Mechanisms of ClpS Reprogramming of the AAA+ Protease ClpAP

by

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Abstract

The proteome of a cell needs to be constantly modified for protein quality control and to respond to environmental and developmental changes. Energy-dependent proteases are key to ensuring the proper composition of the proteome in all kingdoms of life. Because of the irreversible nature of protein destruction, substrates need to be chosen with high accuracy. This selection is achieved in part by the architecture of these proteases, as they have their proteolytic sites sequestered in a chamber inaccessible to the cytosol. As a consequence, folded proteins need to be denatured and translocated into this chamber for degradation to occur. These peptidase chambers can partner with AAA+ (ATPases Associated with various cellular Activities) unfoldases, protein machines that control substrate access to this chamber. An additional level of control is provided by adaptor proteins, which modulate substrate selection by the AAA+ enzymes. I characterized the mechanisms by which the *E. coli* ClpS adaptor protein regulates substrate degradation by its cognate AAA+ protease, ClpAP. I focused my studies on how ClpS inhibits degradation of the ssrA class of substrates while efficiently enhancing degradation of the N-degron substrate class.

I elucidated two strategies that ClpS employs to reprogram ClpA's substrate preference. ClpS weakens, but does not prevent ssrA substrate binding. ClpA, ClpS and ssrA substrate form a ternary complex that is part of the degradation pathway. ClpS also alters the catalytic steps ClpAP employs during ssrA substrate degradation. I report evidence demonstrating that suppression of ClpAP's ATPase rate by ClpS is part of the mechanism by which ClpS inhibits ClpAP's mechanical activities. Finally, I provide completing data establishing that the substrate translocation step directly targeted for ClpS inhibition.

In addition to probing ClpS mechanism, I also dissected the structural features of ClpS required for inhibition. I demonstrate that the unstructured N-terminal extension of ClpS is both necessary and sufficient to inhibit substrate degradation. Engagement of this NTE by the ClpA translocation pore is critical for ClpS-mediated inhibition. I propose a model where ClpS behaves as a substrate mimic to modulate both substrate recognition and processing. Other adaptors that regulate catalysis and substrate choice of their partner AAA+ enzymes may employ features of this model.

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

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Chapter 1

Introduction

1.1 AAA+ Molecular Machines

The AAA+ superfamily of enzymes use a conserved enzyme core to couple the chemical energy from ATP binding and hydrolysis to perform mechanical work across all domains of life. This superfamily is defined by the presence of an ATP-binding module (AAA domain), which consists of a small domain and a large domain (Figure 1-1). Conserved sequence motifs in AAA domains (Walker A, Walker B, Sensor 1, Sensor 2 and Arginine fingers) (Erzberger and Berger, 2006; Hanson and Whiteheart, 2005) are critical for nucleotide binding and hydrolysis. AAA+ enzymes most commonly function as hexamers, with ATP-binding sites nested between the large and small domains of an ATP binding module, at the interface between subunits (Figure 1-1). ATP binding, hydrolysis, and release by AAA+ enzymes cause conformational changes in the enzymes that are transmitted to their protein or DNA substrates. By elaboration of this conserved core with family-specific auxiliary domains, family members have been specialized to direct their activity toward a diverse range of cellular processes, including cell division, DNA repair, microtubule dynamics, protein quality control, DNA replication, and stress-response pathways (Snider et al., 2008). For example, FtsH unfolds and degrades misfolded membrane proteins in chloroplasts, mitochondria and bacteria to control the quality and activity of the membrane proteome (Ito and Akiyama, 2005). NSF disassembles SNARE complexes after membrane fusion is complete, thereby recycling SNARE proteins for the repeated rounds of fusion that are necessary to support vesicular transport (Shah et al., 2015; Whiteheart and Matveeva, 2004). A AAA+ enzyme (the clamp loader) also loads the ring-shaped clamp required for processive DNA replication around the DNA (Davey et al., 2002).

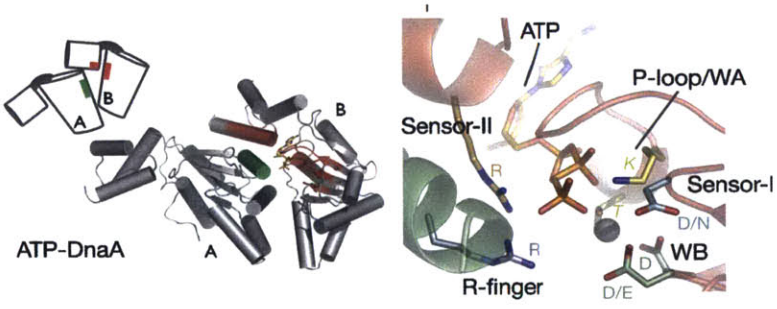

Figure 1-1 Structure of a AAA module. Organization of a single DnaA ATP-binding pocket shows the positions of characteristic motifs, highlighting the interaction between neighboring AAA+ modules. (*left*) Schematic showing the contributions of each module to the ATP-binding sites, highlighted in green and red. (*right*) Active site of ATP-DnaA showing the position of nucleotide-interacting motifs and ATP. Coloring reflects subunit contributions. Figure adapted from Erzberger and Berger, 2006.

ATP-driven conformational changes in AAA+ enzymes can drive substrate remodeling or they can also drive movement. ATP-

coupled conformational change in the AAA+ domains of cytoplasmic dynein, for example, induces movement of its microtubule-binding domains along the microtuble, propelling dynein and its cargo toward the minus ends of microtubules (Bhabha et al., 2016). The bacterial replication initiator DnaA, in contrast, uses its ATP-driven conformational changes to induce local unwinding at DNA replication origins, allowing the replicative helicase DnaB to assemble there (Davey et al., 2002).

AAA+ enzymes can interact with partner or accessory proteins called adaptors. These proteins direct the activity of their cognate AAA+ enzymes to specific functions. For example, eukaryotic NSF binds SNAREs through an adaptor protein called α-SNAP (Barnard et al., 1997; Shah et al., 2015; Whiteheart and Matveeva, 2004). Multiple adaptor protein partners can expand the functional diversity of a single AAA+ enzyme. For example, eukaryotic Cdc48/p97 is known to be involved in a large number of cellular processes. As specified by its adaptors, Cdc48/p97 can remodel or target for proteasomal degradation a variety of proteins to regulate processes such as chromatin remodeling, DNA replication and repair, and autophagy (Jentsch and Rumpf, 2007; Meyer et al., 2012).

1.1.1 AAA+ Unfoldases

A subset of AAA+ proteins uses ATP-driven conformational changes to translocate protein substrates through the central channel of the AAA+ ring, thereby partially or completely unfolding (remodeling) these substrates. Remodeling of protein substrates can serve a variety of purposes, including the disassembly of macromolecular complexes. For example, the tetrameric MuA transposase of phage Mu catalyzes recombination steps necessary for phage DNA transposition. This process ends with a hyper-stable MuA-DNA complex (transpososome), which must be destabilized for replication to proceed. The AAA+ unfoldase ClpX remodels this complex, facilitating MuA disassembly, and releasing DNA for replication (Abdelhakim et al., 2008; Kruklitis et al., 1996; Levchenko et al., 1995; Ling et al., 2015). Interestingly, a recent study showed that yeast mitochondrial ClpX directly activates a key enzyme for heme biosynthesis (aminolevulinic acid synthase, ALAS) by catalyzing incorporation of its cofactor, pyridoxal phosphate (PLP), which is necessary for proper active site formation. Incorporation of

PLP is thought to occur by partial ClpX unfolding of ALAS, thereby making ALAS active sites more accessible to PLP (Kardon et al., 2015).

Another type of remodeling directed by AAA+ unfoldases is the disassembly of protein aggregates, which is critical for survival after heat-stress. Yeast Hsp104 and its bacterial homolog ClpB cooperate with their cognate Hsp70 co-chaperone system to solubilize and refold aggregated proteins (Doyle et al., 2007; Glover and Lindquist, 1998). Although the detailed molecular mechanism of this process remains unclear, numerous lines of evidence support a mechanism requiring Hsp104/ClpB unfolding and translocation activity to remove polypeptides from aggregates (Doyle and Wickner, 2009).

AAA+ unfoldases can also cooperate with peptidases to degrade protein substrates, a critical process in protein quality control. By controlling the intracellular levels of key regulatory proteins protein degradation also plays a crucial role in the regulation of many cellular processes (Sauer and Baker, 2011). To avoid indiscriminate protein degradation, peptidases sequester their active sites within protected chambers. AAA+ unfoldases associate with these self-compartmentalized peptidases to directly couple unfolding of target substrates with translocation into the peptidase chamber and thus degradation. The unfoldase and peptidase components are sometimes encoded in a single polypeptide chain, as in the case of the Lon and FtsH AAA+ proteases.

1.2 Importance of Proteolysis

All cells are densely packed with proteins that need to function at the right time and in the right context to ensure proper cell function. Importantly, the inventory of cellular proteins needs to undergo constant modifications in response to environmental and developmental changes and ATP-dependent proteolysis represents an extreme modification that results in targeted protein removal. For example, proteolysis plays a crucial role in promoting cell-cycle transitions by regulating levels of activators or inhibitors of cyclin-dependent kinases (CDKs). Degradation of the *S. cerevisiae* CDK inhibitor Far1 is essential for execution of the Start checkpoint during the G1 phase of the mitotic cell cycle. In yeast, the Start control point is critical for G1 phase to

proceed. After yeast cells have passed through Start, they are able to replicate their DNA, form buds and duplicate their spindle pole (Fu et al., 2003; Meyer et al., 2012). Proteolysis is also a central player in tuning the control loop of the DNA damage response in bacteria. In *E. coli*, the components of this system --the SOS regulon-- are transcriptionally repressed under normal conditions by the LexA repressor. When cells suffer DNA damage, single-stranded DNA is exposed. The RecA protein binds DNA and stimulates LexA cleavage, which allows for the synthesis of SOS mediators that direct damage repair. Furthermore, once DNA integrity is largely restored, repression resumes and many of the SOS-induced proteins are rapidly degraded (Neher, 2003).

Protein degradation is also crucial for protein quality control. Misfolded proteins and incomplete translation products need to be degraded before they become toxic. As such, organisms have evolved systems to detect and eliminate these aberrant protein forms. For example, the endoplasmic reticulum, which is responsible for the proper folding of secretory proteins in eukaryotes, employs two main strategies to overcome protein misfolding. The ER responds to the presence of misfolded proteins by activating the unfolded protein response (UPR) and the ER-associated degradation pathway (ERAD). In the ERAD, misfolded proteins are specifically recognized and translocated to the cytosol, where they are degraded by the ubiquitin-proteasome system (Bukau et al., 2006). In bacteria, the tmRNA system rescues stalled ribosomes by co-translational addition of an 11-residue sequence that targets the aberrant incomplete polypeptides for degradation by various ATP-dependent proteases (Keiler et al., 1996; Moore and Sauer, 2007).

Because of the irreversible nature of protein degradation, this process needs to be carefully regulated. In bacteria, archaea and most eukaryotic cell compartments, processive protein degradation is carried out by highly specific self-compartmentalized proteases that sequester their proteolytic sites in a protected chamber. These energy-dependent proteases are part of the AAA+ (ATPases Associated with various cellular Activities) superfamily of proteins due to the signature presence of a AAA+ unfoldase ring.

1.3 AAA+ Proteases

AAA+ proteases include ClpXP, ClpAP, ClpCP, HslUV, Lon, FtsH. The 20S peptidase, which is found in all domains of life, can cooperate with different unfoldases, namely Mpa (bacteria), PAN or Cdc48 (archaea) and the Rpt1-Rpt6 ring of the proteasome (eukaryotes) (Finley et al., 2016; Olivares et al., 2016; Sauer and Baker, 2011). Each protease contains at least one AAA unfoldase domain, a protease domain, and a family-specific domain (Figure 1-2).

Proteases can have distinctive architectural features. For example, ClpAP, ClpCP and Cdc48 contain two AAA modules per subunit, adopting a double-ring architecture in the functional hexameric state. In LonA, LonB and FtsH, the AAA and protease domains are encoded in the same polypeptide (Figure 1-2). The auxiliary domains, which are sometimes dispensable for machine function, lack sequence and structure conservation between families. In many cases they serve as docking sites for adaptors and binding-enhancing signals in some substrates.

All AAA+ proteases share a common architecture and mechanism. The AAA+ unfoldase ring associates with the compartmentalized peptidase to form a barrel-shaped complex with a central channel, or pore, that is too narrow for folded proteins to enter (Figure 1-3a). Cycles of ATP binding and hydrolysis alter rotations between large and small AAA+ domains of a subunit.

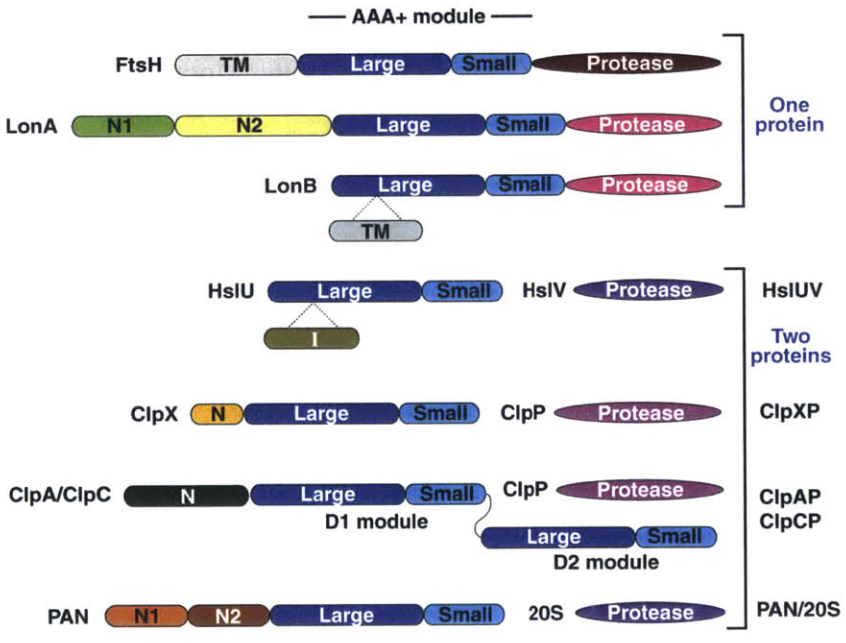

Figure 1-2 Domain structures of AAA+ proteases. AAA+ proteases can be divided into distinct families based upon the sequences of their ATPase, protease, and auxiliary domains. Each protease contains one or two conserved AAA modules, which consist of a large and small domain. In addition, each protease contains a family-specific auxiliary domain. Figure from Sauer and Baker, 2011.

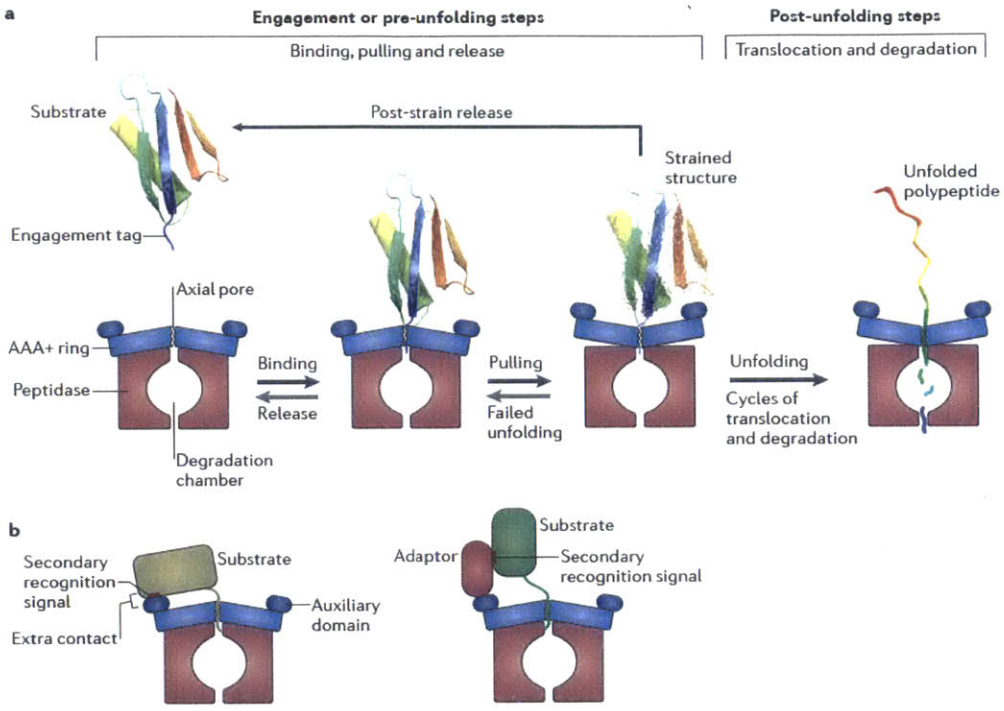

Figure 1-3 Mechanism of AAA+ proteases. **a** A recognition signal (engagement tag or degron) is initially recognized via the AAA+ unfolase. Repetitive cycles of ATP binding, hydrolysis and release drive conformational changes in the AAA+ ring that pull the substrate, which can result in failed unfolding, substrate release or substrate denaturation. After the substrate is efficiently engaged and unfolded the substrate is processively translocated into the peptidase chamber for degradation. **b** Efficient substrate recognition sometimes requires secondary recognition signals, which may directly bind to the AAA+ enzyme (enhancement tags), or through adaptor proteins.
Figure from Olivares et al., 2016)

Multiple failed unfolding attempts, and even substrate release, can occur before the enzyme commits to processively unfolding and degrading a substrate (Olivares et al., 2016; Sauer and Baker, 2011). For simplicity, we summarize the degradation cycle in 5 main steps: binding and engagement (recognition or pre-unfolding steps), unfolding, translocation and proteolysis (processing or post-recognition steps).

