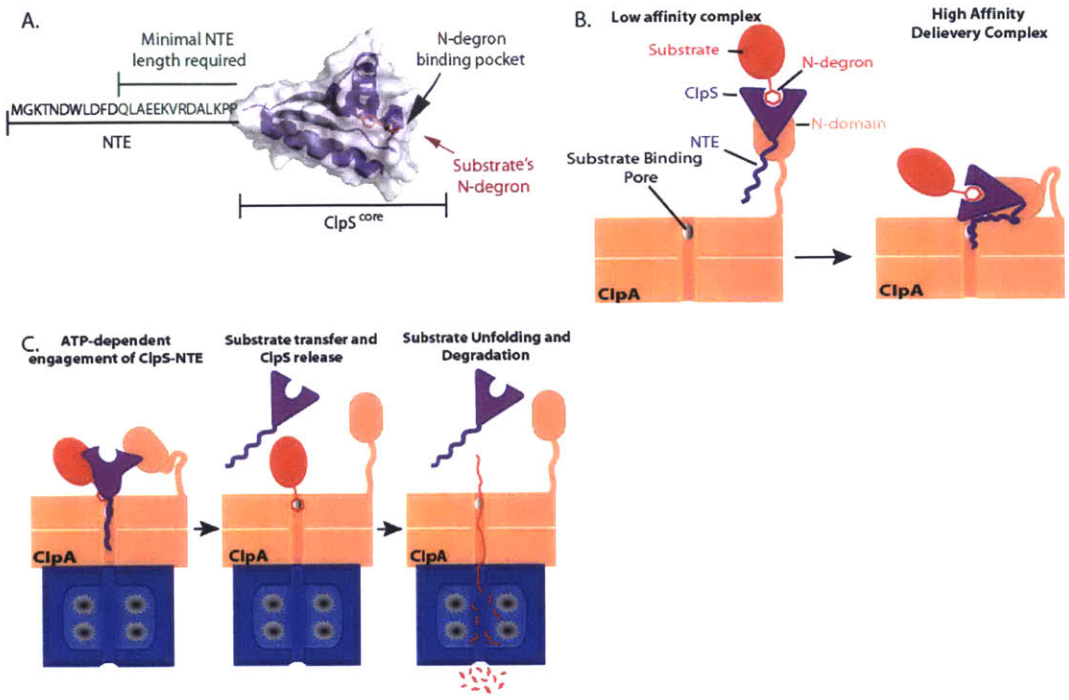

Figure 2.1. Model for the active delivery mechanism employed by ClpS. (A) The adaptor protein ClpS has a long flexible N-terminal region (NTE, residues 1-25) and a folded core domain ($ClpS^{core}$, residues 26-106). $ClpS^{core}$ binds N-degrons (a substrate Tyr in the binding pocket is shown in red) (PDB code 3O1F). Successful substrate delivery requires that the ClpS NTE be at least 14 amino acids long (shown in green). (B) Formation of a high-affinity delivery complex (HADC) between ClpS and ClpA (19) involves formation of additional contacts between ClpA, ClpS, and the N-degron substrate. Assembly of this complex increases the affinity of the substrate for ClpAS ~100-fold. (C) Current model for ClpA-driven disassembly of the HADC and N-degron substrate delivery. Translocation-mediated ClpA "pulling" on the NTE remodels the $ClpS^{core}$ structure, weakens ClpS interactions with the N-degron, and facilitates its transfer to a site in the ClpA pore. Finally, because ClpS cannot be unfolded by ClpA (19), the adaptor escapes the enzyme and the substrate is unfolded by ClpA and subsequently degraded by ClpP.

Results

The ClpS NTE acts in cis during substrate delivery

Multiple ClpS-substrate complexes can dock on the N-domains of a single ClpA hexamer (23-25). As previously established, an NTE of at least 14 amino acids is necessary for ClpS to deliver an N-degron substrate (Fig. 2.1A) (19). However, whether the NTE acts in *cis* to deliver the substrate bound to its own ClpS molecule or in *trans* to activate delivery of a substrate bound to another ClpS molecule is unknown. The optimal ratio of ClpS to ClpA hexamer in the delivery complex is not established, but many ratios yield functional complexes (23-25). To test whether the ClpS NTE acts in *cis* or in *trans*, we monitored delivery of substrates by mixtures of ClpS variants with a full-length functional NTE or truncated non-functional NTE ($ClpS^{\Delta 13}$) and a wild-type or M40A ($ClpS^{M40A}$) N-degron-binding pocket. The $ClpS^{M40A}$ variant recognizes β-branched (Val and Ile) residues, termed *N-degrons, in addition to natural *E. coli* N-degrons (Tyr, Leu, Phe, and Trp) (Fig. 2.2) (20).

In one experiment (Fig. 2.2A; left panel), ClpS and $ClpS^{\Delta 13/M40A}$ were mixed with ClpAP, an N-degron dipeptide (to promote formation of a high-affinity delivery complex (19)), as well as the *N-degron substrate VLFVQELA-GFP. In this experiment, the functional NTE was provided by wild-type ClpS, whereas the *N-degron substrate only bound $ClpS^{\Delta 13/M40A}$ (20). If engagement of the NTE can work in *trans*, then *N-degron substrate delivery would be observed. However, if engagement of the NTE functions only in *cis*, then the absence of a functional NTE in $ClpS^{\Delta 13/M40A}$ would prevent degradation of the *N-degron substrate. Upon addition of ATP, the *N-degron substrate was not efficiently

degraded (Fig. 2.2B, pink trace). Under conditions similar to those used for the degradation experiment, fluorescence-anisotropy experiments established that a fluorescently labeled ClpS$^{\Delta 13/M40A}$ variant bound ClpA tightly (Fig. 2.2C). Hence, the absence of efficient degradation of the *N-degron substrate was not caused by a failure of ClpS$^{\Delta 13/M40A}$ to bind ClpAP. Rather, these data indicate that the NTE does not function *in trans* to trigger substrate delivery. To ensure that ClpS^{M40A} with a functional NTE was able to perform substrate delivery under the conditions of this assay, we mixed it with ClpS$^{\Delta 13}$ (non-functional NTE), ClpAP, N-degron peptide and *N-degron substrate (Fig. 2.2A, right panel). In this case, the *N-degron substrate was efficiently degraded (Fig. 2.2B). Together, these experiments show that delivery requires a functional substrate-binding pocket and a functional NTE within the same ClpS molecule.

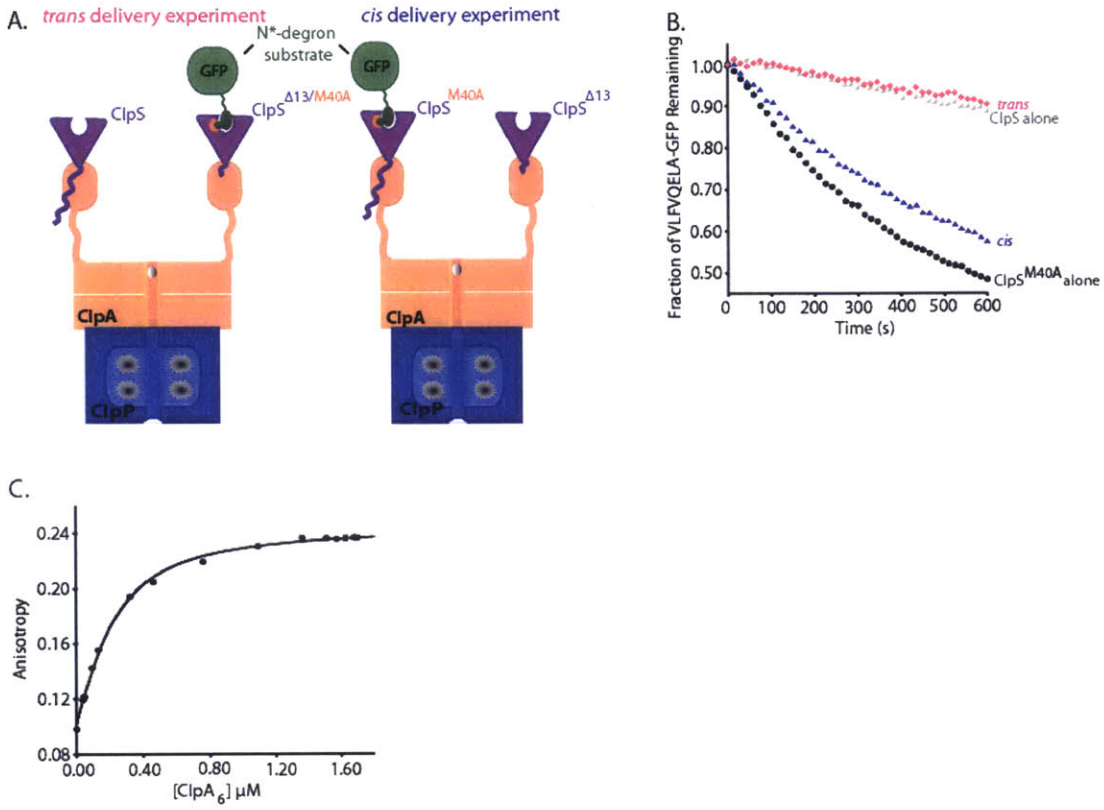

Figure 2.2. The ClpS NTE delivers N-degron substrates *in cis*. (A) Cartoon shows the protein variants for mixing experiments performed to test *cis* vs *trans* activation by the ClpS-NTE. Although present, the N-degron peptide (Phe-Val) is not depicted. (B) Degradation of the *N-degron substrate (VLFVQELA-GFP) by ClpAP. Only when the full-length functional NTE and *N-degron binding pocket were present on the same molecule was this substrate efficiently degraded (*cis* delivery experiment, blue trace). The mixing experiments contained each of the ClpS variants shown in (A) (1.2 μM each), 1 μM of an N-degron peptide and 1 μM of *N-degron substrate. (C) Binding of fluorescein labeled $ClpS^{\Delta 13/M40A}$ to $ClpA_6$ (100 nM) in the presence of ATPγS (2 mM), ClpS (1.2 μM), N-degron peptide (1 μM), and N*-degron peptide (1 μM) as assayed by fluorescence anisotropy (K_D =112 ± 13 nM). Experiments performed by I Rivera-Rivera.

ClpS NTE physically enters the ClpA pore

Previous studies suggested a model in which N-degron substrate delivery requires engagement of the ClpS NTE by the ClpA translocation pore (Fig. 2.1C) (19, 23). To test this model directly, we used Förster resonance energy transfer (FRET) between a

donor fluorophore (EDANS) at the entrance of the ClpP proteolytic chamber (ClpP residue 17, adjacent to the bottom of the ClpA pore, $ClpP^{ED}$) (26) and an acceptor fluorophore (fluorescein) placed at different positions either along the ClpS NTE or on the surface of the folded domain (Fig. 2.3A). The calculated Förster radius for the EDANS-fluorescein pair is ~46 Å. Based on the dimensions of ClpC, a close relative of ClpA, a distance of ~100 Å separates the top of the ClpA pore from the ClpP neck (16). As a consequence, robust FRET would only be expected if a fluorescein dye on ClpS were able to enter the ClpA pore.

When residue 5 of the ClpS NTE was labeled with fluorescein ($ClpS^{5\text{-}Fl}$) and incubated with $ClpAP^{ED}$, N-degron substrate, and ATPgS (Fig. 2.3B), FRET was observed between the donor and acceptor dyes. Excitation of the donor fluorophore in $ClpP^{ED}$ increased acceptor fluorescence (525 nm) and decreased donor fluorescence (475 nm) (Fig. 2.3B, red trace) compared to the sum of the spectra of each component alone (Fig. 2.3B, gray trace). If this signal resulted from FRET between the NTE and $ClpP^{ED}$, then reduced signal would be expected if the fluorescein were placed at position 17 of the ClpS NTE, a more C-terminal location which should be farther from ClpP. Furthermore, little or no FRET would be predicted if the dye were attached to ClpS residue 96, near the N-degron-binding pocket and far from the NTE (Fig. 2.3A). This pattern of FRET signals was observed (Fig. 2.3C), supporting our hypothesis that the ClpS NTE enters the ClpA axial pore with its N-terminal residues reaching close to the ClpA-ClpP complex junction.

To test further if FRET between $ClpS^{5\text{-}Fl}$ and $ClpP^{ED}$ occurs because the NTE is located within the pore, rather than on the surface of the enzyme, we repeated the experiment with donor dye at NTE position 5 in the presence of the solution quencher 4-amino-TEMPO (4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl), which has an anhydrous diameter of ~10 Å and therefore should not diffuse into the ClpA pore efficiently (27). Indeed, fluorescence of free $ClpS^{5\text{-}Fl}$ was quenched ~30% by 4-amino-TEMPO, whereas quenching of the fluorescence of $ClpS^{5\text{-}Fl}$ in complex with $ClpAP^{ED}$ and substrate was less than 5% (Fig. 2.3D). Together, these results support a model in which the ClpS NTE enters the ClpA pore in the ClpAPS-substrate complex.

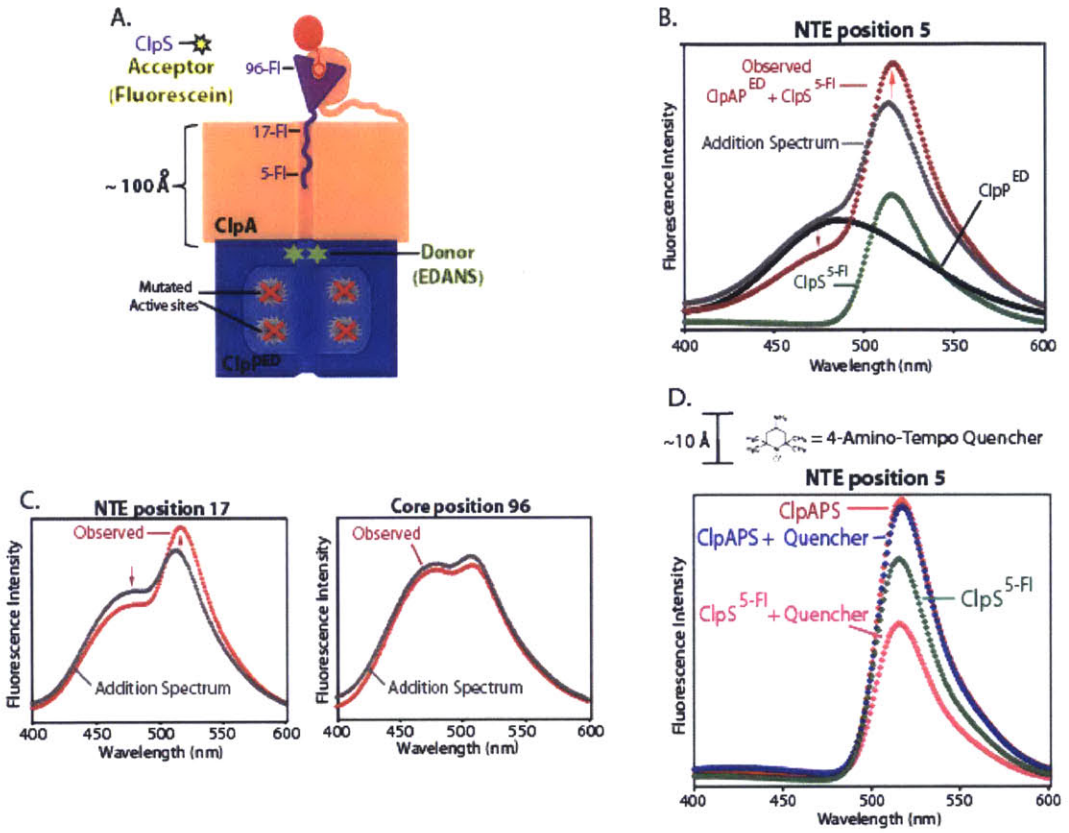

Figure 2.3. The ClpS NTE localizes inside the ClpA pore. (A) Cartoon of the protein variants used in the FRET experiments. Three single-cysteine variants of ClpS were labeled with fluorescein (acceptor fluorophore, yellow star). The labeled positions were C5 and C17, both sites in the ClpS NTE ($ClpS^{5\text{-}Fl}$ and $ClpS^{17\text{-}Fl}$), and C96, which is in the ClpS core domain ($ClpS^{96\text{-}Fl}$). Unlabeled ClpA was used with a ClpP variant in which residue 17 of each subunit was changed to cysteine and labeled with EDANS (donor fluorophore, green star; $ClpP^{ED}$). This ClpP variant also contained the S97A active-site mutation (28). (B) Emission spectra of the donor fluorophore in $ClpP^{ED}$ upon excitation at 336 nm in the presence of $ClpA_6$ and ATPγS (black trace). Emission spectra of the acceptor fluorophore in $ClpS^{5\text{-}Fl}$ upon excitation at 336 nm in the presence of ATPγS (green trace). Addition spectra of the two independent traces obtained from the emission of the donor and acceptor proteins (gray line). Observed emission spectra characteristic of FRET obtained in reactions containing ATPγS, $ClpS^{5\text{-}Fl}$, $ClpAP^{ED}$, and the N-degron substrate YLFVQ- titin I27 (red trace). The red arrow pointing up at ~525 nm marks an increase in fluorescence of the acceptor fluorophore and the red arrow pointing down at ~475 nm depicts the decreased signal of the donor fluorophore. (C) FRET was also observed when the experiment in (B) was repeated with $ClpS^{17\text{-}Fl}$ as the acceptor molecule (red; left panel). In contrast, no FRET was detected when the acceptor molecule was $ClpS^{96\text{-}Fl}$ (red trace; right panel). (D) $ClpS^{5\text{-}Fl}$ fluorescence was insensitive to the fluorescence quencher 4-amino-Tempo when bound in a complex with $ClpAP^{ED}$ and N-degron substrate. Experiments performed by G Román-Hernández.

Increasing the length of the ClpS NTE results in truncation by ClpP

As an orthogonal method to determine if the ClpS NTE enters the ClpA pore during N-degron delivery, we constructed an NTE_2-ClpS variant with a duplicated NTE (Fig. 2.4A). We reasoned that if the NTE enters the ClpAP pore during N-degron delivery, then the longer NTE_2 sequence would enter the ClpP chamber, where it can be cleaved by the ClpP active sites. Control experiments revealed that NTE_2-ClpS delivered the N-degron substrate YLFVQELA-GFP for degradation, albeit somewhat less efficiently than ClpS (Fig. 2.4B). Importantly, during these time-course delivery experiments, the NTE_2-ClpS both delivered N-degron substrate and was truncated by ClpP (Fig. 2.4B-C). Truncation of the NTE_2-ClpS depended on the ATP-driven translocation activity of ClpA, as it did not occur either in the absence of ATP or with the poorly-hydrolyzed analog, ATPγS (Fig. 2.4C). N-terminal sequencing of the smallest truncated ClpS species revealed that 19 amino acids of NTE_2-ClpS had been removed, leaving the native ClpS sequence with an additional 9 N-terminal residues that originated from the NTE duplication (Fig. 2.4C). Such NTE_2-ClpS cleavage by ClpP strongly supports the model in which the NTE of wild-type ClpS is engaged by the ClpA axial pore during delivery of N-degron substrates.

We observed slightly slower N-degron degradation rates for ClpS variants with longer NTEs, including NTE_2-ClpS, H_6-Sumo-ClpS (Fig. 2.4D) ((19), and H_6-DHFR-ClpS (see next section) (29). Slower steady state delivery may be a result of slower engagement of the extended NTE in the ClpS-substrate complex or slower dissociation of the longer NTE ClpS upon substrate delivery. Single molecule experiments show ClpA

translocates unfolded polypepetides at a rate of 30 aa/sec (30), so it is unlikely that longer NTEs significantly decrease the rate of N-degron degradation.

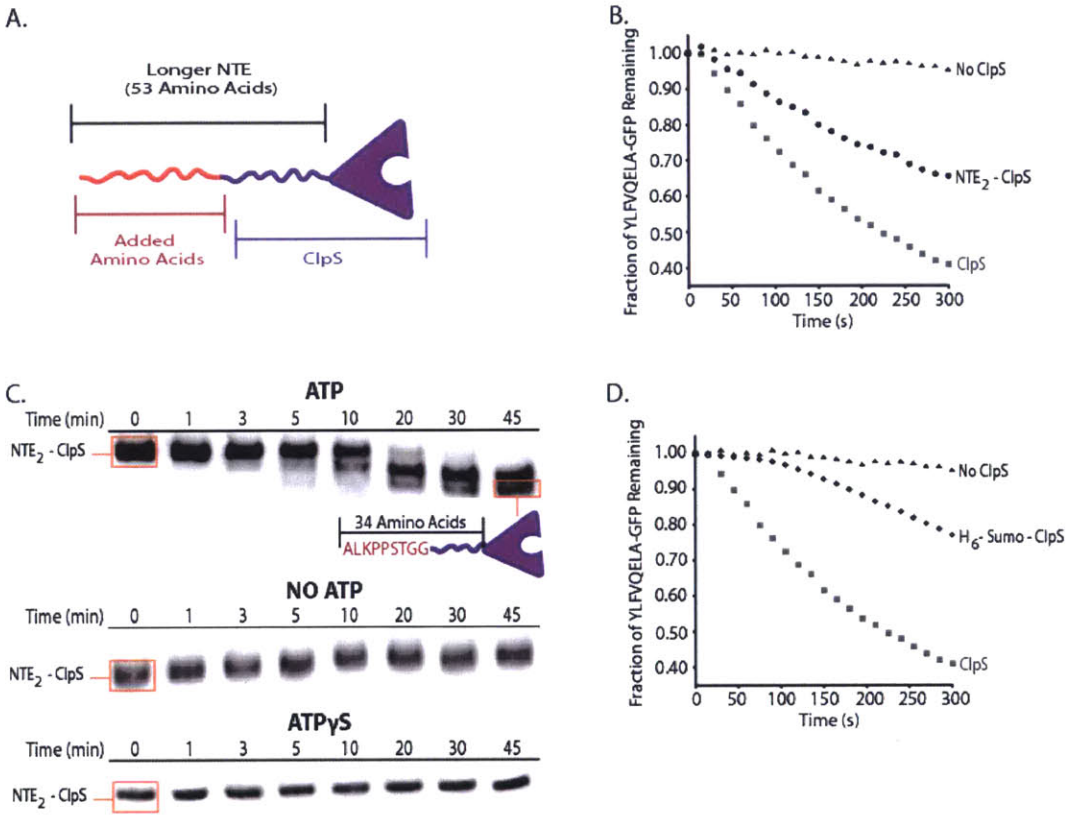

Figure 2.4. ClpAP cleaves an extended ClpS NTE. (A) Cartoon of the NTE_2-ClpS variant. (B) Delivery and degradation of the N-degron substrate, YLFVQELA-GFP, to ClpAP in the absence of ClpS, in the presence of NTE_2-ClpS or in the presence of wild-type ClpS. Degradation was monitored by the decrease in substrate fluorescence. (C) Truncation of NTE_2-ClpS was observed during delivery of N-degron substrates to ClpAP in the presence of ATP but was not observed without ATP or with ATPγS. N-terminal sequencing of the lowest molecular weight product revealed an NTE "tail" of 34 amino acids. This "trimmed" NTE_2-ClpS truncation product is depicted as a cartoon below the top panel. (D) Delivery and degradation of the N-degron substrate, YLFVQELA-GFP, to ClpAP in the absence of ClpS, in the presence of H_6-Sumo-ClpS or in the presence of wild-type ClpS. Degradation was monitored by the decrease in substrate fluorescence. Experiments performed by I Rivera-Rivera.

Antagonizing NTE engagement inhibits N-degron substrate delivery

To probe if entry of the ClpS NTE into the ClpA pore is required for substrate delivery, we constructed a ClpS variant with mouse dihydrofolate reductase (DHFR) attached to the N-terminus of the ClpS NTE (H_6-DHFR-ClpS; Fig. 2.5A). In our experiment, the N-terminal H_6 tag of DHFR served as a ClpA degron (19), and the DHFR domain of this substrate was unfolded and degraded by ClpAP, exposing the ClpS NTE (Fig. 2.5B, left panel). As expected from other studies of DHFR degradation by AAA+ proteases (31), addition of methotrexate stabilized DHFR and prevented truncation of the DHFR-ClpS chimera by ClpAP (Fig. 2.5B, right panel).