1.3.1 Substrate Recognition

Control of protein degradation is crucial to prevent unwanted substrate destruction, and to ensure that important substrates are degraded. Accordingly, substrate recognition is a highly regulated step. Substrate specificity by AAA+ proteases is achieved by a variety of mechanisms, starting with their own architecture. Proteolytic sites are enclosed in a chamber, and the AAA+ ring

controls access to this chamber. Furthermore, the unfoldase needs to actively pull and unfold substrate proteins in an energy-dependent process. As an additional level for controlling substrate recognition, substrates are identified by the AAA+ unfoldase via specific sequence determinants, termed degrons or degradation tags. Moreover, proteases can directly recognize their substrates or they can be assisted by enhancement sequences or adaptor proteins (discussed in section 1.4) (Figure 1-2b).

In eukaryotes, the proteasome recognizes most substrates via ubiquitin tags and an intrinsically disordered region accessible for engagement by the AAA+ ring in the regulatory particle (Prakash et al., 2004). Initial recognition occurs through a complex set of ubiquitin receptors (discussed below). Ubiquitination of substrates starts with recognition by E3-ubiquitin ligases, which catalyze the formation of an isopeptide bond between a lysine residue in the substrate and the C-terminus of ubiquitin. E3 ligases recognize their substrates based on the presence of specific ubiquitination signals, which are usually short regions of primary sequence (*e.g.* N-degrons, discussed below) (Deshaies and Joazeiro, 2009). Thus, proteosomal substrate recognition is controlled by a complex process for assembling the necessary recognition signals on a substrate protein. Notably, ubiquitin-independent proteosomal regognition is also possible (Sauer and Baker, 2011).

Bacteria have no ubiquitination system (with the exception of the ubiquitin-like Pup system in actinobacteria, discussed below). In bacteria and archaea, degrons are typically unstructured peptide motifs that are directly recognized and engaged by the AAA+ ring. These degrons, which are sufficient to target an otherwise native substrate for degradation, become accessible by a variety of mechanisms. For example, proteins can have internal recognition motifs that become exposed after endopeptidase cleavage. Alternatively, substrates may be marked for degradation by post-translational or co-translational addition of a degradation sequence, like the addition of the ssrA "tag"/degron (discussed below). Recognition sequences may be buried in native structures and become exposed when proteins unfold; they may also be only exposed in monomeric subunits but not in complexes, or vice versa. For example, FNR is only recognized by the ClpXP protease during oxidizing conditions, when the $[4Fe\text{-}4S]^{2+}$ cluster that stabilizes FNR dimers is damaged (Mettert and Kiley, 2005). ClpX preferentially recognizes DNA-bound

MuA tetramers relative to MuA monomers, which are also ClpX substrates. Whereas a C-termial degron of MuA is necessary and sufficient for ClpX recognition of MuA monomers and tetramers, enhanced recognition is achieved when the N-domain of ClpX makes three additional contacts provided by additional subunits in a MuA tetramer, which act as enhancement tags (discussed below) (Ling et al., 2015).

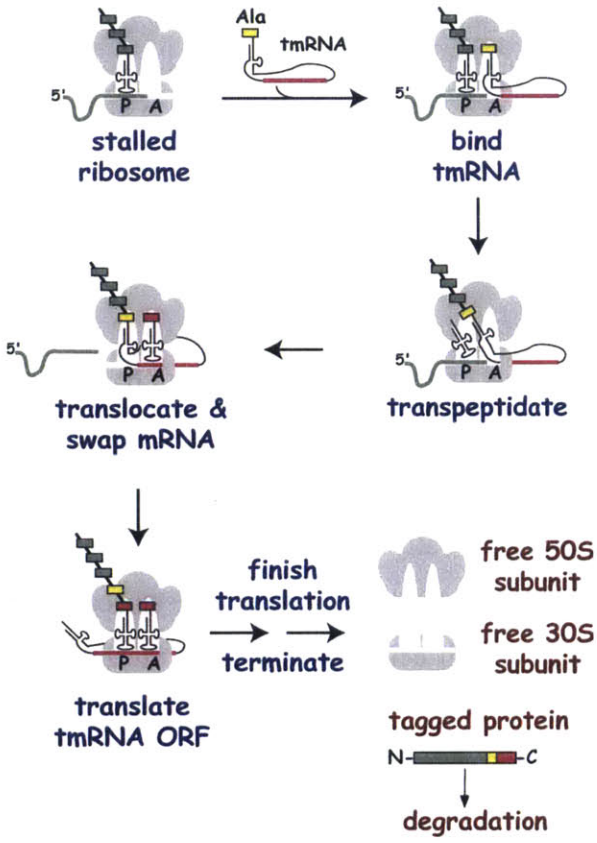

Figure 1-4 Model for tmRNA-mediated tagging and ribosome rescue. Alanyl-tRNA recognizes a stalled ribosome at the end of an mRNA fragment and adds alanine (yellow rectangle) to the nascent polypeptide chain. Following mRNA swapping, the tmRNA open reading frame (red) is translated. Translation termination causes release of the tagged protein for degradation by cellular proteases and liberates ribosomal subunits for new rounds of protein synthesis. Figure from Moore and Sauer, 2007.

ssrA tag

During bacterial protein synthesis, ribosomal stalling can occur for various reasons. For example, termination codons are required to recruit the factors needed for release of a nascent polypeptide from the ribosome. In the absence of a stop codon, ribosomes stall and a partial translation product is generated. Ribosome stalling can also be caused by repeated rare codons, which can deplete the tRNA pool (Roche and Sauer, 1999). All bacterial species have evolved an ssrA-tagging system to remove defective, incomplete translation products from stalled ribosomes. Thus, the tmRNA system, which adds the ssrA tag to incomplete translation products, plays a crucial role in protein-quality control and stalled-ribosome rescue (Moore and Sauer, 2007). It has been estimated that at least 1 in 200 translation products receive an ssrA tag in *E. coli* (Lies and Maurizi, 2008).

SsrA tagging is carried out by a transfer-messenger RNA (tmRNA) molecule, which is charged with an alanine and the ssrA mRNA (Figure 1-4). The tmRNA, in complex with SmpB

(small protein B), recognizes a stalled ribosome and binds to the A site, like a normal tRNA would. Then it donates the alanine to the nascent polypeptide in a transpeptidation reaction. The stalled mRNA is replaced with the ssrA RNA, which is translated as the ssrA degradation signal (Figure 1-4). In *E. coli*, the ssrA tag is the 11-residue sequence AANDENYALAA, which includes the alanine originating from the tmRNA. Translation terminates with a stop codon that is encoded at the end of the ssrA reading frame, and allows the ssrA-tagged polypeptide to be released from the ribosome (Karzai et al., 2000). In the *E. coli* cytosol, ssrA-tagged substrates are degraded by ClpXP, ClpAP and FtsH and Lon (Gottesman et al., 1998; Lies and Maurizi, 2008). SsrA-tagged proteins with signal sequences are exported to the periplasm and degraded by the ATP-independent protease Tsp (Karzai et al., 2000).

SsrA and SmpB are present in all the sequenced bacterial genomes, highlighting the importance of this surveillance system (Karzai et al., 2000). However, the SsrA-SmpB system is not essential for *E. coli* growth, perhaps due to the presence of a redundant system. It is only essential for growth in *N. gonorrhoeae*, *M. genitalium* and *M. pneumoniae*. Whereas the length of the SsrA tag can vary, the sequence is generally conserved across species. The C-terminal residue is always an alanine, and the last 5 residues are usually hydrophobic. *E. coli* ClpX recognizes the C-terminal Ala-Ala-COOH while the adaptor SspB recognizes the N-terminal portion (discussed below). The N-terminal portion, along with central residues of the tag, are important for recognition by ClpA (Flynn et al., 2001).

N-degrons

The N-end rule, which is present in bacteria and eukaryotes, dictates that proteins with certain N-terminal residues are targeted for degradation. These residues, termed N-degrons, are the simplest degradation tag as they can be the sole sequence needed to make a non-substrate protein into an efficiently recognized and degraded substrate. The identity of N-degrons varies across the domains of life. Examples of some N-degrons are listed in Table 1 (Sauer and Baker, 2011). The partner proteins that recognize N-degron-tagged proteins for degradation are called N-recognins. N-recognins fall into two distinct classes: in prokaryotes the recognins are the ClpS adaptor protein for the ClpAP protease whereas in eukaryotes the recognins are pockets within E3 ubiquitin ligases (Varshavsky, 1996) (Figure 1-5).

In well-characterized bacteria the primary N-degrons are the large hydrophobic amino acids: Tyr, Leu, Phe, Trp (Tobias et al., 1991). N-end rule substrates are recognized by the highly specific N-degron pocket of the adaptor ClpS (Roman-Hernandez et al., 2009; Wang et al., 2008b). Additionally, leucyl/phenylalanyl-tRNA-protein transferase (LFTR) can add N-terminal Leu or Phe to proteins bearing Lys or Arg as their N-terminal residue, converting them into N-degron substrates. ClpS recognizes and delivers N-degron substrates to ClpAP for degradation (Erbse et al., 2006; Hou et al., 2008; Roman-Hernandez et al., 2011; Wang et al., 2007). Interestingly, many α-proteobacteria encode a second ClpS, called ClpS2. Recent structural and biochemical studies demonstrate that ClpS2 binds and delivers a more limited set of N-degron substrates to ClpAP (Stein et al., 2016).

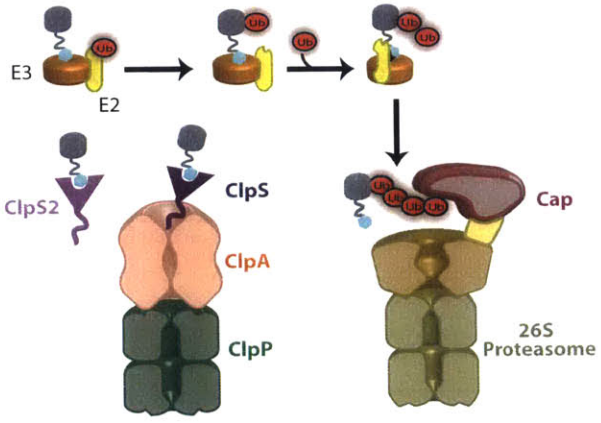

Figure 1-5 N-degron recognition in bacteria and eukaryotes. In bacteria, ClpS recognizes the substrate N-degron and delivers it to the ClpAP protease for degradation. Some bacteria encode a second ClpS (ClpS2), which also recognizes and delivers N-degron substrates to ClpAP. In eukaryotes, a specific E3 ligase recognizes this signal and mediates addition of ubiquitin (Ub), which then leads to recognition and degradation by the proteasome. Figure adapted from Wang et al., 2008.

It remains unclear how bacterial N-degron substrates are generated. These substrates are not primary products of translation or of natural removal of N-terminal formylated-Met (fMet, which is used by bacteria to initiate polypeptide synthesis), as methionine-aminopeptidase, the enzyme responsible for this modification, recognizes substrates bearing small residues at Position 2 (Varshavsky, 2011). Few physiological bacterial N-end rule substrates have been identified. One of them, *E. coli* putrescine aminotransferase (PATase), was shown to require N-terminal addition of Leu by LFTR to its N-terminal Met for ClpS recognition and delivery to ClpAP (Ninnis et al., 2009; Schmidt et al., 2009). This mechanism of tagging proteins with N-terminal Met is surprising and not common, as many proteins retain their initiator Met and do not get degraded. One possibility is that sequence determinants beyond the N-terminal Met specify a recognition signal for LFTR.

The more complex eukaryotic N-degron pathway(s) is part of the ubiquitin-proteasome system, and the N-recognins are E3-ubiquitin ligases, which recognize specific substrates and catalyze their ubiquitination for recognition and degradation by the 26S proteasome (Figure 1-5) (Varshavsky, 2011). A large variety of amino acids can act as primary destabilizing residues in eukaryotes with no further modification required, or as secondary or tertiary destabilizing residues resulting from cotranslational or posttranslational modifications (Table 1). E3 enzymes recognize N-degrons via two types of regions: the type 1 or UBR box class and the type 2 or ClpS-like class. The type 1 region recognizes Lys, Arg and His, whereas the type 2 region recognizes the degrons with large hydrophobic side chains. It is remarkable how the "ClpS-type" binding pocket is key to N-degron recognition in both prokaryotes and eukaryotes, but this pocket resides on two very distinct proteins, an adaptor protein and the E3 ligases (Table 1) (Sauer and Baker, 2011). In eukaryotes, the N-end rule pathway has been implicated in a variety of processes, such as nitric oxide and oxygen sensing, cohesion and segregation of chromosomes, regulation of apoptosis and meiosis, among others (Varshavsky, 2011).

Eukaryokes			
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	

Table 1 N-degrons in eukaryotes (metazoans) and bacteria. Tertiary destabilizing residues (first column) can be modified to produce secondary destabilizing residues (second column) and these are modified to produce primary N-degrons. [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 (Ninnis et al., 2009). Table from Sauer and Baker. 2011.

Ubiquitin

In eukaryotes, the 76-residue ubiquitin (Ub) domain serves as a proteasomal degradation signal when covalently attached to target proteins. It can be conjugated as a single moiety or as a poly-Ub chain to a protein substrate that already has a primary degradation signal, like an N-degron (Varshavsky, 2012). Polyubiquitination and monoubiquitination can serve other signaling functions outside of proteolysis, such as membrane trafficking (Schrader et al., 2009).

The complex process of ubiquitination starts with an ATP-dependent reaction in which the last residue of Ub (Gly76) is linked to a Cys residue of Ub-activating enzymes (E1s). Activated Ub moieties are transferred to a Cys residue of a Ub-conjugating enzymes (E2s). Lastly, ubiquitin protein ligase enzymes (E3s) catalyze formation of an isopeptide bond between the α-carboxyl group of the ubiquitin backbone and the ε-amino group of the substrate Lys (in most cases Lys48) or a Lys of another Ub (to form a poly-Ub chain) (Schrader et al., 2009; Varshavsky, 2012). Depending on the type of E3, ubiquitin may be transferred from the E2 to E3 and then to the substrate, or it can be directly transferred from the E2 to the substrate. Many types of ubiquitin linkages can be formed, as there are seven Lys residues in ubiquitin. There are additional enzymes (called E4 enzymes) that in some cases catalyze multiubiquitin chain assembly in collaboration with E1, E2 and E3 (Hoppe, 2005).

The subunits of the proteasome regulatory particle can directly recognize ubiquitin via its intrinsic ubiquitin receptors or, alternatively, the ubiquitinated substrates can be delivered by adaptors that bind both the proteasome and ubiquitin (discussed below). A tetraubiquitin chain has been thought to be the canonical proteasomal recognition signal, but more recent studies suggest that many other ubiquitination patterns can lead to degradation (Finley et al., 2016). In addition to ubiquitin, an intrinsically disordered region (known as initiation site) is required for engagement by the proteasome (Finley et al., 2016; Prakash et al., 2004; Schrader et al., 2009). The ubiquitin domain and the initiation site can be part of the same substrate protein or they can each be provided by different proteins in a protein complex (Prakash et al., 2004). Ubiquitin resists unfolding, and therefore it is not degraded along with the ubiquitinated substrate. Instead, in order for substrate degradation to proceed, ubiquitin needs to be removed by deubiquitinating enzymes (DUBs) that are part of the regulatory particle. Ubiquitin chains are removed either

before entering the central translocation channel or simultaneously (Finley et al., 2016). Premature removal of ubiquitin can cause the substrate to be released, preventing appropriate substrate degradation (Finley et al., 2016; Schrader et al., 2009).