Importantly, the H_6-DHFR-ClpS adaptor promoted degradation of the N-degron substrate YLFVQELA-GFP in the absence but not the presence of methotrexate (Fig. 2.5C). Interestingly, H_6-DHFR-ClpS stimulated degradation of YLFVQELA-GFP only after a lag of ~100 s, suggesting that degradation of the DHFR domain is prerequisite for NTE engagement and subsequent substrate delivery (Fig. 2.5C). As expected, methotrexate did not inhibit wild-type ClpS delivery of YLFVQELA-GFP to ClpAP (Fig. 2.5C). Furthermore, H_6-DHFR-ClpS assembled normally with ClpAP, ATPγS, and a fluorescent N-degron peptide (LLYVQRSDEC-^{fl}) both in the absence and presence of methotrexate (Fig. 2.5D). Thus, the degradation defect caused by blocking entry of the ClpS NTE into the ClpA pore appears to occur at a step after assembly of the initial substrate-adapter-enzyme ternary complex.

Together, experiments with the H_6-DHFR-ClpS chimera demonstrate that preventing entry of the ClpS NTE into the ClpA pore inhibits delivery and degradation of ClpS-

bound N-degron substrates. These results strongly support a model in which engagement and partial translocation of the ClpS NTE through the ClpA pore is an essential step in the delivery of N-degron substrates.

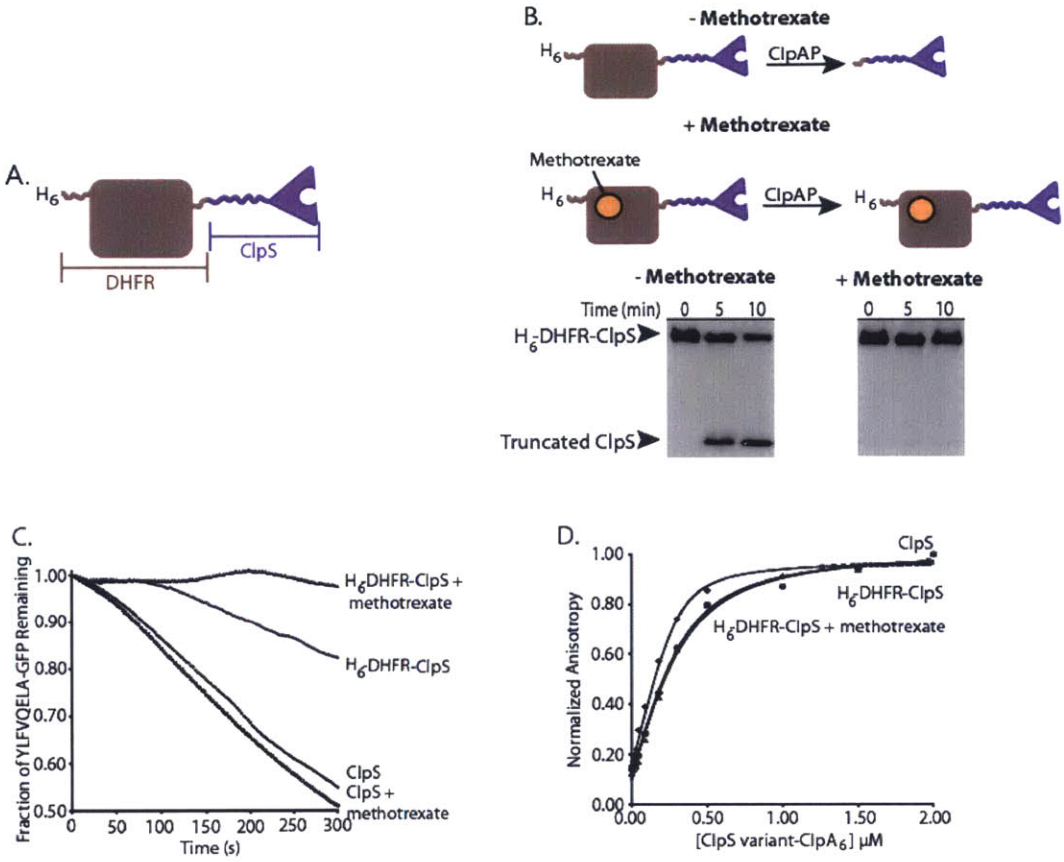

Figure 2.5. Engagement of the ClpS NTE is necessary for delivery of N-degron substrates. (A) Cartoon of the H_6-DHFR-ClpS fusion protein. (B) Cartoon of results obtained upon addition of ClpAP and ATP to H_6-DHFR-ClpS in the absence or presence of methotrexate. Protein processing was monitored by Western blotting of the H_6-DHFR-ClpS protein with anti-ClpS antisera. ClpAP-dependent cleavage of the fusion protein and release of a truncated ClpS adaptor (with an available NTE) was observed in the absence of methotrexate (left), whereas no processing of the fusion protein was detected when methotrexate was present (right). (C) Delivery and degradation of the N-degron substrate YLFVQELA-GFP by ClpAP promoted by either H_6-DHFR-ClpS or ClpS in the presence and absence of methotrexate. (D) Formation of a high affinity delivery complex by ClpS (K_{app}= 35 ± 1 nM), H_6-DHFR-ClpS (K_{app}= 107 ± 17 nM), and H_6-DHFR-ClpS in the presence of methotrexate (K_{app}= 119 ± 21 nM) assayed by anisotropy using a fluorescent N-degron peptide. Experiments performed by I Rivera-Rivera.

Discussion

Regulation of macromolecular complexes is commonly implemented by forming multiple weak binary interactions that synergistically stabilize the complex (19, 32). Stable complexes can serve as checkpoints in a sequential mechanism to enhance specificity but can also make downstream steps slow or inaccessible if stabilizing interactions must be broken before the next step can occur. AAA+ enzymes play important roles in catalyzing both the remodeling and destabilizing macromolecular complexes, including the examples of severing microtubules and promoting both assembly and critical reaction transitions during RNA splicing (1, 2, 19, 32, 33).

Previous studies established that adaptor-mediated recognition of several substrates by AAA+ proteases involves formation of a high-affinity complex between the enzyme, substrate, and adaptor (19, 34-39). The delivery complex consisting of ClpAP, N-degron substrate, and the ClpS adaptor is one such example (Fig. 2.1B) (19). Here, we identify features of the interactions between ClpA and ClpS that are critical for releasing substrate from this high-affinity complex and thus enabling the downstream steps of unfolding and degradation. Our FRET and protein-processing experiments demonstrate that the NTE enters the ClpA pore during substrate delivery. Importantly, we also find that engagement of the NTE by the ClpA pore is essential for ClpS-mediated degradation of N-degron substrates. Consistent with these observations, prior experiments establish that the ClpS NTE can act as a ClpAP degradation tag when attached to other proteins (19).

Why is engagement of the ClpS NTE by the ClpA pore critical for transfer of the N-degron of the substrate from ClpS to the ClpA pore? At the simplest level, ATP-dependent translocation of the ClpS NTE through the ClpA pore pulls the folded domain of ClpS against the pore entrance, distorting the folded structure of ClpS and catalyzing release of the N-degron from the binding pocket (Fig. 2.1C). Because the NTE and N-degron-binding pocket are on opposite sides of the ClpS molecule, however, if the substrate were released far from the entrance to the ClpA pore, it would be poorly positioned for efficient pore capture. As discussed below, one possibility is that conformational changes in ClpS, caused by ClpA pulling, place the N-degron-binding pocket close to the entrance to the ClpA pore and allow transfer of the N-degron or nearby segments of the protein substrate (Fig. 2.6) (40).

One speculative model is that NTE-tugging by ClpA both distorts and inverts ClpS by at least transiently pulling out the β-strand proximal to the NTE (β1-Strand), which is part of a three-stranded β-sheet (Fig. 2.6A-C). Pulling this central strand out of the sheet and into the ClpA pore would flip the remaining ΔβClpS structure relative to ClpA (Fig. 2.6C), positioning the N-degron-binding pocket close to the axial pore for transfer (Fig. 2.6B-D). In this model, ΔβClpS remains stably folded but has reduced N-degron affinity, facilitating transfer of the substrate to ClpA. This model also requires that ΔβClpS not be globally denatured and degraded by ClpAP, as it has been established that ClpS is not degraded during delivery (19). There is precedent for this type of β-strand extraction by AAA+ unfoldases. For example, we note that ClpXP initially extracts a terminal β strand from a sheet in GFP-ssrA without causing global unfolding (41, 42). Moreover, under some conditions, the extracted β-strand appears to slip from the pore of the AAA+

unfoldase, allowing refolding to native GFP (42). For the ClpS-delivery model, we suggest that following transfer of the N-degron, a slipping event could also allow ΔβClpS to refold and therefore restore native ClpS. This reaction would re-invert the structure and favor ClpS escape, as its affinity for the ClpA N domain is weaker without bound N-degron (19).

A strong prediction of any NTE-tugging model is that an NTE would only promote delivery of a substrate bound to the same ClpS molecule, and would not influence delivery of substrates bound to different molecules of ClpS, even if they were bound to the same ClpA hexamer. Our results strongly support this *cis*-only aspect of ClpS NTE function, as we found that only N-degron substrates bound to a ClpS molecule with a functional NTE were degraded by ClpAP. These results support an NTE-pulling model, and argue against models in which the NTE simply serves as an allosteric activator of ClpA (23). During substrate transfer, both the ClpS NTE and the N-terminal residues of the N-degron substrate may need to occupy the ClpA pore. We assume that these multiple polypeptide chains can be accommodated in the ClpA pore as experiments with the related ClpXP enzyme show that pore engagement of multiple polypeptides is possible (43).

Parallels can be drawn between our active handoff model and other protein-degradation systems. For example, the SspB adaptor delivers ssrA-tagged substrates to ClpXP protease via the formation of a high-affinity ternary complex that involves interactions between SspB dimers, the N-domain(s) of ClpX, and a segment of the ssrA-degron (5,

36, 37). In this case, the complex is broken and initiation of substrate degradation proceeds when the ClpX translocation pore engages the ssrA-degron (5, 36, 39, 44). Translocation of this initiation region of the substrate serves to break interactions in the ternary complex, allowing degradation to begin and the adaptor to be recycled. An unstructured initiation region is also required for unfolding and degradation by the proteasome (45-47). Proteins are targeted to the proteasome by a two-part degradation signal, which consists of a disordered region within the substrate and a polyubiquitin tag. The proteasome recognizes the ubiquitin tag and initiates unfolding at the unstructured region within the substrate. Once the proteasome has engaged its substrate, the polyubiquitin tag is cleaved off by de-ubiquitination enzymes, allowing recycling of ubiquitin. For ClpXP-SspB degradation of ssrA-tagged proteins and degradation of substrates by the proteasome, disassembly of the complex occurs when the initiation region on the substrate is engaged. In contrast, for ClpS-mediated delivery, the unstructured region required for complex disassembly is provided instead by the adaptor, which in turn is recycled as it escapes degradation (Fig. 2.6). However, an initiation region in the substrate is also necessary for transfer to ClpA and substrate unfolding (40, 48). In the case of N-degron substrates, this dual-initiation active handoff allows delivery of substrates whose degron is a single N-terminal amino acid that is recognized with high affinity by the ClpS adaptor but only with low affinity by ClpA.

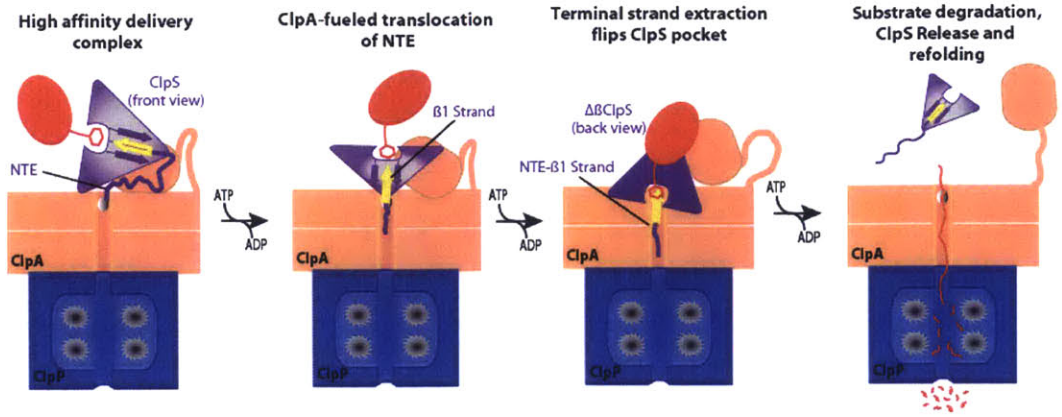

Figure 2.6. Model for ClpA-dependent N-degron substrate transfer. After formation of the high-affinity delivery complex ClpA dependent translocation of the ClpS NTE begins to deform the ClpScore by pulling on the middle β1-strand of the three-stranded β-sheet. Extraction of the β1-strand of ClpS facilitates substrate transfer by inverting the adaptor, thereby positioning the N-degron-binding pocket close to the ClpA pore, and by weakening interactions between the substrate and the ClpS-binding pocket. Subsequently, ΔβClpS resists further unfolding and thus is released from the ternary complex, allowing for refolding of the adaptor and translocation and degradation of the N-degron substrate to commence.

Materials and Methods

Proteins and peptides

Mutants were generated by the QuickChange method (Stratagene) or PCR. ClpS, ClpS mutants, and substrates were purified as described (19). Briefly, ClpS, ClpS mutants, and substrates were initially fused to the C terminus of H_6-Sumo in pet23b (Novagen). Following expression, fusion proteins were purified by Ni-NTA chromatography (QIAGEN) and cleaved with Ulp1 protease. The cleaved H_6-Sumo fragment was removed by passage through Ni-NTA, and the protein of interest was purified by gel filtration on Superdex 75 (GE Healthsciences) and/or ion-exchange chromatography on MonoQ. ClpS variants were concentrated and stored in 20 mM HEPES (pH 7.5), 150 mM KCl, 1 mM DTT, and 10 % glycerol. ClpA, ClpP, and $ClpP^{ED}$ were purified as described (19). As ClpS variants were purified using the Sumo-fusion and Ulp1 cleavage method, the N-terminal methionine of ClpS should be present. Previous publications have either used Sumo-cleavage or native expression for ClpS variants (19, 23). The N-degron (LLFVQRDSKEC) and N^*-degron (ILYVQRDEKEC) peptides were synthesized by standard FMOC techniques using an Apex 396 solid-phase instrument.

Fluorescent Labeling

Peptides were labeled with fluorescein maleimide as described (20). Labeled ClpS variants and $ClpP^{ED}$ were labeled with fluorescein maleimide and EDANS maleimide, respectively as described (19). Briefly, ClpS variants (50 μM) and ClpP containing a single cysteine were incubated with 50 mM DTT in 100 mM TrisCl (pH 8) for 1.5 h at 4° C, buffer exchanged into 100 mM Na_2PO_4 (pH 8), and 1 mM EDTA. The variants were

then singly labeled by addition of 0.3 mg/mL of fluorescein maleimide or EDANS maleimide (Thermo Scientific) for 2 h at room temperature in the dark. Excess reagent was removed by size-exclusion chromatography, and the modified protein was stored in 10 mM HEPES (pH 7.5), 200 mM KCl, and 1 mM DTT.

FRET Experiments

FRET experiments were performed using a Photon Technology Internal Fluorimeter. $ClpA_6$ (200 nM), $ClpP^{ED}$ (200 nM), $ClpS^*$ variants (200 nM), N-degron substrate YLFVQ-titin I27 (500 nM) (48), ATPγS (2 mM), and AT-Quencher (10 μM) when necessary, were incubated for 10 min at 30 °C in reaction buffer (50 mM HEPES [pH 7.5], 300 mM NaCl, 20 mM $MgCl_2$, 0.5 mM DTT, and 10 % glycerol) at 30 °C before taking a spectrum. Samples were excited at 336 nM and emission scans were taken from 400 to 600 nM.

Degradation Assays and Western Blots.

ClpAPS degradation assays were performed as described (48). Briefly, $ClpA_6$ (100 nM), $ClpP_{14}$ (200 nM), and ClpS variants (1 μM) were preincubated in reaction buffer (50 mM HEPES [pH 7.5], 300 mM NaCl, 20 mM $MgCl_2$, 0.5 mM DTT, and 10 % glycerol) with YLFVQELA-GFP or VLFVQELA-GFP (1 μM), and methotrexate (10 μM, Sigma-Aldrich®), when necessary, for 3 min at 30 °C before adding ATP regeneration mix (4 mM ATP, 50 mg/mL creatine kinase, 5 mM creatine phosphate) or ATPγS (2 mM) to initiate assay. GFP degradation was assayed by loss of fluorescence. Reported kinetic parameters

were averages ($n \geq 3$) ± 1 SD. Formation of ClpS truncation products was monitored by SDS-PAGE and western blotting as described (23). Briefly, samples were separated by SDS-PAGE followed by an anti-ClpS western blot. For *cis/trans* experiments, degradation assays were conducted using the same conditions but with 1.2 μM of ClpS or ClpS variants and 1 μM Phe-Val dipeptide.

Binding Assays

Binding assays, monitored by fluorescence anisotropy, were performed using a Photon Technology International Fluorimeter. Data were fitted using a quadratic equation for tight binding. Reported K_{app} values are averages ($n \geq 3$) with errors calculated as $SQRT([K\text{-}K_{avg}]^2/n)$.

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Chapter Three

Delivery of N-degron substrates to the ClpAP protease requires structural rearrangements within the ClpS adaptor protein

Abstract

The ClpS adaptor delivers N-degron substrates to the AAA+ ClpAP protease for degradation, but the molecular mechanism of delivery is poorly understood. We find that ClpA engagement of the ClpS N-terminal extension (NTE) drives structural rearrangements in ClpS and that these rearrangements are important for N-degron substrate delivery. Additionally, our preliminary experiments suggest that ClpS is able to resist degradation by ClpAP due to a combination of a high local stability and a challenging translocation sequence at the junction of the NTE and folded core domain. We propose a model in which ClpA remodels ClpS by translocating the NTE, triggering delivery of the N-degron substrate. Many other AAA+ enzymes that collaborate with adaptor proteins, or remodel/disassemble protein complexes without degradation as the end result may employ similar mechanisms.

Introduction

Regulated protein degradation is involved in biological processes including protein quality control, the initiation of appropriate transcriptional responses to cellular stress, and the control of protein life span, among others (1-4). In all kingdoms of life, AAA+ proteases recognize their substrates and then use cycles of ATP binding and hydrolysis to unfold and translocate the denatured polypeptide into a compartmental peptidase for degradation (5). The ability of these enzymes to recognize their substrates with accuracy is important for efficient degradation of target proteins and minimization of off-target degradation. In bacteria, AAA+ proteases often recognize substrates via short accessible peptide sequences called degradation tags or degrons (4, 5). Substrate degrons are recognized directly by the protease, and, in some cases, accessory adaptor proteins aid in substrate recognition. Adaptor proteins can deliver specific substrates to the protease and/or prevent degradation of other classes of substrates. However, the range of mechanisms used by adaptors to deliver substrates to their partner AAA+ proteases is not yet understood.

The ubiquitious N-degron pathway relates the *in vivo* half-life of a protein to the identity of its N-terminal residue (6). In bacteria, the hydrophobic residues Tyr, Phe, Trp, and Leu serve as primary destabilizing residues (7). Moreover, proteins containing an N-terminal Arg or Lys are converted to N-degron substrates by addition of an N-terminal Phe or Leu in a reaction catalyzed by the Aat aminoacyl transferase (7).

ClpS, a small monomeric adaptor protein, binds and delivers N-degron substrates to the AAA+ ClpAP or ClpCP proteases (7-9). These proteases are composed of ring-shaped

hexamers of the ClpA or ClpC ATPases stacked on barrel-shaped tetradecamers of the ClpP peptidase, which contains the proteolytic chamber (10-12). ClpS shares a region of homology with the hydrophobic residue recognizing N-degron E3-ligase proteins from eukaryotes, strongly suggesting that eukaryotic and prokaryotic systems use a common mode of N-degron recognition (13).

Escherichia coli ClpS has a folded core domain (residues 26-106) and an unstructured N-terminal extension (NTE; residues 1-25; Figure 3.1A) (14-16). Additionally, the ClpS NTE pocesses a region (residues 22-25 of the ClpS NTE; termed junction sequence throughout this chapter) that is substantially conserved among ClpS orthologs, in contrast to the very weak conservation of most of the NTE (Fig 3.1A, 3.4A) (16). The ClpS NTE is engaged by the ClpA translocation pore during substrate delivery and this engagement is necessary for delivery of N-degron substrates (16, 17). The mechanistic underpinnings of this requirement, however, remain incompletely understood. We have proposed an active delivery mechanism in which ClpA-dependent translocation of the ClpS NTE begins to deform the $ClpS^{core}$ by pulling on the middle β-strand (β1-strand) of the three-stranded β-sheet (Fig. 3.1B). Partial or complete extraction of the β1-strand of ClpS, in turn, facilitates substrate transfer by positioning the N-degron–binding pocket close to the ClpA pore while simulatiously weakening interactions between the substrate and the ClpS-binding pocket. Subsequently, ClpS is released from the ClpA pore, allowing for refolding and recycling of the adaptor and translocation and degradation of the N-degron substrate (Fig. 3.1B) (17).

Here, we investigate structural rearrangements within the $ClpS^{core}$ that accompany substrate delivery. We use a method that relies on short distance quenching of a fluorescent dye by a transition metal ion (tmFRET; (18)) to visualize conformational changes in the $ClpS^{core}$ upon N-degron substrate delivery. Furthermore, we demonstrate the importance of these conformational changes in the delivery of N-degron substrates. Additionally, mutational analysis combined with chemical denaturation experiments provide strong evidence that a combination of a high local stability of the β-sheet in ClpS adjacent to a challenging translocation sequence renders the ClpS adaptor resistant to degradation by ClpAP and allows for adaptor recycling.

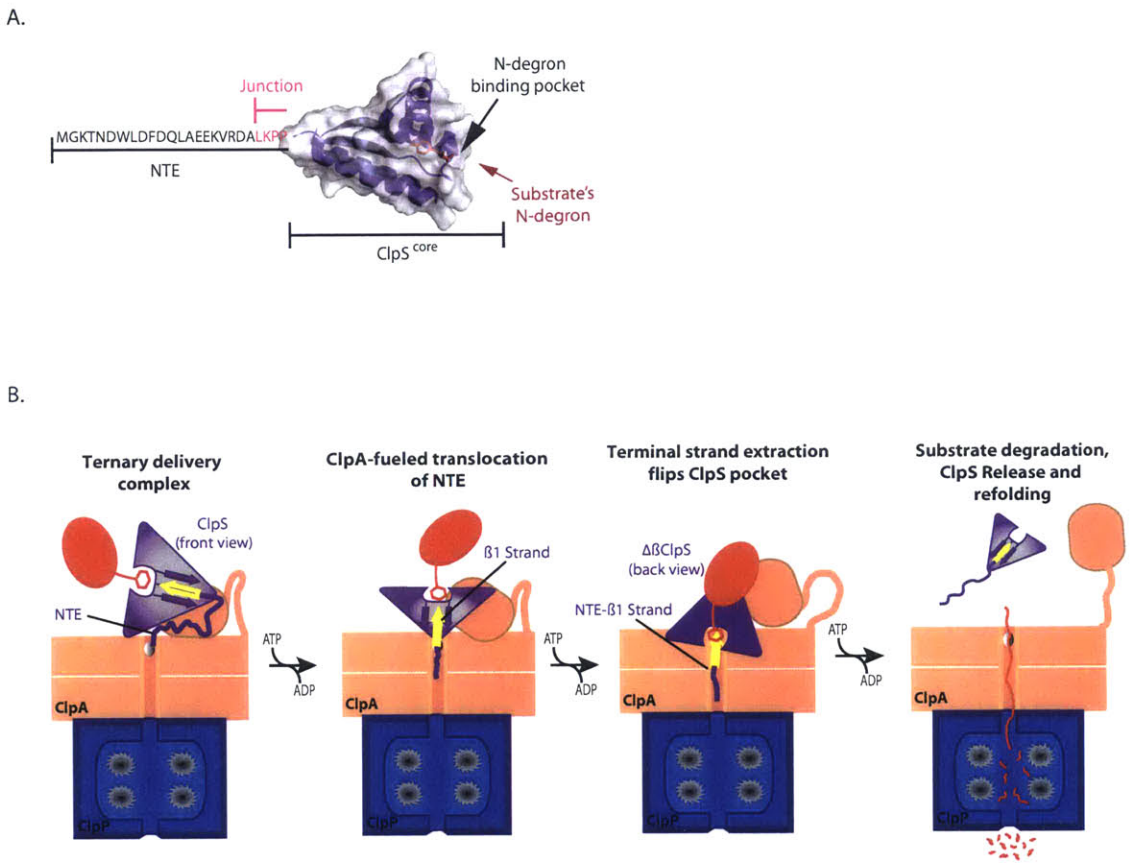


Figure 3.1. Model for ClpA- and nucleotide- dependent N-degron substrate transfer. (A) The adaptor protein ClpS has a long flexible N-terminal region (NTE, residues 1-25) and a folded core domain ($ClpS^{core}$, residues 26-106). $ClpS^{core}$ binds N-degrons (a substrate Tyr in the binding pocket is shown in red) (PDB code 3O1F). The sequence LKPPS, termed the junction sequence, is important for ClpS's undegradability (shown in magenta). (B) After formation of a ternary delivery complex ClpA-dependent translocation of the ClpS NTE begins to deform the $ClpS^{core}$ by pulling on the middle β1-strand of the three-stranded β-sheet. Extraction of the β1-strand of ClpS facilitates substrate transfer by inverting the adaptor, thereby positioning the N-degron-binding pocket close to the ClpA pore, and by weakening interactions between the substrate and the ClpS-binding pocket. Subsequently, the remaining structure (ΔβClpS) resists further unfolding and thus is released from the ternary complex, allowing for refolding of the adaptor and translocation and degradation of the N-degron substrate to commence.