Prokaryotic ubiquitin-like protein (Pup)

Similar to eukaryotic ubiquitin, the prokaryotic ubiquitin-like protein (Pup) is a 64-residue bacterial degron that is post-translationally added to proteasomal substrates in actinobacteria (Pearce et al., 2008). Interestingly, in contrast to ubiquitin, Pup is intrinsically disordered with some helical propensity at its C-terminus (Chen et al., 2009; Liao et al., 2009; Striebel et al., 2014; Sutter et al., 2009). Pup is conjugated, via its C-terminal residue, to specific lysine residues on the substrate. This ligation reaction, which results in an isopeptide bond, is catalyzed by the Pup ligase PafA (proteasome accessory factor A) (Striebel et al., 2014). The depupylation enzyme Dop, which is homologous to PafA in structure and sequence, cleaves the isopeptide bond, releasing Pup. Pup is recognized by the N-terminal domain of Mpa (also known as ARC), the AAA+ unfoldase partner of the bacterial 20S proteasome (Sutter et al., 2009). The N-terminal region of Pup is engaged by Mpa, which translocates Pup along with the substrate for degradation (Striebel et al., 2010). This is in contrast with ubiquitin, which is removed before the substrate is processed. Malonyl-CoA acyl carrier protein transacylase (FabD) and ketopantenoate hydroxymethyltransferase (PanB), which are enzymes required for fatty acid biosynthesis, are examples of proteins that are targets of pupylation (Pearce et al., 2006; Striebel et al., 2009a). Interestingly, Mpa itself is pupylated *in vivo*. Pupylation prevents Mpa from associating with the 20S proteasome, thereby modulating Mpa activity (Delley et al., 2012)

1.3.2 Post-Recognition Steps: Unfolding and Translocation

Polypeptide translocation is the fundamental mechanical activity of AAA+ unfoldases (Baker and Sauer, 2012). Translocation drives polypeptides through the axial channel of the unfoldase ring. This process also generates an unfolding force as the enzyme engages a native protein via its unstructured degron, and pulls it through the narrow translocation channel (Lee et al., 2001b; Prakash et al., 2004; Sauer and Baker, 2011). The susceptibility of a protein substrate to denaturation by these unfoldases depends on the stability of the protein's local structure adjacent to the degradation signal (Cordova et al., 2014; Kenniston et al., 2003; Lee et al., 2001a; Prakash and Matouschek, 2004).

AAA+ proteases undergo conformational changes upon ATP binding, hydrolysis and release, that power mechanical unfolding and translocation of protein substrates. These conformational changes are transmitted to protein substrates mainly via pore loops, which are highly conserved (Figure 1.6). These pore loops, which project into the central pore and contact the substrate, have been shown to play a critical role in unfolding and translocation by many proteases (Hinnerwisch et al., 2005; Park et al., 2005; Sauer and Baker, 2011). Furthermore, crystal structures of single-chain ClpX variants suggest that axial pore loop movements drive unfolding and translocation. Mutations in the aromatic-hydrophobic-glycine motif result in increased slippage and frequent failure to unfold substrates, suggesting that pore loops grip the substrate for unfolding and translocation (Martin et al., 2008a).

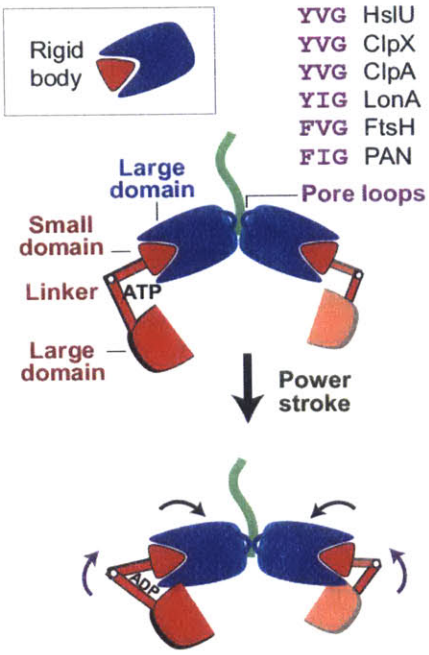

Figure 1-6 Substrate translocation by conserved loops in AAA+ unfoldases. Highly conserved loops in the axial pore of the unfoldase contact the substrate. The power stroke appears to be caused by nucleotide-dependent changes in the rotation between the large and small AAA+ domains of one subunit, which drive rigid-body movements of the entire AAA+ ring and translocate the polypeptide chain. Figure from Sauer and Baker, 2011.

Substrate translocation by AAA+ enzymes can proceed from N terminus to C terminus or vice versa, depending on the location of the recognition tag (Lee et al., 2001b; Prakash et al., 2004; Sauer and Baker, 2011). AAA+ enzymes can translocate many different sequences in single or multiple polypeptide chains. For example, ClpXP can degrade very diverse synthetic peptide substrates (Barkow et al., 2009) and disulfide-bonded polypeptide substrates (Burton et al., 2001). Not surprisingly, the proteasome can also translocate multiple polypeptide chains that are covalently attached via disulfide linkages (Lee et al., 2002). Studies on the bacterial ClpXP protease (discussed in the next section) have revealed important insights into molecular mechanisms that drive unfolding and translocation by AAA+ unfoldases.

Single-molecule studies of ClpXP and ClpAP degradation have revealed detailed information about the mechanisms that drive unfolding and translocation. For example, substrate unfolding

generally occurs in a cooperative or "all-or-none" fashion for many protein substrates. Pre-unfolding dwell times increase with more stable substrates, as they require more power strokes. Consequently, before unfolding stable substrates, the enzyme can hydrolyze many ATPs during failed unfolding attempts. Notably, ATP hydrolysis rates are slower when these enzymes are trying to unfold substrates (Aubin-Tam et al., 2011; Olivares et al., 2016).

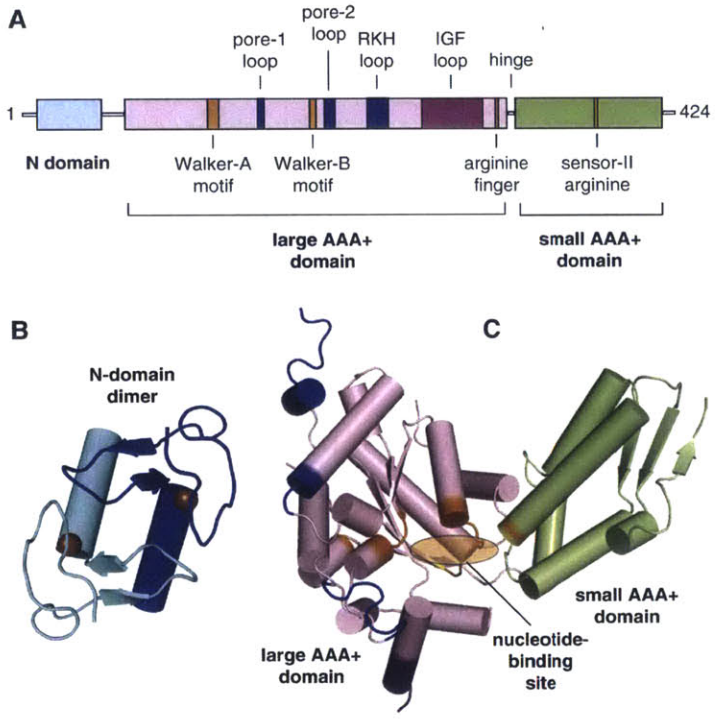

Figure 1-7 Domain structure of ClpX. (A) Arrangement of domains and characteristic motifs are shown for E. coli ClpX. Motifs are colored blue for ssrA-tag binding, orange for ATP binding and hydrolysis, or purple for ClpP binding. The pore-2 loop is also involved in ClpP binding. (B) Structure of the N-domain dimer (PDB code 1OVX). Spheres represent zinc atoms. (C) Structure of a AAA module in a single ClpX subunit (PDB code 3HWS). Nucleotide binds in the cleft between the large and small AAA domains. Motif colors correspond to those in panel A. Figure from Baker and Sauer, 2012

Bacterial Proteases: ClpXP

ClpXP, one of the five ATP-dependent proteases in *E. coli*, is the best-characterized AAA+ protease and serves as a model for understanding other AAA+ proteases. It is formed by the homohexameric ClpX unfoldase and the tetradecameric ClpP peptidase (Baker and Sauer, 2012). ClpX has a family-specific N-terminal domain that is required for recognition of enhancement sequences in some substrates and for adaptor docking (examples discussed below). In addition, each subunit contains a large and a small AAA domain, which together form a AAA module (Figure 1-7). A nucleotide-binding site lies between the small AAA+ domain and the large AAA+ domain of a AAA module. Changes in the orientations of these domains lead to two conformations that predominate in a functional hexamer: a nucleotide-loadable (L) conformation and a nucleotide-unloadable (U) conformation. A subunit with L conformation has a nucleotide-binding cleft (formed by the canonical AAA+ motifs discussed above) between AAA+ domains (Figure 1-7), whereas a subunit with a U conformation does not. Interconversion

between these conformations is critical for ClpX function (Stinson et al., 2013). ATP binding, hydrolysis and product release cause rigid-body movements of the large and small AAA domains, which underlie subunit switching. These conformational changes, which are thought to propagate through movements of the axial pore loops, drive unfolding and translocation (as depicted in Figure 1-6).

Proteomic studies with inactive ClpXP proteases, which were later supported by *in vitro* studies, identified five classes of endogenous *E. coli* ClpXP substrates, which include substrates with N-terminal and C-terminal degrons (Flynn et al., 2003). These sequences are sufficient to target proteins for ClpXP degradation. For example, the C-terminal residues of the MuA transposase target this protein to ClpX, and transfer of this degron to another protein is sufficient to render it susceptible to degradation (Baker and Sauer, 2012). ClpX also recognizes proteins with an ssrA degron. ClpX requires the last two alanines and the alpha-carboxyl group (AA-COOH) for recognition (Flynn et al., 2001). Crosslinking studies demonstrated that the ssrA degron interacts with pore-1 and pore-2 loops in the central channel of the ClpX ring. In addition, mutational studies show that the positively-charged RKH loops at the entrance of the central channel are also important for ssrA recognition (Martin et al., 2008b). Degradation of some ClpXP substrates require the family-specific N-terminal domain of ClpX, as in the case of UmuD', as well as substrates that are delivered by adaptor proteins (discussed below). Thus, ClpX substrates can be recognized via the enzyme's N-domains or via loops that lie in the axial channel.

ClpX can function as a disassembly chaperone in the absence of ClpP. The best-characterized remodeling substrate of ClpX is the phage Mu MuA transposase (Burton and Baker, 2005). ClpX catalyzes the ATP-dependent disassembly of MuA tetramers into monomers, promoting replication initiation (discussed above) (Kruklitis et al., 1996; Levchenko et al., 1995).

E. coli ClpP contains a classical Ser-His-Asp catalytic triad and oxyanion hole, with conformations expected for a functional serine protease (Baker and Sauer, 2012). Even though ClpP cleaves peptides with a wide variety of side chain compositions, there seems to be a preference for non-polar residues. Analysis of GFP-ssrA peptides resulting from ClpXP degradation identified that 80% of these peptides results from cleavage after Leu, Gly, Met, Ala,

and Tyr (Baker and Sauer, 2012). This was also observed on experiments with ClpP assays using model peptide substrates (Thompson and Maurizi, 1994).

The ClpP protease consists of 14 subunits arranged into two homoheptameric rings stacked face-to-face (Kessel et al., 1995), forming a chamber where the proteolytic active sites are sequestered (Wang et al., 1997). The ClpP barrel-like structure contains axial pores located at both ends of the ClpP barrel, at the center of each heptameric ring. When ClpP is not bound to an unfoldase partner, the entrance to its central cavity is blocked, and only small peptides can enter and be cleaved (Thompson and Maurizi, 1994). The turnover number for ClpP peptide cleavage has been estimated to be 800 $min^{-1}ClpP^{-1}$, which is at least 10 times faster than reported rates for the 20S proteasome (Thompson and Maurizi, 1994). It has been proposed that peptides resulting from ClpP cleavage exit the ClpP chamber through windows that open transiently at the ring-ring interface (Sprangers et al., 2005).

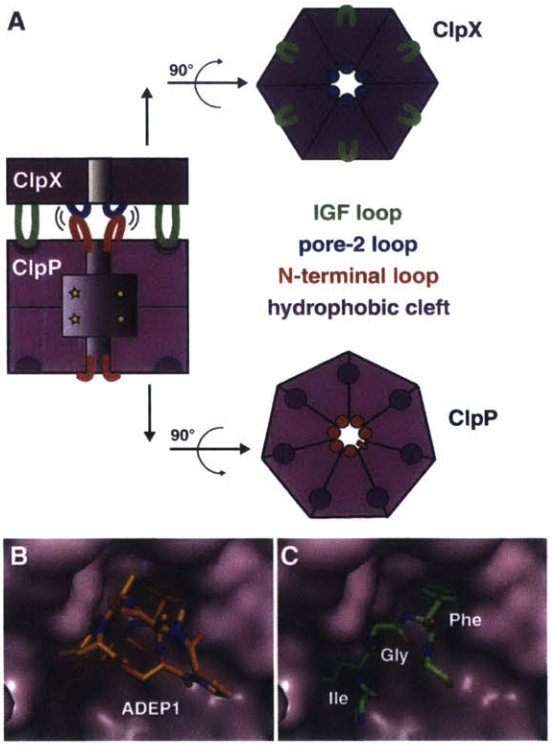

Figure 1-8 Interaction of ClpX and ClpP The ClpXP complex is stabilized by interactions between the conserved IGF loops of ClpX and hydrophobic clefts on ClpP, and by axial interactions between the pore-2 loops of ClpX and the N-terminal stem-loop of ClpP. (B) Structure of an acyldepsipeptide (ADEP1; 3MT6) bound in one of the ClpP clefts. (C) Model of the ClpX IGF peptide binding in the ClpP cleft in a manner analogous to ADEP1.

ClpP can associate with the hexameric ClpX or ClpA unfoldases that align coaxially with the ClpP pore at either or both ends of the barrel to translocate polypeptides into the proteolytic chamber. Assembly of ClpXP and ClpAP complexes, which requires the presence of ATP or ATP analogs (Maurizi, 1991), can lead to formation of singly- or doubly-capped complexes (Grimaud et al., 1998; Kessel et al., 1995; Kress et al., 2009). ClpX (and ClpA) interaction is mediated in part by conserved IGF loops that dock in ClpP hydrophobic pockets, opening the entrance to the degradation chamber and activating the peptidase (Figure 1-8A) (Baker and Sauer, 2012; Effantin et al., 2010; Martin et al., 2007). This interaction can be mimicked by acyldepsipeptide antibiotics

(ADEPs; Figure 1-8B and 1-8C), which constitutively activate ClpP degradation of newly synthesized proteins and unfolded polypeptides (Baker and Sauer, 2012; Li et al., 2010; Olivares et al., 2016). ClpXP complexes are also stabilized by additional interactions between pore-2 loops of ClpX and the N-terminal stem loop residues of ClpP (Figure 1-8A).

This thesis mostly focuses on ClpAP, a AAA+ protease similar to ClpXP, but with differences in architecture and substrate preference (See Section 1.5).

The Eukaryotic Proteasome

Although the proteasome shares the same basic architecture and general operating principles of bacterial AAA+ proteases, it is a much more complex protease. The eukaryotic 26S proteasome, a cylindrical machine of approximately 2.5 MDa, is responsible for the specific degradation of regulatory proteins involved in many cellular processes, such as transcription, apoptosis, and cell cycle control. The proteasome is also responsible for proteolysis of damaged proteins in the cytosol and the nucleus (Groll et al., 1997; Prakash and Matouschek, 2004; Schrader et al., 2009). It is composed of the 20S core particle and a coaxially stacked 19S regulatory particle (Figure 1-9). As discussed above, the degron that targets substrates to the proteasome is formed by the small protein ubiquitin and an unstructured region called initiation site (Prakash et al., 2004).

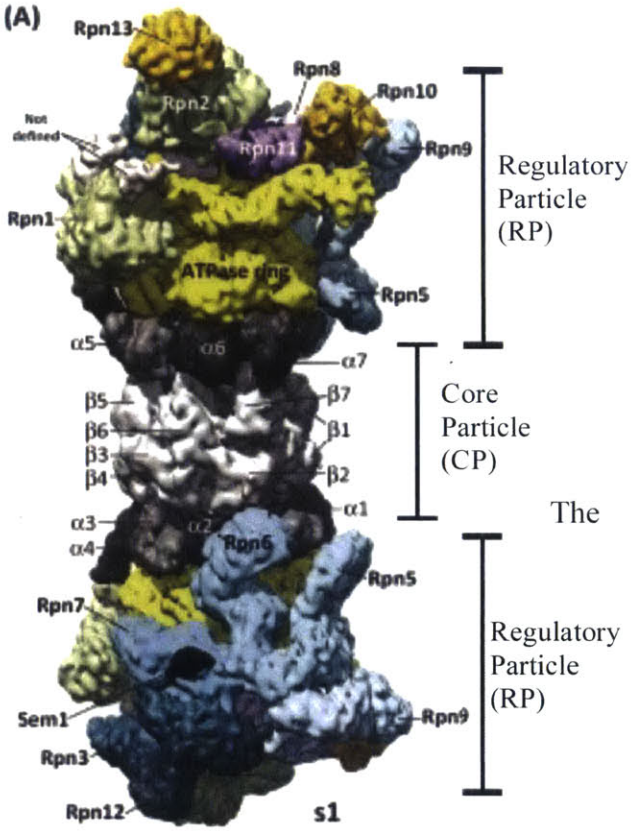

Figure 1-9 Cryoelectron micrograph of the 26S proteasome. Regulatory particles (RP) stack against each end of the core particle (dark and light gray). Top and bottom RPs are rotated relative to each other by approximately 180°.The ATPase rings (yellow) make contacts with the α-rings of the CP. Other lid components are colored blue. Figure adapted from Finley 2016.

The core particle contains 28 subunits that are arranged in four heteroheptameric rings, which contain the proteolytic sites sequestered within a cavity (Figure 1-9) (Finley et al., 2016; Groll et al., 1997). The entrance to this cavity is too narrow for folded proteins to enter. The outer rings are composed of α subunits and the inner rings are composed of β subunits (Figure 1-9). β subunits specifically cleave after hydrophobic, acidic, or basic residues. The core particle is normally autoinhibited by N-terminal tails of α-subunits, which gate the access to the degradation cavity. In the presence of ATP, ATPases in the regulatory particle bind sites in the α-subunits of the core particle, which triggers opening of the gate and facilitates substrate entry (Finley et al., 2016; Lander et al., 2012).

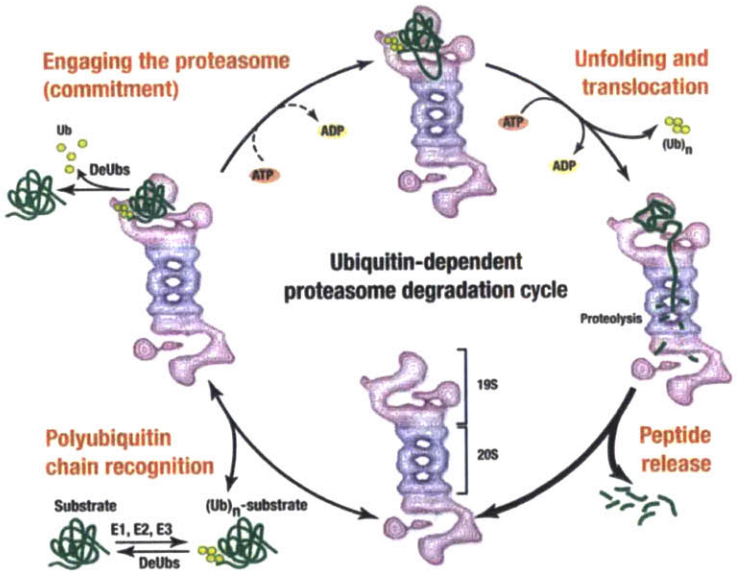

Figure 1-10 Degradation cycle of the proteasome. Substrates are marked for degradation by covalent attachment of polyubiquitin chains by E1, E2 and E3 enzymes. The proteasome regulatory particle subunits recognize ubiquitin chains $(Ub)_n$ in protein substrates. Deubiquitinases (DeUbs) associated to the regulatory particle remove polyubiquitin domains, which can lead to substrate release or substrate degradation. Successful engagement of the unstructured initiation site (top left) leads to unfolding and translocation (top right) into the core particle for proteolysis (bottom right). Figure from Prakash and Matouschek, 2004

The regulatory particle coaxially stacks at both ends of the core particle, forming a narrow channel that leads into the degradation chamber of the core particle (Figure 1-9). The regulatory particle gates entrance into the degradation chamber and plays a role in the recognition, unfolding and translocation of substrates into the 20S core particle (Figure 1-10) (Lander et al., 2012). The regulatory particle is composed of at least 19 subunits and can be subdivided into two subcomplexes, called the lid and the base (Lander et al., 2012). The lid is composed of nine proteins (Rpn3, Rpn5-9, Rpn11, Rpn12 and Sem1 in yeast). Rpn11, which is a deubiquitinating enzyme, is critical for substrate degradation. The base is a heterohexameric ring of AAA+ ATPases (Rpt1-Rpt6) that unfolds and translocates substrates into the core particle for degradation (Figure 1-9). In addition, the base contains four non-ATPase subunits: Rpn1, Rpn2,

Rpn10 and Rpn13. The latter two are ubiquitin receptors. Rpn1 is the docking site for shuttle receptors (discussed below).

In archaea, proteolysis is carried out by the PAN-20S complex, which is a simpler evolutionary precursor of the proteasome. It contains a 28-subunit 20S complex, with subunits arranged in a similar architecture as the eukaryotic core particle. Substrates are recognized, unfolded and translocated in to the core particle by the homohexameric AAA+ unfoldase PAN (Proteasome-Activating Nucleotidase) (Benaroudj and Goldberg, 2000; Finley et al., 2016).

1.4 Regulation of Substrate Degradation

1.4.1 Enhancement Tags

As mentioned above, substrate recognition is likely a highly regulated step. In addition to recognition tags, substrates can bear enhancement sequences. These enhancement sequences make additional contacts to tether the substrate for recognition and degradation via the main degron, which in many cases is a weak signal on its own. For example, in order to be degraded by ClpXP, FNR requires two sequence motifs, located at the N- and C-terminus, respectively. The N-terminal degron is thought to bind to the N domain of ClpX whereas the C-terminal degron binds to the ClpX pore and directs degradation. UmuD/UmuD' is an interesting example where these recognition sequences are provided by different subunits in a complex. UmuD', a subunit of the *E. coli* error-prone translesion DNA polymerase, is a poor ClpXP substrate when it is not in a heterodimer its UmuD precursor. When DNA damage occurs, single-stranded-DNA-bound RecA mediates autocleavage of UmuD between residues 24 and 25, generating UmuD'. UmuD accumulation following DNA repair favors formation of UmuD/UmuD' heterodimers and consequently UmuD' degradation. While UmuD' carries the primary degradation signal, UmuD provides an enhacement tag that binds the ClpX N-domain and tethers UmuD' to ClpXP for degradation (Neher et al., 2003). Thus, this degradation pathway employs enhancement tags to respond to the physiological state of the cell.

The initiation site for proteasomal degradation in eukaryotes can be thought of as an enhancement tag. Although the proteasome recognizes substrates through ubiquitin tags, degradation is initiated by engagement the unstructured inititation site (Finley et al., 2016).

Studies using ubiquitinated substrates show that these substrates are slowly degraded by the proteasome unless they also possess an initiation site (Prakash et al., 2004). Although initiation sites lack defined sequence motifs, there is a preference for certain length and sequence features (Fishbain et al., 2015).

1.4.2 Adaptor Proteins

Another mode of regulation of substrate degradation involves intermediary proteins called adaptors. AAA+ proteases recognize their substrates directly, as mentioned above, or can be assisted by adaptors. Adaptors expand the functional diversity of AAA+ proteins, and also add another level of control of substrate degradation. Some adaptors deliver substrates to their enzymes, while others inhibit degradation of particular substrates.

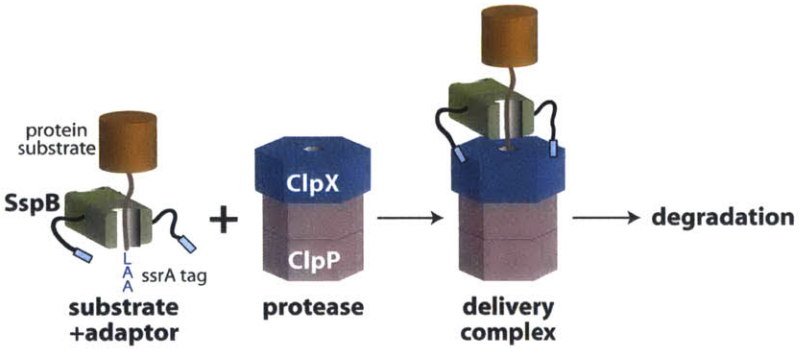

Figure 1-11 SspB delivery of ssrA-tagged substrates to ClpXP. The N-terminal domains of an SspB dimer (green) bind part of the substrate's ssrA tag, and the C-terminal tails bind ClpX, positioning the ClpX recognition sequence directly above the central degradation pore. Figure from (McGinness et al., 2006)

The SspB Molecular Matchmaker

By tethering substrates to enzymes, adaptors can work as "molecular matchmakers". *E. coli* SspB, the best-characterized adaptor of this kind, is a dimeric adaptor composed of two domains, separated by a flexible linker. The N-terminal domain binds ssrA substrates, and the C-terminal tail binds ClpX (Levchenko et al., 2003; Wah et al., 2003). SspB binds N terminal and central residues of the ssrA tag (AANDxxY), whereas ClpX recognizes adjacent C-terminal residues (LAA) (Flynn 2001). By making additional contacts with the N domain of ClpX, SspB tethers the ssrA substrate in a tight ternary complex, acting as a molecular bridge (Figure 1-11). This delivery complex is composed of one or two ssrA proteins, an SspB dimer, and a ClpX hexamer (Wah 2002). SspB docks on the N-terminal domains of ClpX, positioning the substrate directly above the central degradation pore. In this way, SspB increases the effective concentration of substrate relative to ClpX, thereby enhancing degradation efficiency at low substrate concentrations. Importantly, SspB does not get degraded during delivery and does not interfere

with ssrA substrate processing. Notably, SspB binds to a region of the ssrA tag that is required for recognition by ClpA (Flynn et al., 2001) Therefore, SspB acts as a competitive inhibitor of ClpAP degradation of ssrA-tagged substrates. In addition to delivering ssrA substrates to ClpXP, SspB also recognizes the stress-response regulator RseA and delivers this substrate to ClpXP for degradation (Flynn, 2004; Levchenko et al., 2005).

The activating adaptor MecA

B. subtilis MecA is an adaptor that not only delivers substrates to the ClpCP AAA+ protease, but is also required for ClpC assembly and activation (Kirstein et al., 2009). The ComK transcription factor, which regulates competence in *B. subtilis*, is a substrate of the MecA-ClpCP degradation machine. While the C-terminal domain of MecA binds ClpC, the N-terminal domain binds ComK, forming a ternary complex. When MecA delivers ComK, it gets degraded along with the substrate, resulting in disassembly of the protease (Mei et al., 2009). Thus, in non-competent cells, MecA inhibits ComK, thereby inhibiting competence.

When competence is initiated by a quorum-sensing mechanism, the ComS signaling peptide is synthesized. ComS and ComK share a sequence motif that is necessary and sufficient for MecA binding (Kirstein et al., 2009). ComS binds MecA, releasing ComK and therefore preventing its proteolysis. Inhibition by ComS, along with positive autoregulation by ComK, results in activation of transcription of competence development genes (Mei et al., 2009). This is an example of another level of regulation of substrate degradation, in which adaptor proteins are regulated in response to environmental signals.

The RssB adaptor and its anti-adaptors

RpoS (also known as σ^S), the master regulator of stationary phase and stress response genes in *E. coli*, is a ClpXP substrate during exponential phase (Zhou, 2001). RpoS protein levels increase in response to many stresses and return to normal levels once growth resumes. Moreover, RpoS levels increase as cells enter stationary phase. These changes in protein levels are largely achieved via ClpXP degradation.

However, RpoS degradation by ClpXP also requires the RssB adaptor protein. RssB competes with core polymerase for binding to RpoS, and then delivers RpoS to ClpXP. Interestingly,

RssB, RpoS and ClpXP form a tighter affinity ternary complex relative to either RssB or RpoS alone with ClpXP, thus favoring delivery of RpoS to ClpX. After delivery, RssB does not get degraded, and instead is released to carry out multiple cycles of RpoS delivery (Zhou, 2001). RssB function is also subject to posttranslational regulation, as phosphorylation of RssB is thought to cause a conformational change that exposes a ClpX recognition sequence, thereby enhancing the RpoS-ClpX interaction (Stüdemann et al., 2003). It has been shown that the sensor kinase ArcB, which is less active during energy starvation, can phosphorylate RssB. Thus, during energy starvation, RssB phosphorylation is decreased, and RpoS proteolysis decreases as a result.

In addition to post-translational modifications, adaptor proteins can be negatively regulated by anti-adaptors in response to different external signals. This paradigm is best exemplified by RssB, which has several associated anti-adaptors that act to stabilize RpoS. For example, in response to phosphate starvation, the *E.coli* anti-adaptor IraP binds RssB, inducing releasing RpoS. As a consequence, RpoS-dependent genes are expressed. IraM works in a similar way to IraP, but in response to Ca^{2+} or Mg^{2+} starvation, whereas IraD responds to DNA-damaging conditions (Kirstein et al., 2009). Thus, RpoS protein stability is positively regulated by anti-adaptors during stress conditions, thereby allowing expression of stress-response genes.

E3 ligases and proteasomal adaptors

Proteasomal specificity is derived mainly from E3 ligases, which recognize specific substrates and catalyze their ubiquitination. Some E3 ligases directly bind the proteasome, acting like the bacterial adaptors that recognize and deliver substrates to their cognate protease (Elsasser and Finley, 2005). There are at least a thousand distinct E3 enzymes in the mammalian genome (Varshavsky, 2012). Control of ubiquitination, which is the most regulated step of proteasomal degradation, occurs mainly through E3 enzymes. Substrate mimics lacking lysines that can bind E3 enzymes, preventing them from targeting their substrates, represent one interesting example of regulation of E3 enzymes.

Ubiquitination is a very dynamic process, which adds another level of control to substrate recognition. Ubiquitin chains in substrates are continuously being modified by ubiquitin ligases

or deubiquitinating enzymes (DUBs), before and during proteasomal recognition. Ubiquitin receptors may influence ubiquitination dynamics. For example, it was shown that the Rad23 receptor (discussed below) inhibits elongation of polyubiquitin chains, suggesting that these adaptors can bind ubiquitin chains to prevent further modifications by ubiquitin ligases and DUBs (Elsasser and Finley, 2005). Regulatory particle DUBs like Uch37 can also regulate degradation by causing premature substrate deubiquitination (Schrader et al., 2009).

The proteasome regulatory particle can directly recognize ubiquitin chains through its Rpn13 and Rpn10/S5a (yeast/mammalian) subunits, or it can bind shuttle receptors that act in a similar way as bacterial adaptors. These receptors (Rad23, Dsk2 and Ddi1) contain an ubiquitin-like domain (UBL) that binds the proteasome ubiquitin receptors and an ubiquitin-associated domain (UBA) that binds ubiquitin chains (Elsasser and Finley, 2005; Finley et al., 2016). For example, degradation of the Sic1 cyclin-dependent kinase inhibitor requires Rad23. Some shuttle receptors may bind specific ubiquitin ligases. For example, Ddi1 directly interacts with the E3 enzyme Ufo1 and is required for degrading the Ufo1 target, HO endonuclease (Elsasser and Finley, 2005).

Some ubiquitinated substrates are recruited to the eukaryotic AAA+ ATPase Cdc48 (also known as VCP or p97) in steps upstream of proteasomal recognition. Cdc48 binds to a different class of ubiquitin receptors, called UBX-UBA receptors. In addition to having a UBA domain, these receptors have a UBX domain that, similar to the UBL domain, is structurally related to ubiquitin. Cdc48 can act in cooperation with the proteasome to degrade many ubiquitinated substrates, although the underlying mechanism is not clear (Elsasser and Finley, 2005). Cdc48 has been proposed to extract polyubiquitinated substrates from membranes for subsequent delivery to the proteasome, and to assist the proteasome in unfolding certain substrates (Elsasser and Finley, 2005; Meyer et al., 2012). Interestingly, archaeal Cdc48 was recently shown to partner with the 20S peptidase to form a functional proteasome (Barthelme and Sauer, 2012). Notably, a striking number of Cdc48 adaptors have been identified but are not well understood. Most of these adaptors contain a UBX motif (Meyer et al., 2012), and include deubiquitinating enzymes. As dictated by its adaptors, Cdc48 can influence many cellular processes, such as endoplasmic reticulum associated degradation (ERAD) pathway, autophagy and vesicle fusion (Elsasser and Finley, 2005; Meyer et al., 2012).

1.5 The ClpAP Protease and Its Adaptor ClpS

1.5.1 The ClpAP Protease

E. coli ClpA was the first member of the Clp/Hsp100 family characterized to degrade proteins *in vitro* (Katayama et al., 1988). Like ClpX (discussed above), ClpA is a hexameric AAA+ unfoldase that can partner with ClpP to catalyze ATP-dependent protein degradation in the bacterial cytosol (Katayama et al., 1988; Maurizi, 1991). ClpA uses an IGL loop, similar to the IGF loop of ClpX, to bind ClpP (Figure 1-8). (Effantin et al., 2010; Kim et al., 2001; Sauer and Baker, 2011; Singh et al., 2001). ClpA also contains the conserved YVG sequence motif that resides in the translocation loops in the ClpX channel, which are involved in substrate recognition and translocation (Figure 1-6) (Siddiqui et al., 2004). Crosslinking studies using GFP-ssrA and RepA suggest that degradation of these substrates requires binding and translocation by the same channel-facing loops (Hinnerwisch et al., 2005).

In vitro, ClpAP can recognize and degrade proteins with N- or C-terminal degrons, such as N-degron substrates (Tobias et al., 1991), RepA (Hoskins et al., 2000), ssrA-tagged proteins (Gottesman et al., 1998), RecN (Neher et al., 2006) and ClpA itself (Gottesman et al., 1990). ClpAP also recognizes alpha-casein, and other model substrates that lack defined secondary or tertiary structure (Hoskins et al., 2000; Susi et al., 1967), thus inspiring the idea that ClpAP might work to degrade unfolded substrates.