Results

The β1-strand of ClpS is mobile during N-degron substrate delivery

Engagement of the ClpS NTE by the translocation machinery of ClpA is necessary for delivery of N-degron substrates (16, 17, 19). We have hypothesized that nucleotide-fueled tugging of the NTE partially or completely extracts the β1-strand of ClpS to remodel the N-degron binding pocket and facilitate substrate transfer (Fig. 3.1B) (17). This model predicts ClpA- and nucleotide-dependent conformational changes within the $ClpS^{core}$. To test for structural rearrangements in the $ClpS^{core}$ we performed tmFRET experiments (18) (Fig. 3.2), which rely on short-distance quenching of a fluorescent dye by a transition-metal ion (18). In our experiments, quenching is determined by the distance between a fluorescein dye, attached via maleimide chemistry to S26C in the NTE of ClpS, and a Cu^{2+} ion chelated by an α-helical His-X3-His motif in a proximal α-helix of the $ClpS^{core}$ (Fig. 3.2A) The His-X3-His motif was engineered by introducing A78H and E82H mutations, and the removal of the endogenous reactive ClpS cysteines (C73V/C101S). We refer to the resulting variant as $ClpS^{tmFRET}$. Importantly, unlabeled $ClpS^{tmFRET}$ delivered an N-degron substrate with wild-type-like activity in the absence of Cu^{2+} (Fig. 3.2B) and $ClpS^{tmFRET}$ labeled with fluorescein formed a stable complex with ClpA and N-degron substrates in the presence of Cu^{2+} (Fig. 3.2C). The calculated distance for half-maximal quenching, R_0, for the Cu^{2+}-fluorescein pair is ~16 Å, and the modeled distance between fluorescein and Cu^{2+} in the native conformation of $ClpS^{tmFRET}$ (based on PDB 3O1F) is ~13 Å. Thus, we expected robust quenching of unperturbed $ClpS^{tmFRET}$ and decreased quenching upon β1-strand extraction.

First, we took emission spectra of fluorescein at increasing concentrations of Cu^{2+} to monitor the Cu^{2+}-dependent fluorescence quenching of $ClpS^{tmFRET}$. As expected, we

observed that fluorescence decreased as the concentration of Cu^{2+} increased (Fig. 3.2D). We observed a Cu^{2+} concentration-dependent quenching similar to that reported by Taraska *et al.* suggesting that observed quenching is due to Cu^{2+} binding to the His-X3-His motif.

Figure 3.2E, shows fluorescence emission spectra of $ClpS^{tmFRET}$ in complex with N-degron substrate and ClpA, in the presence of either ATPγS (blue trace) or ATP (green trace). Notably, addition of ATPγS resulted in less quenching than in the absence of nucleotide and ClpA and at a lower Cu^{2+} concentration (red trace), suggesting that $ClpS^{tmFRET}$ is restructured in this condition. Indeed, ATPγS supports engagement of the ClpS NTE deep within the ClpA pore based on previous FRET experiments probing the engagement of the ClpS NTE by the ClpA translocation pore (17). Addition of ATP, which is hydrolyzed much more quickly than ATPγS and supports higher levels of machine function, resulted in a modest but highly reproducible decrease in quenching of $ClpS^{tmFRET}$ (green trace). Importantly, addition of nucleotide had no effect on $ClpS^{tmFRET}$ fluorescence in the absence of Cu^{2+} (Figure 3.2F). Our results are consistent with ClpA- and nucleotide-dependent remodeling of the $ClpS^{core}$ structure.

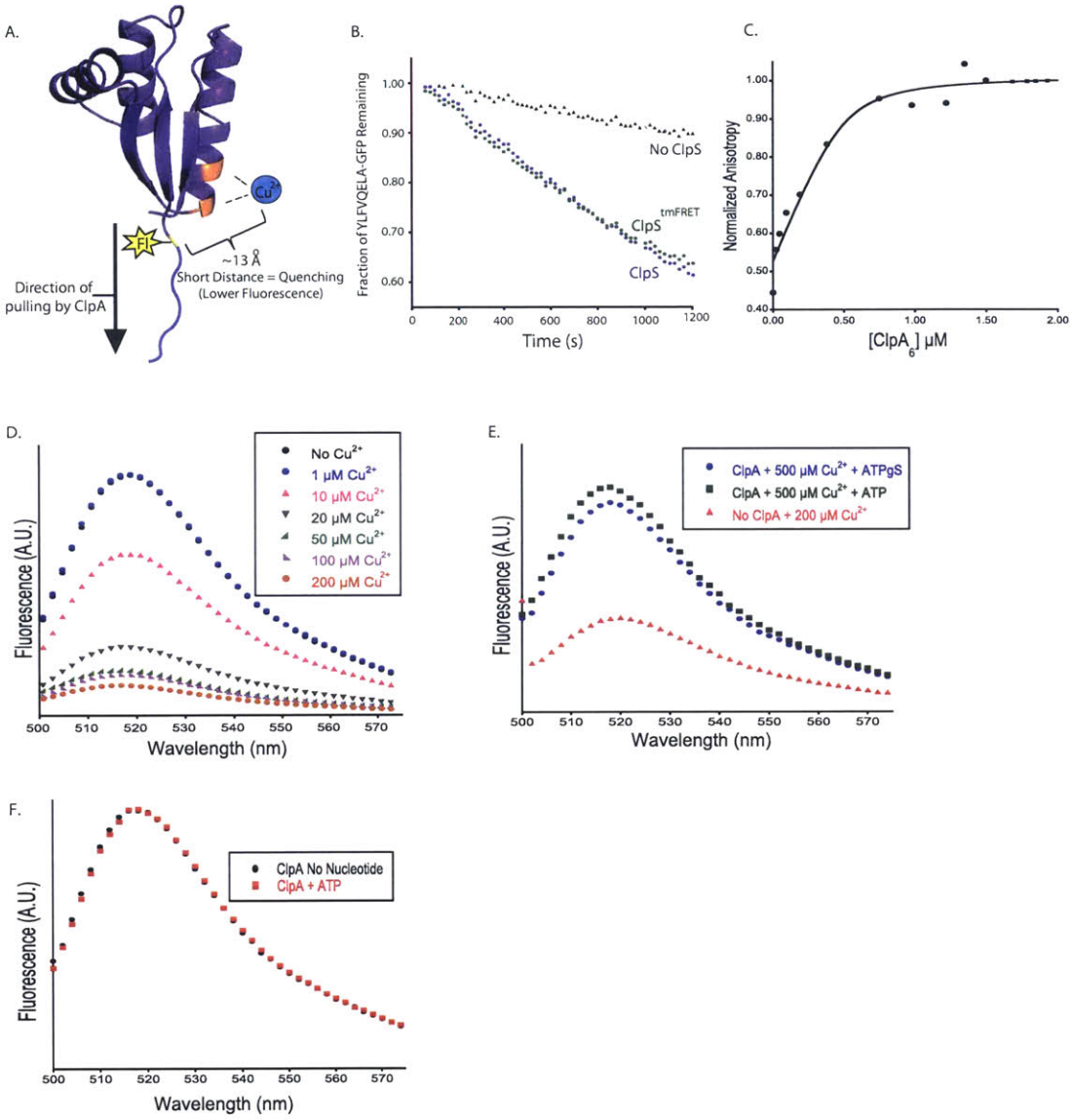


Figure. 3.2. The β1-strand of ClpS is remodeled during substrate delivery. (A) Cartoon of $ClpS^{tmFRET}$. Positions 78 and 81 of ClpS (orange) were mutated to histidine for chelation of Cu^{2+} (blue circle). Fluorescein is shown at position 26 of the NTE (Fl). (B) Delivery and degradation of the N-degron substrate YLFVQELA-GFP to ClpAP in the absence of ClpS, in the presence of $ClpS^{tmFRET}$ or in the presence of WT ClpS. Degradation was monitored by the decrease in substrate fluorescence. (C) Binding of $ClpS^{tmFRET}$ (500 nM) to $ClpA_6$ in the presence of ATPγS (2 mM), N-degron peptide (1 μM) and, $CuSO_4$ (500 μM), measured by fluorescence anisotropy (K_D < 200 nM; because K_D < ClpS concentration K_D is not well determined). (D) Quenching of $ClpS^{tmFRET}$ depends on the concentration of Cu^{2+}. Emission spectra of fluorescein on 500 nM $ClpS^{tmFRET}$ upon excitation at 495 nm at increasing concentrations of $CuSO_4$. (E) Emission spectrum of 500 nM $ClpS^{tmFRET}$ upon excitation at 495 nm in the presence of (blue) $ClpA_6$ (1 μM), N-degron peptide (1 μM), ATPγS (2mM), $CuSO_4$ (500 μM), and NTA (4 mM); (green) $ClpA_6$ (1 μM), N-degron peptide (1 μM), ATP (2mM), $CuSO_4$ (500 μM), and NTA (4 mM); (red) $CuSO_4$ (200μM). (F) Emission spectrum of 500 nM $ClpS^{tmFRET}$ upon excitation at 495 nm in the presence of (black) $ClpA_6$ (1 μM), N-degron peptide (1 μM), ATP (2mM), and NTA (4 mM); (gray) $ClpA_6$ (1 μM), N-degron peptide (1 μM), and NTA (4 mM).

Restricting movement of the β1-strand inhibits N-degron substrate delivery

As an orthogonal test of structural rearrangements within the $ClpS^{core}$, we sought to restrict the mobility of the β1-strand by introducing a disulfide crosslink between the β1-strand and the adjacent α-helix. We searched the crystal structure of ClpS and found one pair of cysteine substitutions with good geometry (Y28C/A81C). We cloned and purified this ClpS variant, henceforth termed locked-ClpS, under denaturing conditions and refolded either under oxidizing (by addition of copper phenanthroline) or reducing conditions (by addition of DTT). Locked-ClpS that had been refolded under reducing conditions displayed wild-type-like delivery of the N-degron substrate YLFVQELA-GFP (Fig 3.3B). By contrast, locked-ClpS refolded under oxidizing conditions displayed a significant defect in delivery of the N-degron substrate, as assayed by degradation (Fig 3.3B). Importantly, the oxidized locked-ClpS formed a stable complex with ClpA, with wild-type-like affinity, suggesting that the defect in N-degron substrate degradation is in the delivery step rather than in the formation of the delivery complex (Fig. 3.3C). The observed ~20% activity of oxidized locked-ClpS relative to wild type could correspond to non-oxidized contaminant or the less efficient intrinsic activity of the disulfide-crosslinked variant. Future quantification of crosslinking efficiency will discriminate between these possibilities.