The ClpA unfoldase can work as a molecular chaperone *in vitro* (Pak and Wickner, 1997; Wickner et al., 1994). In *E. coli*, plasmid P1 replication requires the initiator protein RepA. In its monomeric form, RepA binds to P1 replication origin (oriP1) DNA with high affinity, whereas RepA dimers are inactive for DNA binding. ClpA binds inactive RepA dimers and converts them into active monomers in a reaction that requires ClpA ATPase activity (Pak and Wickner, 1997). Furthermore, ClpA can target RepA for degradation by ClpP. Interestingly, RepA bound to origin DNA was protected from degradation by ClpA (Wickner et al., 1994). Additionally, Wickner and colleagues suggest that ClpA prevents luciferase aggregation when present during heat-treatment but cannot reverse it once aggregated (Wickner et al., 1994).

The principal physiological role(s) of ClpAP are not very well elucidated. ClpA is only present in bacteria, although some bacteria, such as *B. subtilis*, and plant chloroplasts have the ClpA homolog ClpC. ClpC regulates competence development in *B. subtilis* (discussed above) and is essential in *M. tuberculosis* (although its function is not clear) (Ollinger et al., 2012). In *E. coli*, the *clpP* and *clpA* genes are not essential, and *clpA*- mutants grow well under many conditions tested (*i.e.* starvation, heat shock, UV-induced DNA damage) (Katayama et al., 1988; Olivares et al., 2016; Weichart et al., 2003). Few ClpAP physiological substrates have been identified, and include ClpA itself (Katayama et al., 1988; Maurizi et al., 1990). The MazE protein of the MazEF toxin:antitoxin module, is also a ClpAP substrate in *E. coli* (Aizenman et al., 1996; Engelberg-Kulka et al., 2005). The *mazEF* toxin:antitoxin module is responsible for programmed cell death in response to nutrient starvation. MazF is a long-lived toxin and MazE, which is short lived, is an antitoxin that antagonizes MazF. Therefore, MazE needs to be continuously expressed to prevent cell death. ClpAP can also degrade ssrA-tagged substrates *in vivo*, although under the conditions investigated, ClpXP plays a larger role in degradation of ssrA-tagged proteins (See below).

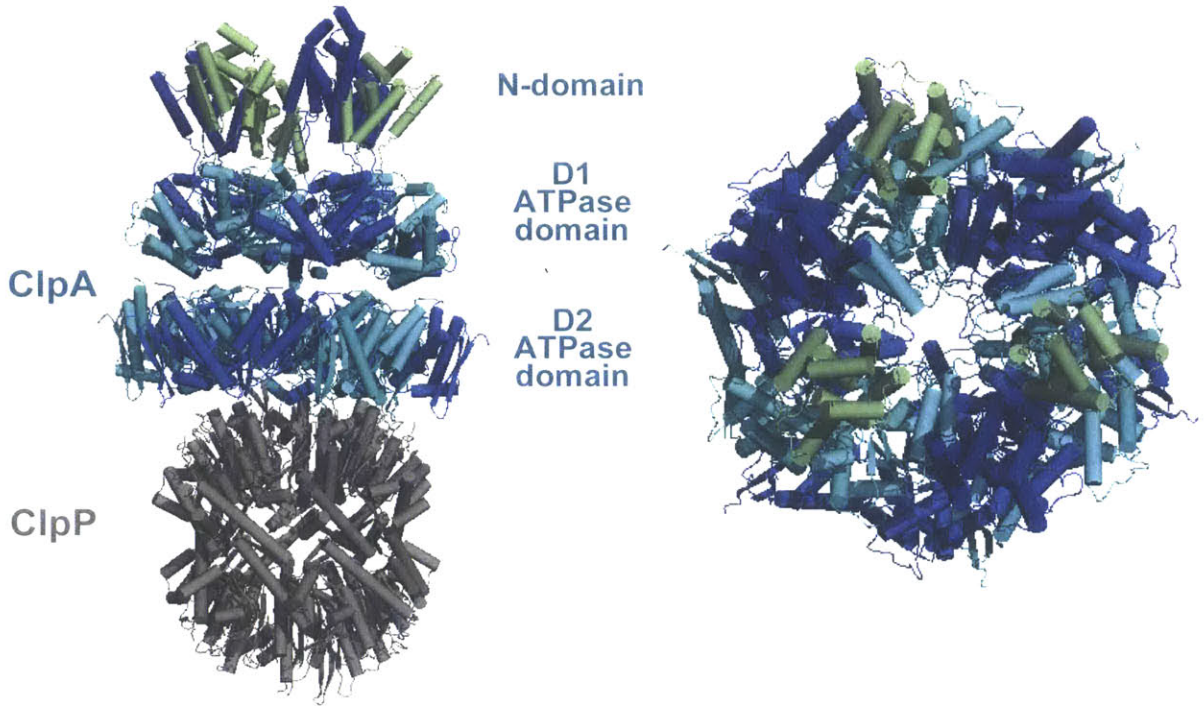

Figure 1-12: Homology model of ClpA. Model of *E. coli* ClpA using ClpA PDB 1R6B modeled using ClpC-MecA structures 3PXG and 3PXI. (*left*) A side view of the ClpA double-ring hexamer is shown. The N-domain (dark blue and green) is attached to the ATPase domains (light shades of blue) via flexible linkers. The bottom D2 AAA ring, which is larger than the D1 ring, is more homolous to the ClpX AAA domain, and also interacts directly with ClpP. The ClpP barrel is shown in gray. (*right*) Top view of the ClpA hexamer. This ClpA model was kindly provided by Adrian Olivares (MIT)

Studies have shown that ClpA and ClpP levels increase as cells enter stationary phase. Farrell and colleagues estimated that, during exponential growth, there are enough intracellular ClpA molecules to form 40-50 hexamers, whereas in stationary phase ClpA levels increased to roughly 150 hexamers per cell. ClpP levels also increased from about 100 $ClpP_{14}$ molecules during exponential phase to 250-300 $ClpP_{14}$ molecules as cells entered stationary phase. In addition to the few protein substrates identified, ClpAP, like ClpXP, can degrade ssrA substrates *in vivo* (Gottesman et al., 1998; Lies and Maurizi, 2008). Degradation of ssrA substrates increases as ClpAP levels increase in stationary phase (Farrell et al., 2005; Lies and Maurizi, 2008). However, in a *clpA*- strain ClpXP was sufficient to degrade most of the GFP-ssrA. Thus, ClpA is not a large contributor to degradation of ssrA proteins and ClpXP is responsible for most of their *in vivo* degradation. However, this result does not rule out the possibility that ClpAP is important for ssrA degradation under as of yet unstudied growth conditions.

One main architectural difference between ClpA and ClpX is that each ClpA subunit contains two nonidentical but highly homologous AAA modules (for a total of 12 ATP binding sites in the hexamer), whereas ClpX has one AAA module (Guo, 2002a). ClpA, therefore, forms a double-ring hexamer in the presence of ATP or ATPγS (Beuron et al., 1998; Kessel et al., 1995; Singh et al., 2001) (Figure 1-12). The N- and C-terminal AAA modules are termed D1 and D2, respectively. Interestingly, D2 is more homologous to ClpX than D1, and also interacts directly with ClpP (Grimaud et al., 1998). These AAA modules can be inactivated individually by mutations in the Walker B motif (E286A in D1 and E565A in D2), providing a means of studying the contributions of the D1 and D2 rings. These mutations, which replace the ATPase active site glutamate by alanine, impair ATP hydrolysis but not binding (Kress et al., 2009). ClpA hexamers with Walker B mutations form stable complexes with substrates (Erbse et al., 2008). Studies inactivating one or both of these modules suggest that the D2 ring is responsible for most of ClpA ATPase and unfolding/translocation activity whereas D1 is thought to play a role in oligomerization (Kress et al., 2009; Singh and Maurizi, 1994). However, both ATPase domains contribute to the degradation of proteins with high structural stability. Adding an additional layer of complexity and regulation, ClpA ATPase activity can also be modulated by binding partners. For example, ClpP stimulates ClpA ATP hydrolysis whereas ClpS suppresses ATPase activity (Hinnerwisch et al., 2005; Hou et al., 2008; Kress et al., 2009).

Single molecule studies of ClpA revealed mechanistic differences in substrate processing by this double-ring machine, as compared to ClpX (Olivares et al., 2014). Even though ClpA is a slower translocase, ClpA unfolds substrates significantly faster than ClpX. Additionally, ClpA translocates substrates with steps that are smaller and more regular as compared to ClpX steps, even though the fundamental unit step size of both enzymes is ~1 nm (Aubin-Tam et al., 2011; Olivares et al., 2014). Olivares and colleagues propose a model where ClpA "grips" substrates stronger than ClpX, by making additional contacts provided by additional axial pore loops, accounting for its faster unfolding of model domains.

In addition to two AAA+ modules, ClpA has a family-specific N-terminal domain that is attached to the D1 domain via a flexible linker (Figure 1-12) (Guo, 2002a), and it is expected to be highly mobile, based on cryo-electron microscopy studies (Beuron et al., 1998; Ishikawa et al., 2004). The N-domain is dispensable for unfolding and translocation by ClpA (Cranz-Mileva et al., 2008; Lo et al., 2001; Maglica et al., 2008; Singh et al., 2001). Studies with different N-domain deletion variants suggest that the N-domain is involved in modulating ATPase and degradation activities (Lo et al., 2001; Singh et al., 2001). Moreover, studies suggest that the N-domain aids in recognition of some substrates. For example, the N-domain is required for recognition of casein (Seol et al., 1994). Importantly, the N-domain is also the docking site for the ClpS adaptor and is therefore necessary for ClpAP degradation of N-degron substrates that are delivered by ClpS (Dougan et al., 2002b; Roman-Hernandez et al., 2011). The length of the linker connecting the N-domain and D1 AAA domains affects the efficiency of this ClpS-dependent delivery of N-degron substrates (Cranz-Mileva et al., 2008; Roman-Hernandez et al., 2011).

To date, only two regulators of ClpA substrate recognition have been identified. One of them is the SspB adaptor, which, as discussed above, binds to ssrA tag residues that are critical for ClpA recognition of this degron. Thus, SspB competitively inhibits ClpAP ssrA substrate degradation. The ClpS adaptor, which is the focus of this thesis, has a more active role in regulating ClpAP substrate degradation. ClpS binds N-degron substrates and delivers them to ClpAP for degradation (Erbse et al., 2006; Hou et al., 2008; Rivera-Rivera et al., 2014; Roman-Hernandez et al., 2011; Stein et al., 2016; Wang et al., 2007) Like SspB, ClpS also inhibits ClpAP substrate

degradation (Flynn et al., 2001). In fact, ClpS inhibits degradation of all non-N-degron substrates tested to date (ClpA, casein, RepA) (De Donatis et al., 2010; Dougan et al., 2002b).

1.5.2 The ClpS Adaptor

ClpS is a small, 12 KDa monomeric adaptor protein that regulates the ClpAP protease (Dougan et al., 2002b; Guo, 2002b). ClpS appears to be an important player in the bacterial N-end rule pathway and has been shown to deliver N-degron substrates to ClpAP *in vitro* (Erbse et al., 2006; Wang et al., 2007). Interestingly, ClpS is distantly related to an ~100 residue region present in some eukaryotic E3 ubiquitin ligases; this ClpS-like region can act as an N-recognin for hydrophobic N-degrons in the eukaryotic N-end rule pathway (Lupas and Koretke, 2003; Varshavsky, 1996). ClpS is widely distributed throughout bacteria. In particular, it is well conserved throughout proteobacteria, where it is generally encoded by a gene that is upstream of the *clpA* gene (Dougan et al., 2002b). ClpS homologs also are found in more distant genera, such as actinobacteria, cyanobacteria and plant chloroplasts (Lupas and Koretke, 2003). A second, paralogous *clpS* gene exists in α-proteobacteria and cyanobacteria. This gene encodes the ClpS2 protein, which was recently found to cooperate with ClpAP in *A. tumefaciencis* and with ClpCP3/R in *S. elongatus* to degrade N-degron substrates, with a distinct degron preference compared to the canonical ClpS (ClpS1) (Stein et al., 2016).

The 3-dimensional structures of ClpS from several species have been solved in the past decade. These structures reveal the "core"domain, but generally lack a visible N-terminal extension

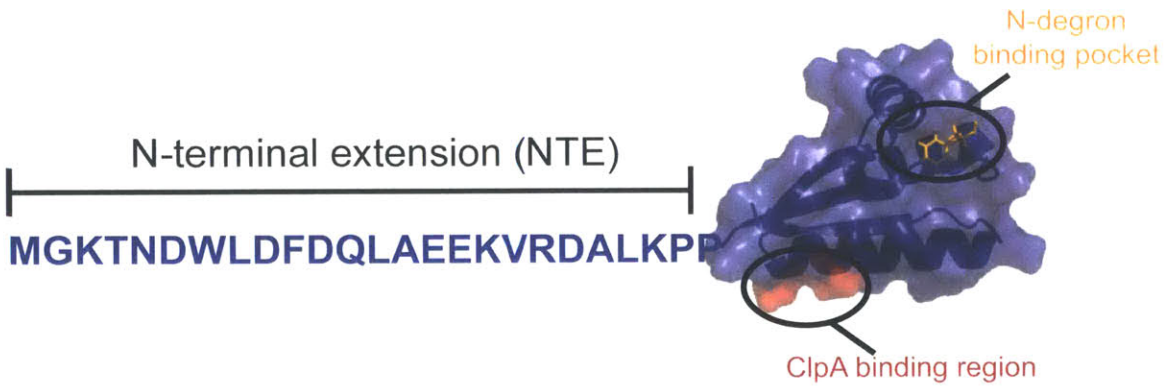

Figure 1-13 Structure of the ClpS adaptor protein bound to a Phe N-degron (PDB code 3O2B). The ClpS adaptor has an unstructured N-terminal extension (NTE, residues 1-25) and a core domain (residues 2-26), which harbors a binding pocket for N-degrons (orange). A surface of ClpS that binds to the N-terminal domain of ClpA is highlighted in red.

(NTE), suggesting that the N-terminal region is flexible and not ordered in the crystals (Figure 1-13). The structures reveal distinct features of the "core" domain, including a region that binds the N-domain of ClpA and a hydrophobic substrate-binding pocket (Guo, 2002b; Zeth et al., 2002). ClpS binds to the ClpA N-domain and as such there are six potential binding sites in a ClpA hexamer (Guo, 2002b; Hou et al., 2008). The ClpS core harbors a hydrophobic binding pocket for N-degrons (Roman-Hernandez et al., 2009; 2011; Wang et al., 2008b) (described below). The ClpS NTE, which lacks evolutionary sequence or specific length conservation (Figure 1-14), is not needed to efficiently bind substrates or ClpA (Hou et al., 2008; Roman-Hernandez et al., 2011). However, the work presented here and elsewhere demonstrates that the NTE is critical for ClpS function (Hou et al., 2008; Rivera-Rivera et al., 2014; Roman-Hernandez et al., 2011)

ClpS represents a unique class of adaptors because it can regulate ClpA activity positively and negatively. *In vitro* biochemical studies of ClpAP degradation of GFP-ssrA and YLFVQ-titin^{i27} in the same reaction show that addition of ClpS modifies substrate preference. In the presence of ClpS, the YLFVQ-titin^{i27} (a model N-degron substrate) degradation K_M is tightened, whereas the GFP-ssrA (a model ssrA substrate) degradation K_M is weakened as compared to ClpAP alone (Hou et al., 2008). However, ClpS does not simply act as a binary switch, as different levels of ClpS relative to $ClpA_6$ allow a "fine tuning" of ClpAP substrate preference. For example, when there are two ClpS molar equivalents per $ClpA_6$ in solution, ClpAP degrades N-degron substrates as well as ssrA substrates. When this ratio increases to 4-6 ClpS molar equivalents per $ClpA_6$,

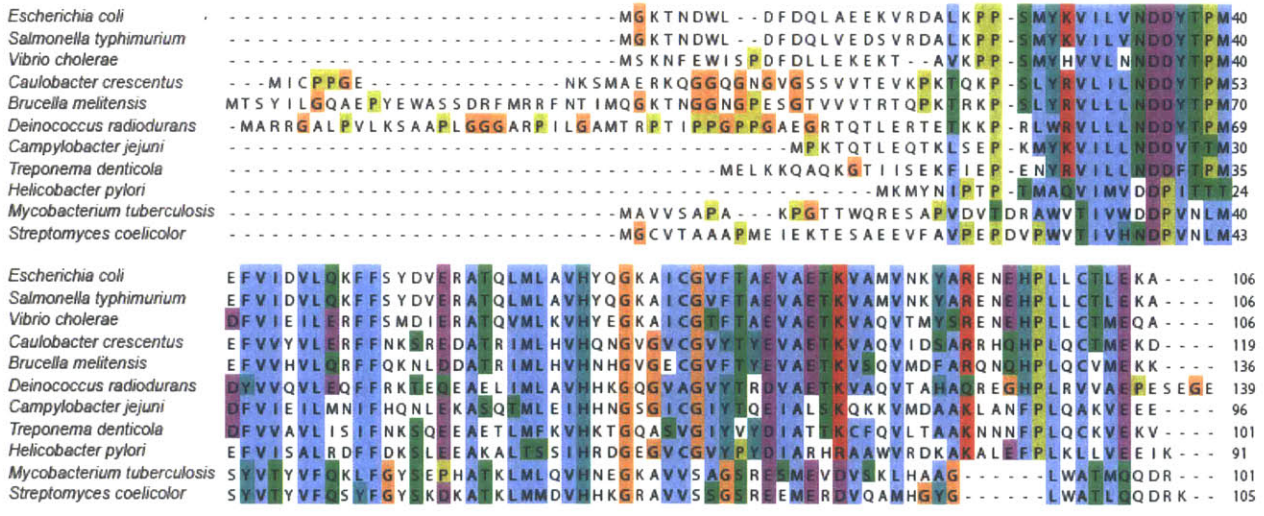

Figure 1-14 Alignment of ClpS from various bacterial species. Residues similar in nature are colored. Figure adapted from Hou *et al.*, 2008.

ssrA substrate degradation is minimal and N-degron substrate degradation is strongly favored (Hou et al., 2008). *In vivo*, ClpS levels remain constant across growth phases (~300 molecules per cell) (Farrell et al., 2005). However, ClpA levels increase from ~45 molecules per cell to ~150 molecules per cell as cells enter stationary phase. Thus, ClpAPS transitions from conditions that strongly favor N-degron substrate degradation (ClpS:$ClpA_6$ ratio of 6) to conditions were the ClpAP substrate repertoire is expanded (ClpS:$ClpA_6$ ratio of 2). Indeed, in a reporter assay for ssrA substrate degradation, increased ClpAP expression during stationary phase was shown to cause an increase in GFP-ssrA degradation (Farrell et al., 2005).

ClpS has been shown to enhance ClpAP N-degron substrate degradation while inhibiting degradation of all ClpAP non-N-degron substrates tested, including ssrA substrates and ClpA itself (De Donatis et al., 2010; Dougan et al., 2002b; Hou et al., 2008). Interestingly, ClpS employs different strategies to enhance and inhibit ClpAP substrate degradation. Much biochemical work has been done using model N-degron substrates to characterize the mechanism of ClpS delivery (Erbse et al., 2006; Hou et al., 2008; Rivera-Rivera et al., 2014; Roman-Hernandez et al., 2011; Wang et al., 2007). ClpS binds N-degron substrates and delivers them to ClpA for ClpAP degradation (Erbse et al., 2006; Hou et al., 2008; Roman-Hernandez et al., 2011; Wang et al., 2007).

The ClpS core domain has a hydrophobic pocket where N-degrons (Tyr, Leu, Phe, Trp in bacteria) are tightly packed. Crystal structures of *E. coli* and *C. crescentus* ClpS bound to N-degron peptides show that the side-chain of the N degron is buried in the hydrophobic pocket and the α-amino group and first peptide bond make additional contacts with ClpS (Roman-Hernandez et al., 2009; 2011; Wang et al., 2008b; Xia et al., 2004). When ClpS delivers N-degron substrates to ClpA, the substrate needs to be transferred from the ClpS binding pocket to the ClpA translocation pore (Figure 1-15). ClpA, ClpS and N-degron substrate form a ternary complex that leads to substrate delivery. In this complex, the ClpS-substrate and the ClpS-ClpA binding affinities are increased 75-fold and 9-fold, respectively, relative to when the third component is missing. Formation of this high-affinity ternary complex involves interactions mediated by residues of the ClpS NTE, the ClpS binding pocket residue His^{66}, the N-degron residue of the substrate, and the D1 ring of ClpA. Notably, a sufficiently long linker between the

ClpA N- and D1 domains is required for efficient substrate delivery. Importantly, experiments using ClpS NTE deletion variants show that the NTE needs to be 14-residues long for delivery and this same length is required for suppression of ClpAP ATPase rate (Hou et al., 2008; Roman-Hernandez et al., 2011). Notably, although the NTE lacks significant sequence or length conservation, the shortest NTE among ClpS from various bacterial species appear to meet this requirement (Figure 1-14). Recent studies further characterizing the mechanism of ClpS substrate delivery demonstrate that the ClpS NTE is engaged by the ClpA translocation machinery during delivery (Figure 1-15). Numerous lines of evidence support a model where pulling of the NTE by ClpA drives remodeling of the ClpS core (which resists degradation). This remodeling is thought to destabilize the ternary complex, facilitating substrate transfer to ClpA (Rivera-Rivera et al., 2014).

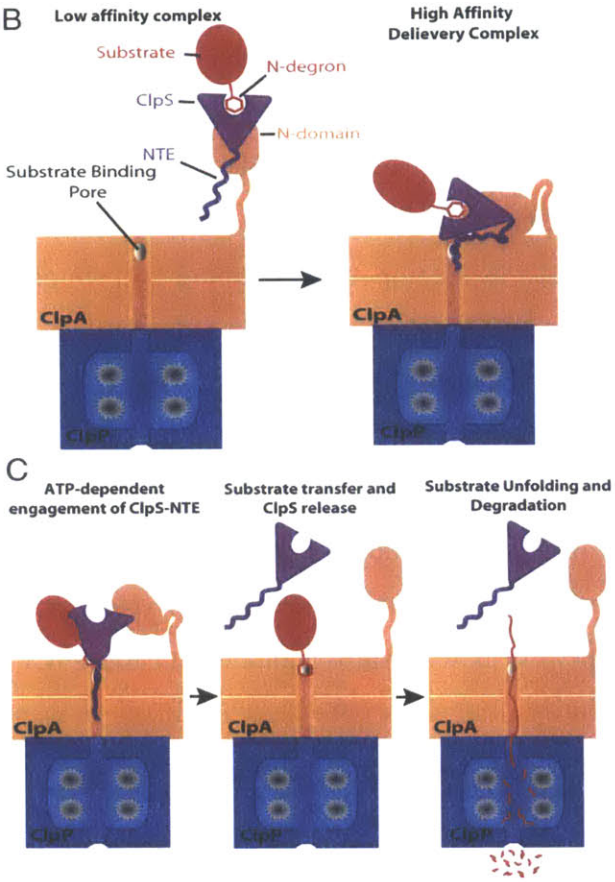

Figure 1-15 Model for the active delivery mechanism used by ClpS. B) Formation of high-affinity delivery complex (HADC) between ClpS, substrate, and ClpA involves formation of additional contacts between ClpA, ClpS, and the N-degron substrate. (C) The current model for ClpA-driven disassembly of the HADC and N-degron substrate delivery. Translocation- mediated ClpA "pulling" on the NTE remodels the ClpS core structure, weakens the interactions of ClpS with the N-degron, and facilitates its transfer to a site in the ClpA pore. Because ClpS cannot be unfolded by ClpA, the adaptor escapes the enzyme, and the substrate is unfolded by ClpA and subsequently degraded by ClpP. Figure adapted from Rivera-Rivera *et al.*, 2015.

The mechanism of ClpS inhibition, which has remained poorly understood, is the focus of this thesis. Previous studies argue that ClpS prevents substrate binding to ClpA. Deletion of the N-terminal 17 residues of ClpS impairs inhibition (Dougan et al., 2002b), but this mutant still binds ClpA N domain tightly (Guo, 2002b; Roman-Hernandez et al., 2011). Hou and colleagues made ClpS variants with truncated NTEs and found that, whereas an 8-residue NTE was deficient for inhibition, an NTE with one residue longer

inhibited ssrA substrate degradation efficiently (Hou et al., 2008) Thus, all previous studies implicate the NTE in the mechanisms of inhibition.

Some of these earlier studies propose models for inhibition. Based on solution binding experiments evaluating ClpS's effect on substrate binding, Dougan and colleagues proposed that ClpS act as a competitive inhibitor and simply prevents ssrA substrate binding to ClpA (Dougan et al., 2002b). In contrast, based on their NTE truncation experiments, Hou and colleagues concluded that this short NTE length is unlikely to occlude ssrA substrate binding. This observation and the fact that ClpS suppresses ClpAP's ATPase rate led Hou and colleagues to conclude that ClpS is not a strict competitive inhibitor. Instead, they propose an allosteric model in which ClpS binding causes conformational changes that favor an N-degron binding site and weaken ssrA binding interactions (Hou et al., 2008). Later, based on binding and kinetics experiments with various model substrates, De Donatis *et al.* proposed that the ClpS NTE makes additional contacts with the ClpA ring, preventing substrates from entering the axial channel of ClpA (De Donatis et al., 2010).

Here we dissect the mechanism employed by ClpS to reprogram the ClpAP protease, with emphasis on the negative regulatory role that ClpS exerts on non-Ndegron substrate degradation. In contrast to many previous studies, we find that ClpS affects substrate recognition and processing, acting in a non-competitive mechanism. We show evidence for an ssrA-ClpAP-ClpS ternary complex that leads to productive degradation by ClpAP. Furthermore, we show that ClpS slows substrate unfolding/translocation through suppression of the ATPase rate of ClpAP. Finally, by probing the ClpS structural features important for inhibition, we demonstrate that the ClpS NTE is necessary and sufficient for inhibition.

Chapter 2

The Intrinsically-Disordered N-terminal Extension of the ClpS Adaptor Reprograms Its Partner AAA+ ClpAP Protease

This chapter has been written as a manuscript for publication. I performed all the experiments. R.T. Sauer performed simulations for the data in Figure 2-6.

2.1 Abstract

Adaptor proteins modulate substrate selection by AAA+ proteases. The ClpS adaptor delivers N-degron substrates to ClpAP but inhibits degradation of substrates bearing ssrA tags or other related degrons. How ClpS inhibits degradation of such substrates is poorly understood. Here, we demonstrate that ClpS impedes recognition of ssrA-tagged substrates by a non-competitive mechanism and also slows subsequent unfolding/translocation of these substrates as well as N-degron substrates. This suppression of mechanical activity is largely a consequence of the ability of ClpS to repress ATP hydrolysis by ClpA, but several lines of evidence show that ClpS inhibition of substrate binding and ATPase repression are separable activities. Using ClpS mutants and ClpS-ClpA chimeras, we establish that engagement of the intrinsically disordered N-terminal extension (NTE) of ClpS by ClpA is both necessary and sufficient to inhibit multiple steps of ClpAP-catalyzed degradation. These observations reveal how an adaptor can simultaneously modulate the catalytic activity of a AAA+ enzyme, efficiently promote recognition of some substrates, and suppress recognition/degradation of other substrates. We propose that similar mechanisms are likely to be used by other adaptors to regulate substrate choice and the catalytic activity of molecular machines.

2.2 Introduction

Energy-dependent AAA^+ proteases, are critical in all domains of life, functioning to maintain proteostasis and to regulate many cellular processes (Hanson and Whiteheart, 2005; Olivares et al., 2016). These proteases consist of a AAA+ ring hexamer that recognizes, unfolds, and translocates protein substrates into the degradation chamber of an associated peptidase (Olivares et al., 2016; Sauer and Baker, 2011; Striebel et al., 2009b). In prokaryotes, protease-associated AAA+ unfoldases recognize small, accessible peptide sequences, called degrons or degradation tags, typically located near the N or C terminus of a protein substrate (Baker and Sauer, 2006). For example, the ssrA degron (AANDENYALAA-COOH), which is co-translationally added to the C termini of proteins when translation is compromised, targets ssrA-tagged proteins for degradation by the ClpXP and ClpAP proteases of *Escherichia coli* (Gottesman et al., 1998; Karzai et al., 2000; Keiler et al., 1996). N-degrons are singe N-terminal amino acids (F, W, Y and L in *E. coli*) that target substrates for degradation by ClpAP via the ClpS adaptor (Tobias et al., 1991; Wang et al., 2007).

Adaptor proteins alter the substrate repertoire of AAA+ enzymes and therefore influence many cellular processes (Baker and Sauer, 2006). *E. coli* SspB, one of the best-characterized adaptors, delivers ssrA-tagged substrates to ClpXP. SspB binds to ClpX and to part of the substrate ssrA tag, providing a molecular bridge between the enzyme and substrate. By tethering the ssrA-tagged substrate to ClpXP, SspB increases the effective concentration of the degron with respect to the axial pore of ClpX, which must engage the tag to begin unfolding and translocation (Wah et al., 2003). Like SspB, most known adaptors influence the substrate-recognition step (Dougan et al., 2002a; Elsasser and Finley, 2005). However, the *Bacillus subtilis* MecA adaptor regulates both assembly of the ClpCP protease and recognition of the ComK transcription factor (Kirstein et al., 2006; Mei et al., 2009; Turgay et al., 1998).

Here, we dissect the molecular mechanism by which the ClpS adaptor negatively controls degradation of ssrA-tagged substrates by ClpAP, a protease consisting of the hexameric AAA+ ClpA unfoldase and the tetradecameric ClpP peptidase (Figure 2-1a). ClpA subunits contain an N-terminal domain and two AAA+ modules (D1 and D2), which assemble into a double-ring homohexamer with an axial translocation pore that aligns with the pore of ClpP (Figure 2-1a)

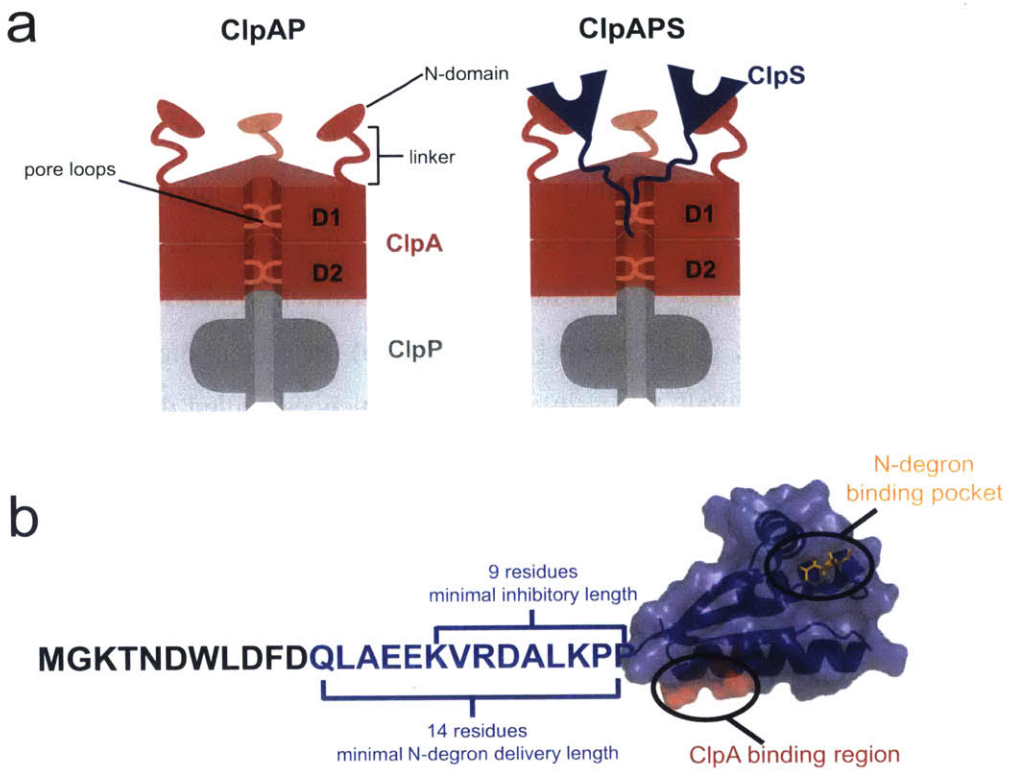

Figure 2-1 The ClpAPS complex. (**a**) The ClpAP protease. (*left*) The ClpA hexamer consists of D1 and D2 AAA+ rings, with N-terminal domains connected to the D1 domain of each subunit by a flexible linker. Conserved loops in the ClpA translocation pore grip substrates and mediate translocation and unfolding. (*right*) The ClpS adaptor binds the N-domain of ClpA. (**b**) Structure of *E. coli* ClpS bound to a Phe N-degron (PDB code 3O2B). The ClpS adaptor has an unstructured N-terminal extension (NTE, residues 1-25) and a core domain (residues 26-106), which harbors a binding pocket for N-degrons (orange). A region of ClpS that binds to the N-terminal domain of ClpA is highlighted in red. The portion of the NTE required for ClpS function is color blue.

(Kessel et al., 1995). As shown in Figure 2-1b, ClpS contains a tightly folded core domain (residues 26-106), in which a hydrophobic pocket binds N-degrons and another surface binds the N-terminal domain of ClpA, and an intrinsically disordered N-terminal extension or NTE (residues 2-25) (Dougan et al., 2002b; Hou et al., 2008; Roman-Hernandez et al., 2011; Zeth et al., 2002). ClpS variants with NTEs shorter than nine residues fail to inhibit ClpAP degradation of ssrA-tagged substrates (Dougan et al., 2002b; Hou et al., 2008), and NTEs of 14 residues or longer are needed for efficient delivery of N-degron substrates and suppression of ATP hydrolysis by ClpAP (Roman-Hernandez et al., 2011). Current evidence supports a model in which ClpS enhances degradation by active "handoff" of the N-degron of a substrate to the translocation pore of ClpA (Rivera-Rivera et al., 2014; Roman-Hernandez et al., 2011). This handoff involves a mechanism in which the NTE of ClpS is engaged, like a degron, by the translocation machinery of the ClpA axial pore. In fact, the NTE functions as a degron in

chimeric proteins. For example, NTE-GFP is a good substrate for ClpAP degradation, whereas GFP alone is not (Roman-Hernandez et al., 2011). Unlike protein substrates, however, the core domain of ClpS resists ClpA unfolding and thus NTE engagement does not result in ClpS denaturation or degradation (Rivera-Rivera et al., 2014).