Taken together, the tmFRET and locked-ClpS experiments suggest that conformational changes within the $ClpS^{core}$ occur during the ClpA translocation of the ClpS NTE for delivery of N-degron substrates and that these conformational changes are important for delivery.

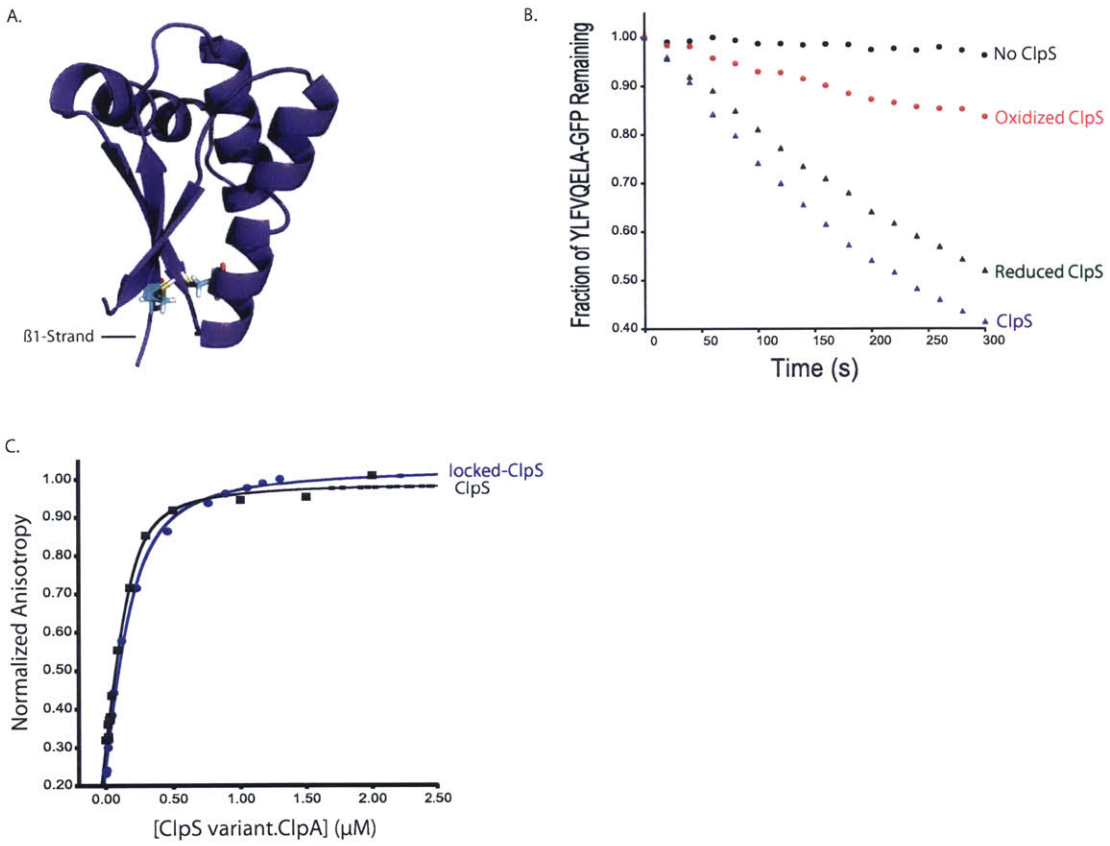


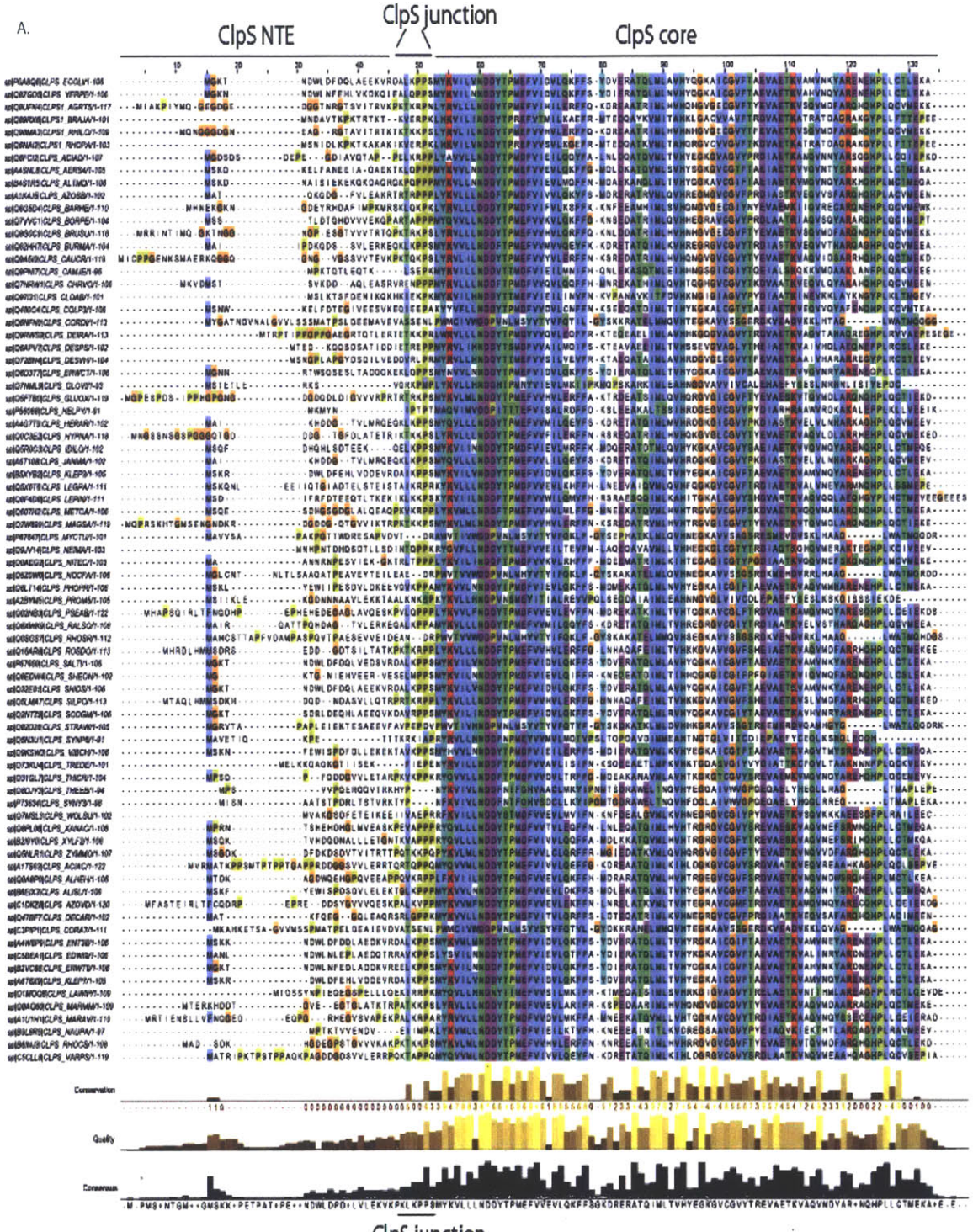
Figure 3.3. Crosslinking the β1-strand inhibits delivery of N-degron substrates. (A) Cartoon of locked ClpS. Cysteine mutations are depicted in cyan. (B) Delivery and degradation of the N-degron substrate YLFVQELA-GFP to ClpAP in the absence of ClpS, in the presence of oxidized locked-ClpS, in the presence of reduced locked-ClpS, or in the presence of WT ClpS. Degradadtion was monitored by the decrease in substrate fluorescence. (C) Formation of a stable complex by oxidized locked-ClpS (K_{app} = 70 ± 12 nM) and ClpS (K_{app} = 58 ± 7 nM assayed by anisotropy using a fluorescent N-degron peptide.

The ClpS junction sequence contributes to degradation resistance

Although the ClpS NTE enters the ClpA pore during substrate delivery (17) and the data presented above demonstrate a ClpA- and nucleotide-dependent conformational change in the $ClpS^{core}$, ClpS is resistant to degradation by the ClpAP protease (16, 20). What molecular features promote ClpS resistance to degradation, and is degradation-resistance important for substrate delivery? To answer these questions, we performed site-directed mutagenesis to produce degradable ClpS variants. Given that the rate at which AAA+ proteases unfold/degrade different substrates correlate with the local mechanical stability of the structural element(s) most directly adjacent to the peptide sequence being pulled (21, 22) we focused on the region where ClpA should initially encounter ClpS's folded structure: the junction between the NTE and $ClpS^{core}$ (Fig.3.1A). Additionally, the junction sequence is substantially conserved among ClpS orthologs, in contrast to the very weak conservation of most of the NTE, suggesting that it may play an additional functional role in adaptor mechanism (Fig. 3.4A).

We introduced alanine substitutions at P24 and P25 to generate $ClpS^{PP/AA}$. When added to a reaction containing ClpAP, and ATP, $ClpS^{PP/AA}$ was degraded (Fig. 3.4B, top panel). We also introduced P24N and P25N substitutions to generate $ClpS^{PP/NN}$. Similarly to wild type, $ClpS^{PP/NN}$ resisted degradation by ClpAP (Fig. 3.4C). Taken together, these results suggest that the specific sequence of the ClpS NTE-core junction plays an important role in determining susceptibility to degradation. Interestingly, addition of N-degron peptide substrate significantly inhibited $ClpS^{PP/AA}$ degradation (Fig. 3.4B, bottom panel), suggesting that N-degron substrate binding stabilizes the $ClpS^{core}$ structure. Furthermore, $ClpS^{PP/AA}$ was able to degrade an N-

degron substrate, albeit slower than wild type ClpS, suggesting that degradation-resistance is not essential for adaptor function (Figure 3.4D).


A.
ClpS NTE
ClpS junction
ClpS core
Conservation
Quality
Consensus
ClpS junction

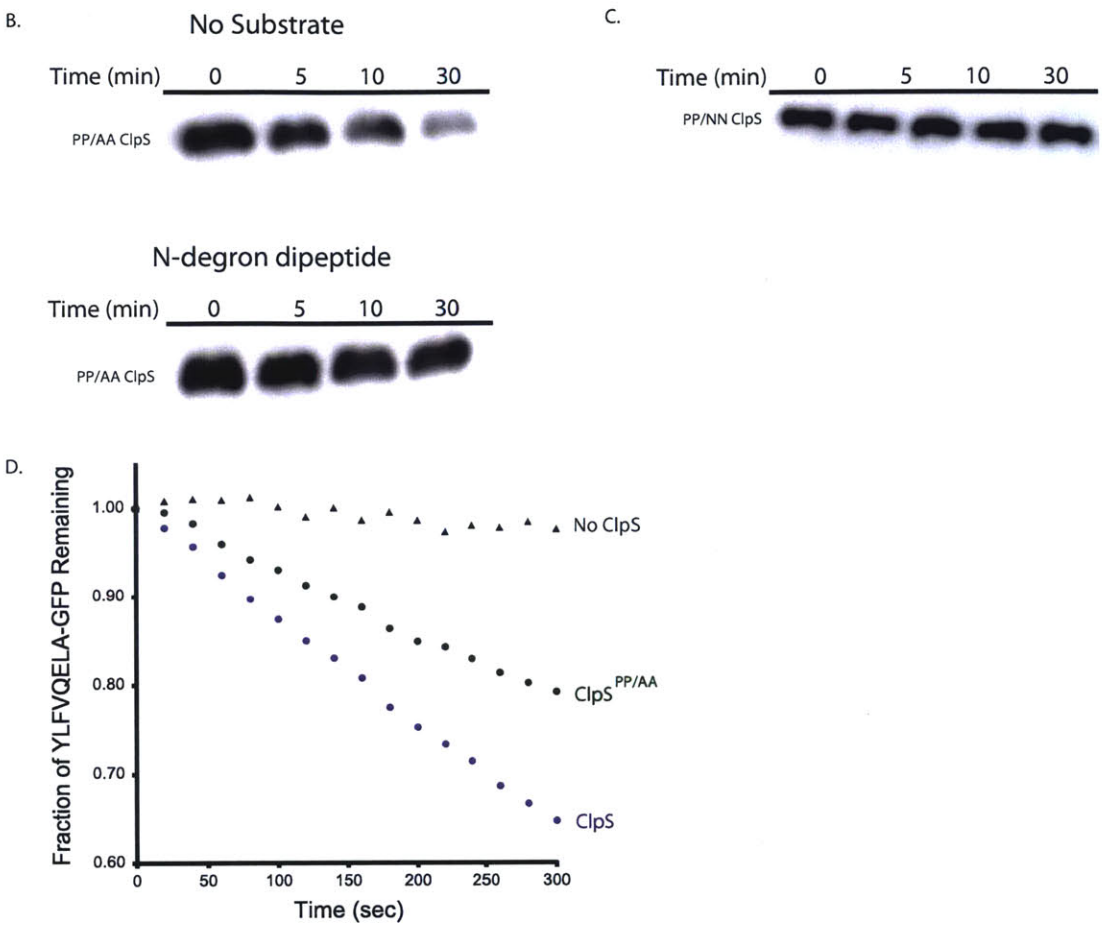


Figure. 3.4. Mutations in the ClpS junction render the adaptor susceptible to degradation by ClpAP. (A) Sequence alignment of ClpS homologs. Sequences were obtained from the Universal Protein Resource (UniProt 2010) and aligned with ClustalW. Taken from Román-Hernández *et. al.* 2011. (B) Western Blot analysis of PP/AA ClpS degradation in the absence (top) or presence (bottom) of N-degron peptide. (C) Western Blot analysis of PP/NN ClpS degradation in the absence of N-degron peptide. (D) Delivery and degradation of the N-degron substrate YLFVQELA-GFP to ClpAP in the absence of ClpS, in the presence of $ClpS^{PP/AA}$, or in the presence of WT ClpS. Degradadtion was monitored by the decrease in substrate fluorescence.