Although ClpS delivery of N-degron substrates to ClpA has been actively studied (Erbse et al., 2006; Rivera-Rivera et al., 2014; Roman-Hernandez et al., 2011; Wang et al., 2007), less is known about the mechanism of ClpS inhibition of ClpAP degradation of ssrA-tagged and related substrates. Some models propose that the NTE directly competes with recognition of ssrA-tagged substrates (De Donatis et al., 2010; Dougan et al., 2002b). Here, however, we show that ssrA-tagged substrates do bind, albeit weakly, to ClpAPS but are degraded very slowly as a consequence of reductions in the rates of substrate unfolding and translocation. We also demonstrate that the NTEs from multiple ClpS molecules are needed to efficiently inhibit degradation and show that the ability of the NTE to suppress the maximal rate of degradation parallels its activity in repressing ATP hydrolysis by ClpAP. We discuss the ways in which the ClpS NTE acts as a "degron mimic", compare the inhibitory and stimulatory activities of ClpS, and consider the implications of our results for general adaptor function.

2.3 Results

2.3.1 SsrA-tagged substrates bind to ClpAPS but with weakened affinity.

Does ClpS prevent ClpA binding to ssrA-tagged substrates by a competitive mechanism or reduce ClpA-substrate affinity by a non-competitive mechanism? To facilitate binding assays using fluorescence anisotropy, we used fluorescein maleimide to label an ssrA-tagged variant of the DNA-binding domain of λ repressor containing one cysteine (λ^{*fl}-ssrA) (Gottesman et al., 1998; Keiler et al., 1996) or to label a variant of ClpS containing one cysteine ($ClpS^{*fl}$) (Roman-Hernandez et al., 2011). ClpA bound λ^{*fl}-ssrA with an affinity of ~0.7 μM (Figure 2-2a) in the presence of an ATP analog (ATPγS) that is not hydrolyzed (Reid et al., 2001). Next, binding of ClpA to $ClpS^{*fl}$ was assayed in the presence of 30 μM λ-ssrA. Under these conditions, ClpA bound $ClpS^{*fl}$ with an affinity of ~0.16 μM (Figure 2-2b), whereas an affinity of ~0.18 μM was previously measured for binding of ClpA to $ClpS^{*fl}$ in the absence of ssrA-tagged substrates (Roman-Hernandez et al., 2011). These results are inconsistent with a model of strict competition, which predicts that 30 μM λ-ssrA should decrease the apparent affinity of ClpA for ClpS by a factor of (1 + 30 μM/0.7 μM) ≈ 44.

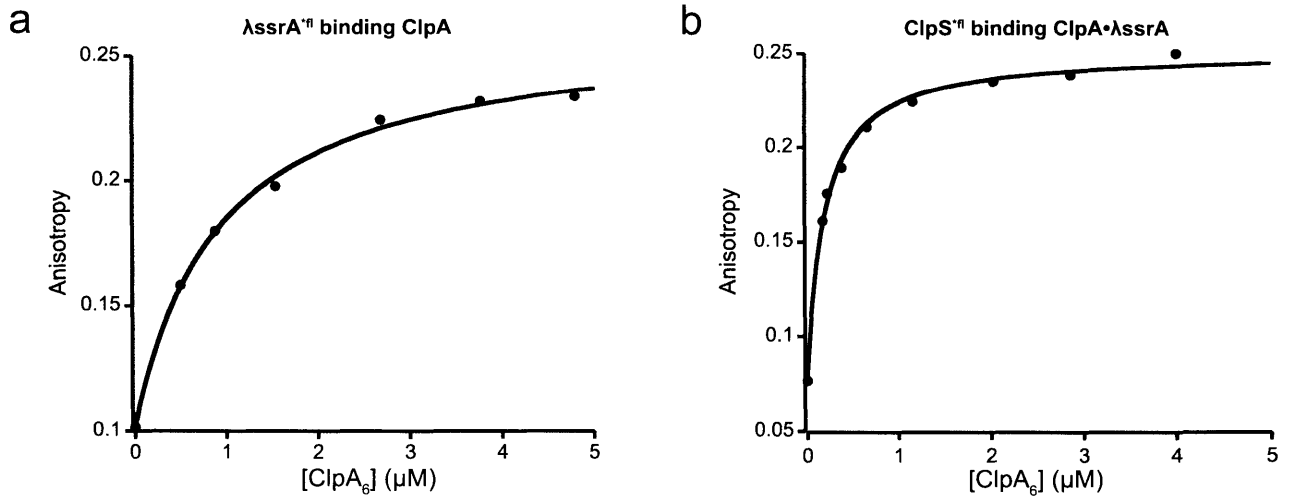

Figure 2-2 Binding of λ^{*fl}-ssrA or $ClpS^{*fl}$ to ClpA. (**a**) Binding of ClpA to λ^{*fl}-ssrA (0.15 μM) in the presence of 2 mM ATPγS, as assayed by fluorescence anisotropy. The line is fit to a hyperbolic equation with 50% binding (K_D) at 740 ± 190 nM. (**b**) Binding of ClpA to $ClpS^{*fl}$ (0.2 μM) in the presence of λ-ssrA (30 μM) and ATPγS (2 mM), as assayed by fluorescence anisotropy. The line is fit to a quadratic equation for near stoichiometric binding with 50% binding (K_D) at 160 ± 51 nM. The K_D values in (a) and (b) are averages ± SD (n=3). Data are representative of three independent experiments.

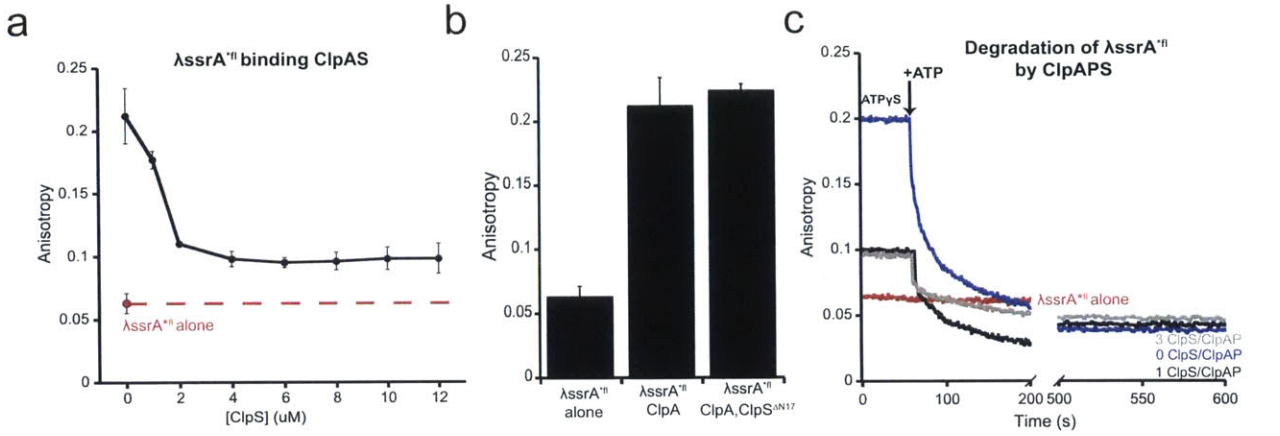

Figure 2-3 Binding of λ^{*fl}-ssrA to ClpAS and degradation by ClpAP or ClpAPS. (**a**) λ-ssrA*^{fl} (0.15 μM) was mixed with ClpA (2 μM) and ATPγS (2 mM) without and with increasing concentrations of ClpS. Binding was assayed by equilibrium levels of fluorescence anisotropy. The red dashed line marks the anisotropy of free λ^{*fl}-ssrA. Values are averages ($n \geq 3$) ± 1 SD. (**b**) Fluorescence anisotropy of λ-ssrA*^{fl} (0.15 μM) binding ClpA in the absence (2 μM $ClpA_6$) or presence of $ClpS^{\Delta N17}$ (4.5 μM $ClpA_6$, 18 μM $ClpS^{\Delta N17}$) and 2mM ATPγS. (**c**) Effects of different amounts of ClpS on the kinetics of ClpAP degradation of λ^{*fl}-ssrA, as assayed by fluorescence anisotropy. λ^{*fl}-ssrA was pre-incubated with ClpAP or ClpAPS and ATPγS (2 mM). After ~60s, degradation was initiated by addition of 8 mM ATP. Red trace, λ^{*fl}-ssrA alone. Blue trace, λ^{*fl}-ssrA with ClpAP (2 μM $ClpA_6$, 4 μM $ClpP_{14}$). Grey trace, λ^{*fl}-ssrA with ClpAPS (2 μM $ClpA_6$, 4 μM $ClpP_{14}$, 2 μM ClpS). Black trace, λ^{*fl}-ssrA with ClpAPS (2 μM $ClpA_6$, 4 μM $ClpP_{14}$, 12 μM ClpS).

If λ-ssrA and ClpS compete for ClpA binding by a non-competitive binding mechanism, then excess ClpS should fail to completely displace λ-ssrA from ClpA. To test this prediction, we mixed a small amount of λ^{*fl}-ssrA with a concentration of ClpA sufficient to give ~75% binding (2 μM) and then added increasing concentrations of ClpS (Figure 2-3a). Importantly, ClpS in two-fold or higher excess over ClpA reduced the anisotropy to a stable plateau that was higher than the anisotropy of λ^{*fl}-ssrA alone. At this plateau ~20% of the λ^{*fl}-ssrA remained bound to ClpAPS and ~80% was free, confirming that ClpS and λ^{*fl}-ssrA can bind ClpA at the same time, the hallmark of non-competitive binding. Based on this bound/free ratio, we calculate an affinity (K_D) of ~8 μM for the binding of ClpAPS to λ^{*fl}-ssrA. Thus, ClpS binding weakens ClpA affinity for λ^{*fl}-ssrA ~11-fold. $ClpS^{\Delta N17}$, which lacks the N-terminal 17 residues of wild-type ClpS, bound ClpA with an affinity of ~0.16 μM (Figure 2-4) but did not displace λ^{*fl}-ssrA from ClpA (Figure 2-3b), establishing that the missing NTE residues are required for non-competitive inhibition.

To test if the intermediate λ^{*fl}-ssrA anisotropy observed in the presence of ClpAPS corresponds to a productive complex, we pre-assembled ClpAP or ClpAPS complexes with λ^{*fl}-ssrA in the

presence of ATPγS and monitored anisotropy for ~60 s before adding ATP to initiate degradation. Both with no ClpS and with ClpS in three-fold excess over ClpAP, the anisotropy decreased to a value lower than that of free λ*^{fl}-ssrA following addition of ATP (Figure 2-3c), as expected if λ*^{fl}-ssrA was degraded into peptides. Following addition of ATP, the loss of anisotropy was biphasic, likely because ATP hydrolysis causes λ*^{fl}-ssrA dissociation in addition to supporting degradation. We note that non-competitive inhibition of λ*^{fl}-ssrA binding to ClpA saturated at a 2:1 ratio of ClpS:ClpA (Figure 2-3a), whereas a 4:1 ratio of ClpS:ClpA is required for strong degradation inhibition (Dougan et al., 2002b; Hou et al., 2008). Thus, the mechanisms by which ClpS weakens substrate binding and inhibits degradation appear to be somewhat different.

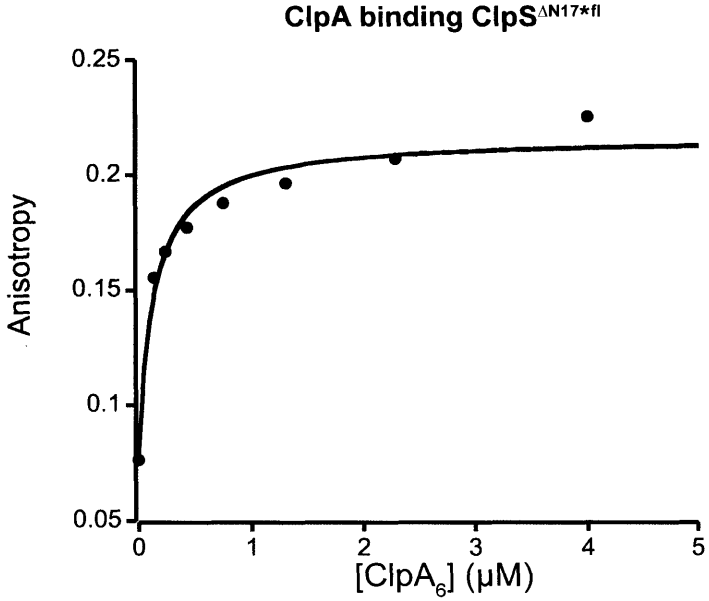

Figure 2-4 Binding of ClpA to ClpS$^{\Delta N17*fl}$ (0.2 μM) in the presence of λ-ssrA (30 μM), 2 mM ATPγS, as assayed by fluorescence anisotropy. ClpS$^{\Delta N17*fl}$ is a ClpS variant that contains a single cysteine and lacks the N-terminal 17 residues. The line is fit to a quadratic equation for near stoichiometric binding with 50% binding (K_D) at 160 ± 13 nM. The K_D is an average ± SD (n=2). Data are representative of three independent experiments.

2.3.2 ClpS increases K_M and decreases V_{max} for ^{SF}GFP-ssrA degradation

To analyze ClpS inhibition of enzyme function, we assayed the effects of ClpS on the steady-state kinetics of ClpAP degradation of super-folder GFP with an ssrA tag (^{SF}GFP-ssrA) (Nager et al., 2011; Pédelacq et al., 2005). Rates of initial degradation of different concentrations of GFP-ssrA were determined by loss of native fluorescence and fit to the Michaelis-Menten equation. With ClpS present in six-fold excess over ClpAP, ^{SF}GFP-ssrA was degraded with an 8-fold weaker K_M and 5-fold slower V_{max} compared to degradation by ClpAP alone (Figure 2-5a). Thus, ClpS decreased V_{max}/K_M, the second-order rate constant for degradation, by a factor of ~40-fold.

Addition of Phe-Val, an N-degron dipeptide that stabilizes ClpAPS complexes (Roman-Hernandez et al., 2011), did not result in substantially stronger inhibition (Figure 2-5a), suggesting that ClpA is already saturated with ClpS under the conditions of this experiment. The observed changes in K_M and V_{max} parameters support a classical mixed-inhibition model that is fully consistent with non-competitive binding. Moreover, the V_{max} decrease suggests that ClpS negatively affects one or more mechanical activities of ClpAP.

ClpS depression of V_{max} has also been observed for ClpAP degradation of N-degron-tagged variants of the I27 domain of human titin (N-titin^{I27}) (Wang et al., 2007). Likewise, compared to ClpAP, we found that ClpAPS displayed a reduced V_{max} for degradation of the N-degron substrate YLFVQ-GFP, even as it enhanced recognition by lowering K_M (Figure 2-5b). In combination, these results support a model in which ClpS reprograms ClpAP to alter substrate specificity but at a cost of slowing mechanical unfolding and/or translocation of the substrate. For both ssrA-tagged and N-degron tagged substrates, the tags are the first part of the substrates to be degraded by ClpAP. As a consequence, it seems unlikely that the tags themselves rather than ClpS are responsible for reprogramming translocation of the entire substrate.