One possible explanation for the susceptibility of ClpS$^{PP/AA}$ to degradation is a decrease in global stability of the ClpS^{core} upon introduction of the alanine substitutions. Preliminary equilibrium chemical denaturation experiments show no significant difference between the curves for wild type ClpS and ClpS$^{PP/AA}$ suggesting that, upon completion of the experiments, we would obtain similar free energies of unfolding for wild-type ClpS and ClpS$^{PP/AA}$. This, in turn, suggests that the alanine substitutions have a negligible effect on global ClpS stability (Fig. 3.5A).

ClpS orthologs frequently contain a one to three residue long proline motif at the junction (Fig. 3.4A) (16). Measurements of peptide translocation by a AAA+ ring hexamer suggest that proline-rich sequences are translocated relatively slowly (23). We tested the contribution of a potentially "difficult" proline stretch at the junction by inserting one to four alanines between the junction sequence and the ClpS^{core} (Fig.3.1A). We observed that insertion of one alanine between the junction sequence and the ClpS^{core} preserved ClpS degradation resistance (Fig. 3.5B). However, increasing the number of inserted alanines from 2 to 4 resulted in increased ClpS degradation by ClpAP. Like ClpS$^{PP/AA}$, degradation of the 2 to 4-ala-add-ClpS variants was inhibited by the addition of N-degron substrate, (Fig.3.5B). Together, our results from perturbing the ClpS junction sequence indicate that the specific amino acid sequence in the unstructured junction sequence, where the unfoldase is bound when it encounters a folded obstacle, plays a critical role in determining susceptibility to unfolding and degradation.

Local ClpS stability governs degradation susceptibility

Our results suggest that a "difficult" to translocate junction sequence contributes to ClpS resistance to degradation. We hypothesized that degradation resistance further depends on the stability of the local structure directly C-terminal of the junction, where ClpA must initiate its unfolding. To test the contribution of local ClpS stability, we engineered a ClpS variant where unfolding by ClpAP initates from the protein's C terminus rather than the N terminus by appending the ssrA-degradation tag to the C-terminus of ClpS (ClpS-ssrA, Fig. 3.5C). Because the K_M of ssrA-tagged substrates is lower (~1 μM) than that of NTE-tagged substrates (~15 μM) (16, 24, 25), we reasoned that ClpA would preferentially engage the ssrA-tag and subsequently be challenged by local structure differing from that normally encountered during N-terminal engagement. Based on the crystal structure of ClpS (PDB 3O1F; (16)) the C-terminus of ClpS possess an unstructured region. Therefore, addition of an ssrA tag to the non-structured C-terminus is unlikely to affect the global stability of the adaptor, and we will test this assumption in future chemical denaturation experiments. Notably, ClpS-ssrA was readily degraded by ClpAP, both in the presence and absence of N-degron peptide (Fig. 3.5C). This result strongly supports the mechanism that suggests that local stability, and not global stability, dictates ClpS degradation resistance by ClpAP. This type of mechanism for resistance to degradation by AAA+ proteases has been previously proposed by Matouschek *et al.* 2003 (26).

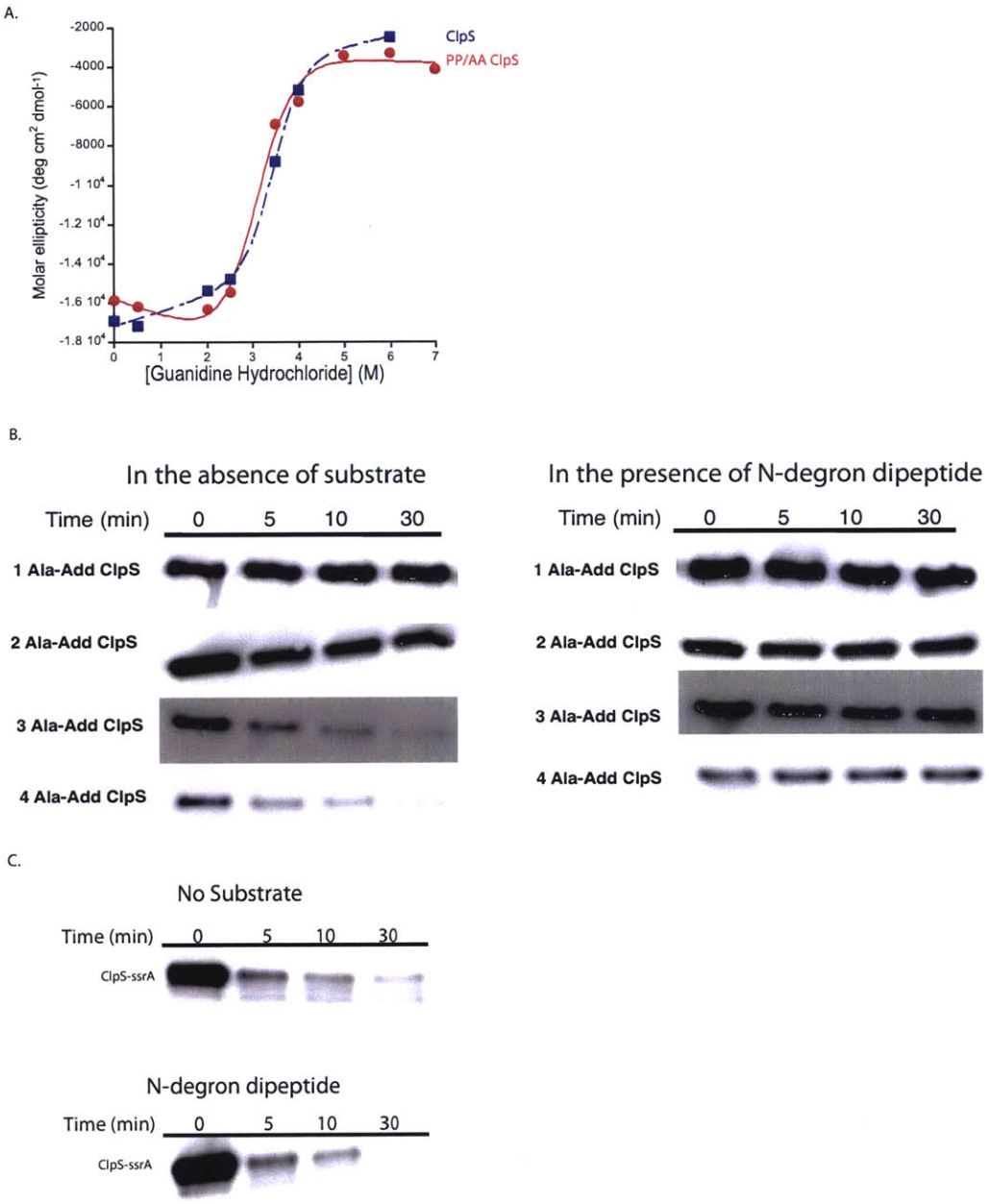


Figure 3.5. Preliminary data suggest that global stability of ClpS is not affected by mutations in the junction sequence. (A) Chemical denaturation of WT ClpS and the ClpS$^{PP/AA}$ variant. Denaturation was followed by circular dichroism at 220 nm. (B) Western Blot analysis of the 1-4 Ala-Add ClpS variants in the absence (left) and presence (right) of N-degron substrate. (C) Western Blot analysis of ClpS-ssrA in the absence (top) or presence (bottom) of N-degron peptide.

Discussion

The work presented in this chapter addresses important mechanistic details of ClpS delivery of N-end rule substrates to the ClpAP protease. Our data support a model, shown in Figure 3B, in which ClpA-dependent engagement of the ClpS NTE remodels the $ClpS^{core}$, resulting in partial or complete extraction of the ClpS β1-strand. Such remodeling may facilitate substrate delivery to ClpA by positioning the substrate near the ClpA pore, weakening the ClpS-substrate interaction, or both. Indeed, bound N-degron substrates stabilized some ClpS variants against degradation, suggesting that substrate binding and structural stability are thermodynamically linked. After substrate delivery, ClpS resists global unfolding and might be released by the ClpA pore, allowing translocation and degradation of the N-degron substrate and refolding/recycling of ClpS.

The ClpS/ClpA-mediated active delivery model we propose shares common features with the mycobacterial pupylation pathway. Vast arrays of substrates are covalently modified by the Pup protein, which also interacts with the N-terminal domain of the PAN AAA+ ring. Pup is recognized by the PAN pore and translocated, along with the substrate, into the proteolytic chamber of the proteasome for degradation (27). Thus, Pup acts as both an adaptor by tethering the substrate to the ATPase ring and as a degradation signal for proteins that may lack a degradation tag that can be recognized directly by PAN (27). Similarly, the ClpS adaptor binds simultaneously to N-degron substrates and to the N-domain of ClpA, and the unstructured ClpS NTE is recognized and translocated by the unfoldase (15-17, 28, 29). In contrast to Pup, ClpS is remodeled and recycled after substrate transfer (16, 20). ClpS recycling may be important if N-

degron degradation functions under stress conditions in which synthesis of new ClpS molecules is poor.

Partial substrate unfolding by AAA+ enzymes serves important biological roles. For example, the bacterial chaperone ClpB lacks a robust unfoldase activity but is critical for protein homeostasis (30). Experiments with protein fusions of misfolded and native domains show that even partial unfolding of a misfoded protein by ClpB can be sufficient to solubilize aggregates (30). Recently, mitochondrial AAA+ ClpX has been proposed to partially unfold ALA synthase to catalyze the insertion of its essential cofactor, PLP. This partial unfolding protein remodeling reaction is essential for robust heme biosynthesis, thereby allowing cofactor binding and enzyme activation (31). We propose a similar mechanism in which partial unfolding of the ClpS adaptor promotes N-degron substrate transfer. Our tmFRET and crosslinking experiments suggest that ClpA-dependent partial unfolding in the $ClpS^{core}$ occurs during substrate delivery and that this remodeling is important for the delivery of N-degron substrates. Notably, our tmFRET experiments suggest that ClpS binding to ATPγS-loaded ClpA results in considerable structural rearrangements in ClpS, and further remodeling occurs upon introduction of ATP. Because ATPγS supports little to no ClpA machine function (but supports substrate-adaptor binding and at least partial engagement), our results raise the possibility that binding to ClpA primes ClpS for further ATP-dependent remodeling to promote substrate delivery.

Although AAA+ proteases can unfold substrates with diverse structures and stabilities, some substrates are only degraded partially or not at all (21, 22, 32-37). Inhibitory or

challenging sequences, highly stable domains, or stable-unfolding intermediates all could contribute to resisting degradation. Our mutational and preliminary chemical denaturation experiments suggest that ClpA simultaneously encounters a stable secondary structure and a translocation-challenging sequence at the ClpS junction, thereby allowing ClpS to escape unfolding and degradation. Future hydrogen-deuterium exchange experiments will more directly test local stability near the ClpS junction.

Materials and Methods

Proteins and Peptides

Mutants were generated by the QuickChange method (Stratagene) or PCR. ClpS, ClpS mutants, and substrates were purified as described (17). Briefly, ClpS, ClpS mutants, and substrates were initially fused to the C terminus of H_6-Sumo in pET23b (Novagen). Following expression, fusion proteins were purified by Ni-NTA chromatography (QIAGEN) and cleaved with Ulp1 protease. The cleaved H_6-Sumo fragment was removed by passage through Ni-NTA, and the protein of interest was purified by gel filtration on Superdex 75 (GE Healthsciences) and/or ion-exchange chromatography on MonoQ (GE Healthsciences). ClpS variants were concentrated and stored in 20 mM HEPES (pH 7.5), 150 mM KCl, 1 mM DTT, and 10 % glycerol. ClpA and ClpP were purified as described (16). As ClpS variants were purified using the Sumo-fusion and Ulp1 cleavage method, the N-terminal methionine of ClpS should be present. Previous publications have either used Sumo-cleavage or native expression for ClpS variants (16, 19). The N-degron (LLFVQRDSKEC) was synthesized by standard FMOC techniques using an Apex 396 solid-phase instrument.

Fluorescent Labeling

Peptides were labeled with fluorescein maleimide as described (29). Labeled ClpS variants were labeled with fluorescein maleimide as described (16). Briefly, ClpS variants (50 μM) containing a single cysteine were incubated with 50 mM DTT in 100 mM TrisCl (pH 8) for 1.5 h at 4 °C, buffer exchanged into 100 mM Na_2PO_4 (pH 8), and 1 mM EDTA. The variants were then singly labeled by addition of 0.3 mg/mL of fluorescein maleimide (Thermo Scientific) for 2 h at room temperature in the dark.

Excess reagent was removed by size-exclusion chromatography, and the modified protein was stored in 10 mM HEPES (pH 7.5), 200 mM KCl, and 1 mM DTT.

Fluorescent assays

TmFRET experiments were measured using a PTI QM-20000-4SE spectrofluorimeter (excitation 495 nm; emission: 500 - 575 nm). Unless noted all assays contained 500 μM Cu^{2+} and 4 mM NTA. NTA was included because it binds Cu^{2+} and reduces its affinity for free and ClpA bound nucleotides but does not prevent binding to the His-X_3-His motif.

Degradation Assays and Western Blots

ClpAPS degradation assays were performed as described (38). Briefly, $ClpA_6$ (100 nM), $ClpP_{14}$ (200 nM), and ClpS variants (1 μM) were preincubated in reaction buffer (50 mM HEPES [pH 7.5], 300 mM NaCl, 20 mM $MgCl_2$, 0.5 mM DTT, and 10 % glycerol) with YLFVQELA-GFP (1 μM) for 3 min at 30 °C before adding ATP regeneration mix (4 mM ATP, 50 mg/mL creatine kinase, 5 mM creatine phosphate) or ATPγS (2 mM) to initiate assay. GFP degradation was assayed by loss of fluorescence. Reported kinetic parameters were averages ($n \geq 3$) ± 1 SD. Degradation of ClpS variants was monitored by SDS-PAGE and western blotting as described (19). Briefly, samples were separated by SDS-PAGE followed by an anti-ClpS western blot.

Binding Assays

Binding assays, monitored by fluorescence anisotropy, were performed using a Photon Technology International Fluorimeter. Data were fitted using a quadratic equation for

tight binding. Reported K_{app} values are averages (n ≥ 3) with errors calculated as SQRT($[K-K_{avg}]^2$/n).

Oxidations and reductions

Residue positions for engineered disulfides were identified using the program Disulfide by Design (39) by searching the ClpS structure (PDB 3O1F) (16). Denaturing purifications were performed as described (19). Refolding of the ClpS variants was performed with either 10 mM Copper Phenanthroline for oxidative conditions or 10 mM DTT for reducing conditions. ClpS variants were then concentrated and stored in 20 mM HEPES (pH 7.5), 150 mM KCl, 10 % glycerol, and 1 mM DTT for the reduced variant.

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CHAPTER FOUR

Perspectives and Future Directions

Introduction

Work presented here and elsewhere demonstrates that the ClpS adaptor does not simply tether substrates to the ClpAP protease, but collaborates with ClpA to employ a novel active delivery mechanism (1-3). Structural and biochemical data have revealed the interactions between ClpS and both N-degron substrates and ClpA (2, 4, 5). Three components of the ClpS structure interact with ClpA to form an active substrate delivery complex. *i)* The ClpS body or core, which contains the N-degron binding pocket, binds to the N-domain of ClpA (4, 6, 7), but this binding is not sufficient for delivery of N-degron substrates (1, 2). *ii)* The long and flexible N-terminal extension (NTE) is engaged by the ClpA translocation pore during substrate delivery (3). Furthermore, we show that, for successful delivery, substrates must be bound to a ClpS molecule whose NTE is actively engaged (3). (2, 7). *iii)* The junction between the N-terminal extension and the $ClpS^{core}$ is moderately conserved among ClpS orthologs (2). Previous data revealed that residues of the junction serve as tethering signals to bring the adaptor-substrate complex near the ClpA pore (2), and we provide evidence that this region contributes to the resistance of ClpS to degradation by ClpAP.

Our data show engagement-dependent structural rearrangements of the $ClpS^{core}$ that are important for substrate delivery. Additonally, our data suggest that local stability, likely proximal to the β1-strand, and not global stability plays an important role in the ability of ClpS to excape degradation by ClpAP. We propose that these structural rearrangements may facilitate substrate transfer by a combination of placing the N-degron binding pocket closer to the ClpA processing pore and by weakening interactions between the binding pocket and the N-degron substrate. After delivery,

ClpS resists degradation by ClpAP and the adaptor is released.

It will be important to probe the complexes involved in the ClpAS delivery mechanism to obtain a higher resolution view of the specific protein conformational changes that accompany, and are responsible for, efficient N-degron substrate delivery. The first stage important to focus on is remodeling that occurs in the $ClpS^{core}$ upon engagement by ClpA. Hydrogen-deuterium (H/D) exchange experiments can measure dynamics of solvent accessibility with residue resolution. H/D exchange measurements of ClpS will provide a powerful tool to probe for nucleotide- and ClpA-dependent conformational changes in the $ClpS^{core}$.

Physiological Role of the N-degron pathway in E. coli

In eukaryotes, the N-end rule pathway controls myriad processes including peptide import, chromosome stability, cardiovascular development and nitric oxide detection (8). However, the physiological roles of the N-degron pathway in *E. coli* are poorly understood and remain an important area of future research.

In bacteria, almost all newly synthesized proteins contain an N-terminal N-formylmethionine, which is a stabilizing residue in the N-degron pathway. Therefore, the best current evidence strongly indicates that all N-degron substrates must be generated post-translationally. For example, multiple leucine residues, which are

destabilizing N-degrons, are covalently attached to the initiating methionine of *E.* coli PATase, a ClpS substrate isolated by a ClpS affinity column (9), by L/F aminoacyl transferase (Aat protein; ((9)). PATase catalyses the aminotransferase reaction from putrescine to 2-oxoglutarate to generate L-glutamate and 4-aminobutanal as part of the arginine biosynthesis pathway (10). Notably, typical Aat substrates bear an N-terminal lysine or arginine, to which a single leucine or phenylalanine is attached. Why PATase is an exception is unknown.

Another pathway for the generation of N-degron substrates involves endoproteolytic cleavage of substrates. A recent study by Humbard *et al.* using a ClpS affinity column coupled with mass spectrometry and N-terminal sequencing found dozens of ClpS-interacting proteins that appeared to be the product of cleavage by unknown proteases or peptidases (11). Furthermore, previous studies found the DNA protection during starvation (Dps) protein to be an N-degron substrate generated by removal of five N-terminal residues to reveal a leucine residue (9, 11). The responsible Dps endopeptidase is unkown, nor has it been clearly demonstrated that this reaction is physiologically relevant. The currently known spectrum of substrates suggests a possible physiological role for the N-degron pathway in protein quality control as well as cell division, translation, transcription, and DNA replication given the array of protein identified in studies. For example, some of the proteins identified in the studies are the translation initiaition factor 2, the lactose inhibitor LacI, the transcriptional repressor and membrane-associated enzyme PutA, and the DNA gyrase subunit GyrB, among others (9, 11).

Notably, the large excess of internal peptides limits the identification of N-terminal sequences produced by endoproteolytic cleavage by mass spectrometry. Kim *et al.* have presented a method to enrich N-terminal peptides from complex mixtures that involves several blocking and chemical derivatization steps that selectively convert protein N-termini into reactive thiol groups amenable to capture by thiol affinity resins. These authors demostrated near-quantitative yields of thiol derivitization and initial application of their strategy to a whole cell lysate of *Aspergillus niger* followed by LC-MS/MS revealed high N-terminal peptide enrichment efficiency (12). This type of regorious and near quantitative proteomic studies are still needed in *E.coli*, and other bacteria, in identifying and characterizing ClpS N-degron substrates and their biological roles.

Do interactions with the N-domain of ClpA stabilize ClpS?

Our studies show that binding of an N-degron substrate to the ClpS binding pocket stabilizes ClpS and prevents degradation of some ClpS variants that are readily degraded in the absence of N-degron substrates. ClpS also binds to the N-terminal domain of ClpA to form a stable complex in the presence of ATPγS (2, 7). Whether this interaction also contributes to ClpS degradation resistance, other than just recruiting ClpS, is unknown. Using ClpA lacking the N-domain ($ClpA^{\Delta N}$), which would potentially degrade substrates delivered by ClpS (albeit, if possible, it would be much slower), we will measure degradation of ClpS variants that are efficiently degraded by wild-type ClpA. Furthermore, comparisons of ClpS H/D exchange measurements conducted

without ClpA, with wild-type ClpA, with $ClpA^{\Delta N}$, and with the isolated ClpA N-domain will reveal contributions of the N-domain to the structural dynamics of ClpS.

Is ClpS always a part of the ClpAP machine?

ClpS and ClpA form a nucleotide-dependent complex, but it is unknown whether the complex persists for multiple rounds of substrate delivery or whether ClpS is released after one round of substrate delivery. Future ClpS competition experiments measuring fluorescence anisotropy of a labeled ClpS variant will address this question. We will form a high anisotropy ClpS-substrate-ClpA complex in the presence of ATPγS and initiate substrate delivery by adding ATP, N-degron substrate, and an excess of unlabeled ClpS. To ensure a stoichiometry of one ClpS per ClpA hexamer in the pre-formed complex, ClpA will be present in excess of ClpS. If ClpS persists in the complex for multiple rounds of delivery, we expect complex lifetime (τ_c), as measured by decay of the anisotropy signal, to be significantly longer than the N-degron substrate degradation time (τ_{deg}), defined as the inverse of the steady-state degradation rate. Conversely, a τ_c similar to τ_{deg} would suggest that ClpS is released from ClpA upon substrate delivery, and a τ_c less than τ_{deg} would suggest that ClpS usually releases from ClpA before substrate is delivered.

Partial substrate unfolding by AAA+ enzymes

Our model for N-degron substrate delivery by the ClpS adaptor posits that partial unfolding of the ClpS adaptor promotes delivery of N-degron substrates. Models of

partial protein unfolding have been proposed for enzymes involved in other biological processes. For example, partial unfolding of a misfolded protein by the bacterial AAA+ ClpB chaperone can solubilize aggregates (13). Additionally, a recent study suggests that partial unfolding of ALA synthase, an enzyme essential for heme biosynthesis, by mitochondrial AAA+ ClpX plays an important role in cofactor binding and enzyme activation (14). Thus, mechanisms involving partial protein unfolding may not be restricted to protein degradation and the protein quality control network, but also may fulfill other important roles.

The ubiquitous N-degron degradation pathway contributes to the network of regulated proteolysis, an essential process in all cells that relies on a high degree of substrate specificity. Adaptor proteins are important regulators of substrate specificity. Thus, understanding the breadth of molecular mechanisms used by adaptors is essential to fully understand both recognition and function of AAA+ proteases and other AAA+ machines.

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