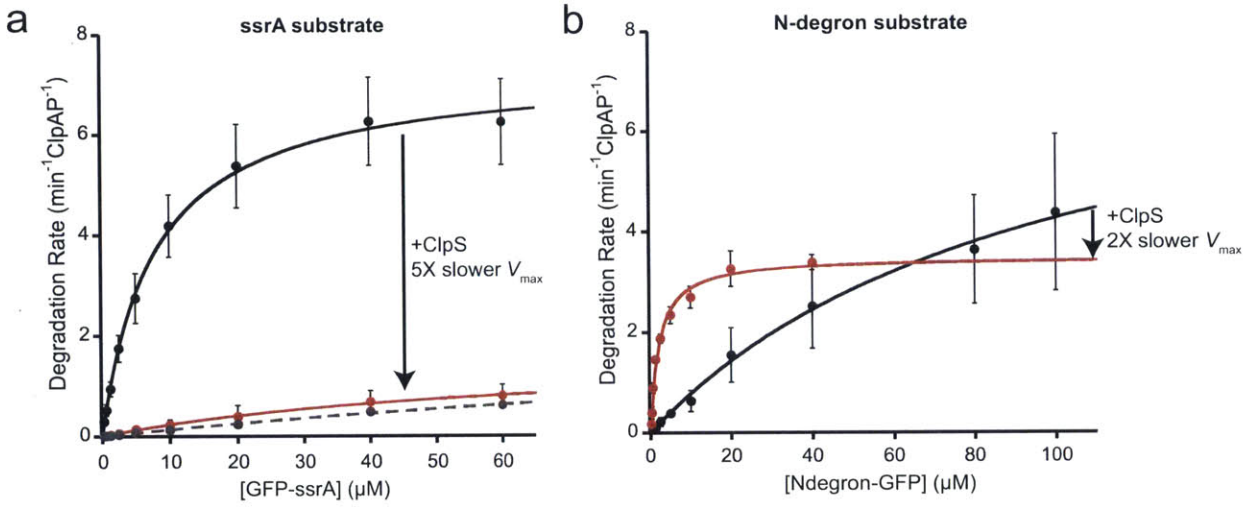

Figure 2-5 ClpS inhibits recognition and degradation of an ssrA-tagged substrate. (**a**) Michaelis-Menten analysis of steady-state degradation of ^{SF}GFP-ssrA by ClpAP (black circles) or ClpAPS (red circles). ClpAP (0.4 μM $ClpA_6$, 0.8 μM $ClpP_{14}$) degraded ^{SF}GFP-ssrA with a K_M of 7.8 μM and a V_{max} of 7.3 min^{-1} ClpAPS^{-1}. ClpS (2.4 μM) weakened K_M to 59 μM and reduced V_{max} to 1.6 min^{-1} ClpAP^{-1}. In the presence of the N-degron dipeptide Phe-Val (10 μM), ClpAPS degraded ^{SF}GFP-ssrA with a K_M of 167 μM and a V_{max} of 2.4 min^{-1} ClpAPS^{-1} (**b**) Degradation of the N-degron substrate YLFVQ-GFP by ClpAP (black circles) or ClpAPS (red circles). ClpAP (0.2 μM $ClpA_6$, 0.4 μM $ClpP_{14}$) degraded YLFVQ-GFP with a K_M of 98 μM and V_{max} of 8.4 min^{-1} ClpAP^{-1}. ClpS (1.2 μM) tightened the K_M to 2.1 μM (47-fold) and slowed V_{max} to 3.5 min^{-1} ClpAPS^{-1}. In both panels, values are averages (n = 3) ± 1 SD, and solid lines are fits to the Michaelis-Menten equation.

2.3.3 ClpS inhibits substrate unfolding and translocation

Following ClpAP binding and engagement, substrates must be unfolded and translocated through the axial pore of ClpA to allow entry into ClpP. We sought to determine if ClpS affects these mechanical activities, which require ATP hydrolysis. To do this, we studied degradation of a previously described multi-domain substrate, CFP-GFP-titin^{V15P}-ssrA (Figure 2-6a), in which the CFP and GFP domains have comparable time constants for enzymatic unfolding and translocation (Martin et al., 2008c). Because degradation of this substrate proceeds from the C-terminus to the N-terminus, GFP fluorescence is lost before CFP fluorescence, and the lag between the GFP and CFP curves depends on the rate at which unfolded GFP (~240 amino acids) is translocated and the CFP domain is unfolded. Notably, the lag for ClpAPS degradation was approximately twice as long as the lag for ClpAP degradation (Figure 2-6b), suggesting that ClpS slows translocation and/or unfolding. Moreover, single-molecule estimates of the average times for GFP unfolding and translocation by ClpAP indicate that the majority of the lag in both of the Figure 2-6b experiments is likely to represent translocation (Olivares et al., 2014). Indeed, the solid lines in Figure 2-6b represent a simulation in which ClpAPS both unfolded and translocated the GFP and CFP domains at half of the rate of ClpAP. These results support the idea that ClpS slows the mechanical unfolding and translocation of ssrA-tagged protein substrates. Consistently, when we

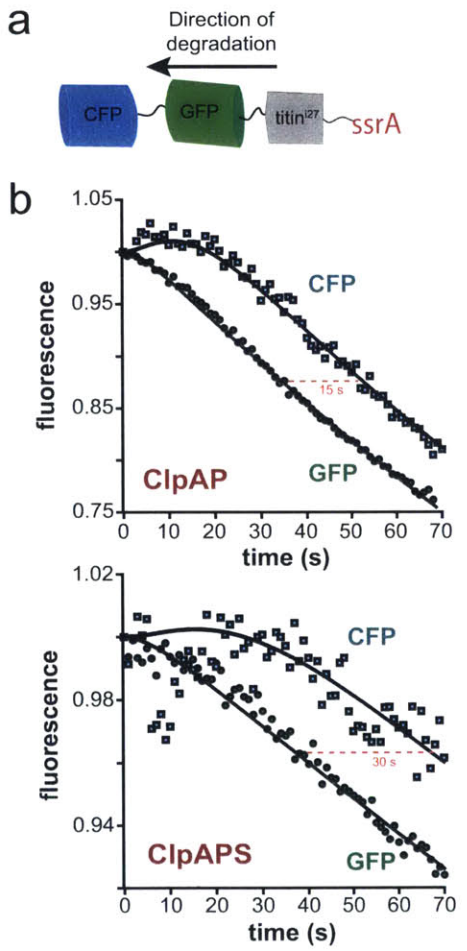

Figure 2-6 ClpS inhibits post-engagement mechanical steps during ClpAP degradation. (**a**) Cartoon of the multi-domain substrate CFP-GFP-titin^{V15P}-ssrA. (**b**) Degradation of the GFP and CFP domains of CFP-GFP-titin^{V15P}ssrA (0.5 μM) by ClpAP (4.5 μM $ClpA_6$, 9 μM $ClpP_{14}$) in the absence (top) or presence (bottom) of ClpS (27 μM). The curves shown are representative of three independent experiments. The lines are kinetic simulations for a model with first-order rate constants for binding, engagement, and degradation of the titin^{V15P} domain (k_1), for unfolding of the GFP/CFP domains (k_2), and for translocation of the GFP/CFP domains (k_3). For the ClpAP simulation, the values of k_1, k_2, and k_3 were 0.00435 s^{-1}, 0.25 s^{-1}, and 0.15 s^{-1}, respectively. For the ClpAPS simulations, these constants were 0.0012 s^{-1}, 0.125 s^{-1}, and 0.075 s^{-1}, respectively. The initial increase in CFP fluorescence results from loss of FRET upon unfolding of the GFP domain.

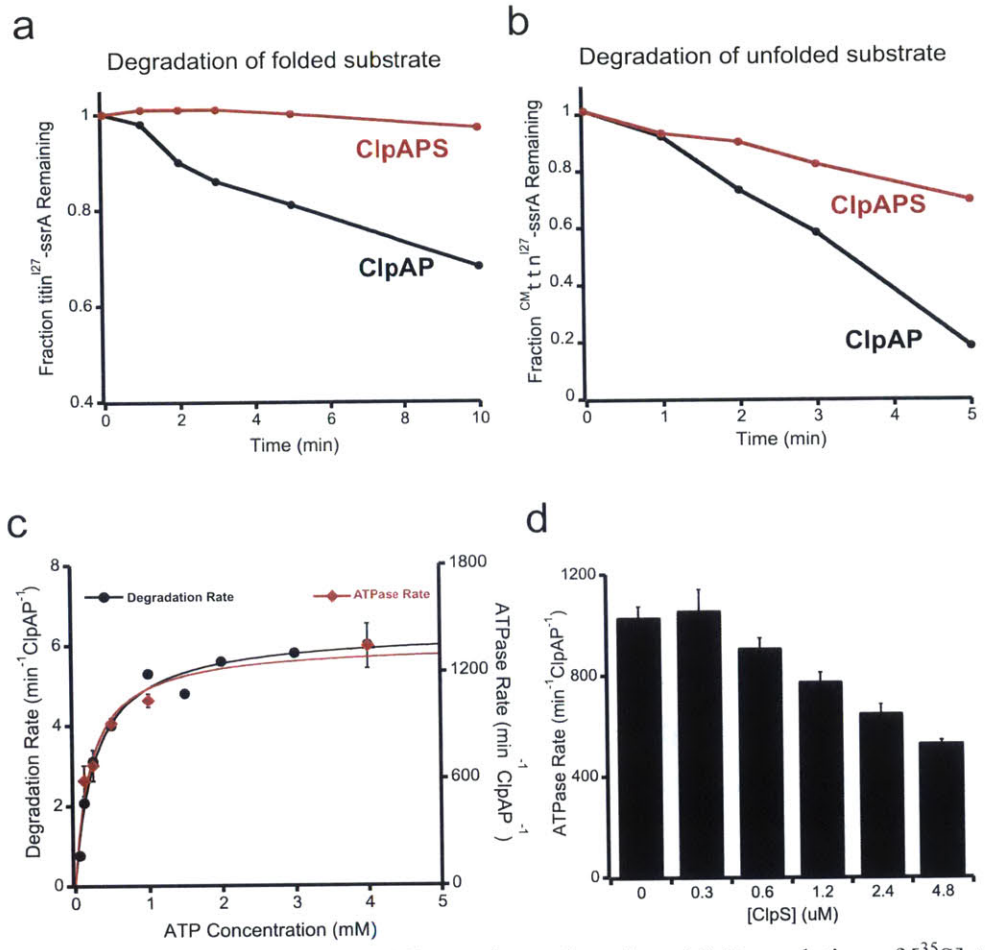

Figure 2-7 ClpS inhibits substrate undolding and translocation. (**a**) Degradation of [^{35}S]-titin^{I27}-ssrA (40 μM) by ClpAP (0.2 μM $ClpA_6$, 0.4 μM $ClpP_{14}$) in the absence (black circles) or presence (red circles) of ClpS (1.2 μM). (**b**) Degradation of [^{35}S]-^{CM}titin^{I27}-ssrA (40 μM) by ClpAP (0.2 μM $ClpA_6$, 0.4 μM $ClpP_{14}$) in the absence (black circles) or presence (red circles) of ClpS (1.2 μM). Data in (c) and (d) are representative of three independent experiments. (**c**) Covariation of rates of substrate degradation and ATP hydrolysis. Rates of ^{SF}GFP-ssrA degradation (black circles) and ATP hydrolysis (red diamonds) by ClpAP (0.4 μM $ClpA_6$, 0.8 μM $ClpP_{14}$) were determined at different ATP concentrations. Values for ATP hydrolysis rates are averages (n = 3) ± 1 SD. (**d**) Suppression of ClpAP ATPase rate by ClpS. ATP hydrolysis rates by ClpAP (0.4 μM $ClpA_6$, 0.8 μM $ClpP_{14}$) were determined in the presence of 30 μM λ-ssrA at increasing ClpS concentrations. Values are averages (n = 3) ± 1 SD.

assayed ClpAP and ClpAPS degradation of a stable native substrate (titin^{I27}-ssrA) and the same protein unfolded by carboxymethylation of cysteines normally buried in the hydrophobic core (^{CM}titin^{I27}-ssrA) (Kenniston et al., 2003), ClpS reduced the rate of degradation of the native substrate more than that of the unfolded substrate (Figures 2-7a, 2-7b).

ClpS slowing of mechanical activities is likely to result, at least in part, from suppression of the rate of ATP hydrolysis of ClpAP (Hou et al., 2008). In support of this model, we found that the rates of ATP hydrolysis and degradation of ^{SF}GFP-ssrA were highly correlated over a wide range of concentrations of ATP (Figure 2-7c). Moreover, when we assayed ATP hydrolysis by ClpAP

in the presence of high concentrations of λ-ssrA, increasing concentrations of ClpS reduced ATPase activity ~2-fold (Figure 2-7d). Interestingly, concentrations of ClpS that did not lead to additional weakening of λ-ssrA binding did cause additional reduction in ATPase activity (compare Figures 2-3a and 2-7d), suggesting that these activities of ClpS are separable.

2.3.4 Inhibition requires ClpA access to the ClpS NTE

The length but not the sequence of the ClpS NTE is critical for inhibiting ClpAP degradation of ssrA-tagged substrates and for delivering N-degron substrates to ClpAP, with the latter activity requiring engagement of the NTE by the ClpA translocation machinery (Dougan et al., 2002b; Guo, 2002a; Hou et al., 2008; Rivera-Rivera et al., 2014; Roman-Hernandez et al., 2011). To test the importance of NTE access in inhibition, we fused H_6-tagged mouse dihydrofolate reductase to ClpS (H_6-DHFR-ClpS) (Rivera-Rivera et al., 2014). The H_6 tag of this protein serves as a degron for ClpA, but access to the full NTE is impeded unless ClpAP can unfold and degrade DHFR, which occurs slowly in the presence of methotrexate (MTX) (Lee et al., 2001a). Figure 2-8a shows ClpAP degradation of ^{SF}GFP-ssrA with and without wild-type ClpS (open and closed

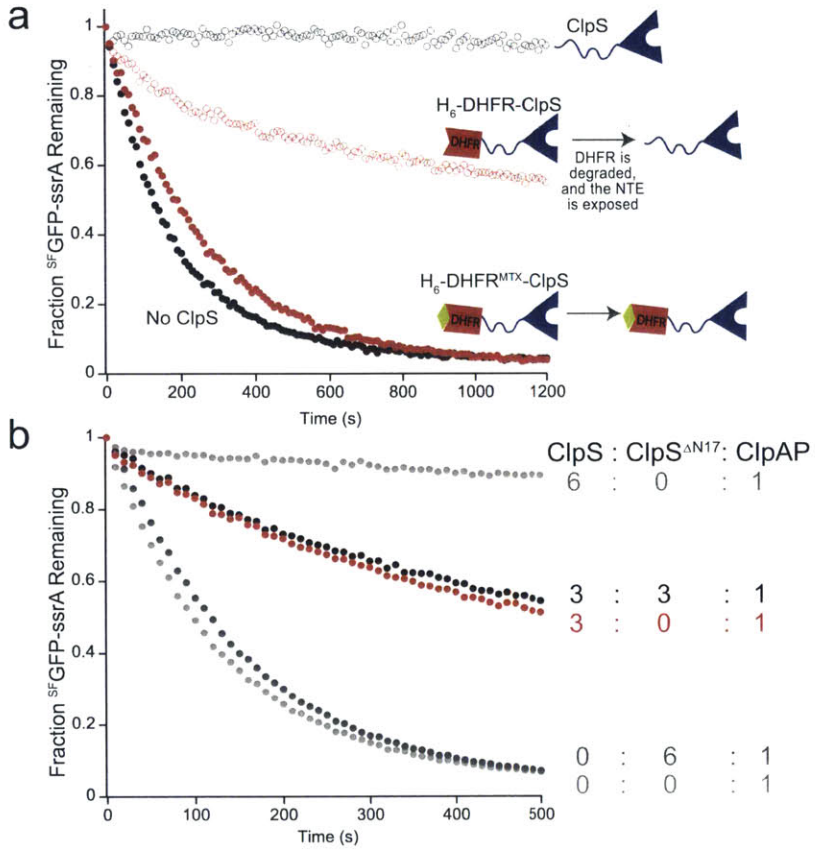

Figure 2-8. The NTE is critical for inhibition. (**a**) Degradation of ^{SF}GFP-ssrA (5 μM) by ClpAP (0.2 μM $ClpA_6$, 0.4 μM $ClpP_{14}$) alone (solid black circles) or in the presence of 1 μM ClpS (empty black circles), 1 μM H_6-DHFR-ClpS (solid red circles), or 1 μM H_6-DHFR^{mtx}-ClpS and 10 μM MTX (empty red circles). Data are representative of 3 independent experiments. (**b**) Inhibition of degradation of ^{SF}GFP-ssrA (5 μM) by ClpAP (1 μM $ClpA_6$, 2 μM $ClpP_{14}$) with ClpS alone (3 μM or 6 μM), ClpS^{ΔN17} alone (6 μM), or a mixture of ClpS (3 μM) and ClpS^{ΔN17} (3 μM). Curves are representative of 3 independent experiments.

black symbols, respectively) and with H_6-DHFR-ClpS in the absence or presence of MTX (open and closed red symbols, respectively). When MTX was present, H_6-DHFR-ClpS inhibited substrate degradation only marginally compared to no ClpS. In the absence of MTX, by contrast, inhibition by H_6-DHFR-ClpS required ~300 s to reach a steady-state level, which was less inhibitory than wild-type ClpS. Nevertheless, the delay in reaching steady-state inhibition by H_6-DHFR-ClpS suggests that the H_6-DHFR portion of the adaptor must be degraded before the NTE can be engaged by ClpA.

Four to six molar equivalents of ClpS per ClpA hexamer are required for maximum inhibition (Hou et al., 2008). To test if the same number of NTEs are required for inhibition, we performed ^{SF}GFP-ssrA degradation assays with mixtures of ClpS and the truncated ClpS$^{\Delta N17}$ variant, which binds ClpAP but fails to inhibit (Figure 2-8b). As observed previously (Hou et al., 2008), three ClpS molecules per $ClpA_6$ provided ~50% of the inhibition achieved with six ClpS molecules per ClpA hexamer. Strikingly, inhibition did not improve when three ClpS$^{\Delta N17}$ molar equivalents and three ClpS molar equivalents per ClpA hexamer were present. This result suggests that binding of ClpS cores to the N-domains of ClpA is important only because of their attached NTEs, which directly mediate inhibition.

2.3.5 The NTE is sufficient for inhibition

To test if the ClpS NTE is sufficient for inhibition, we initially constructed an NTE-ClpA$^{\Delta N}$ fusion (ΔN deletes ClpA residues 1-168, which are not required for degradation of ssrA-tagged proteins (Lo et al., 2001; Singh et al., 2001) but this protein was subject to severe autodegradation in the presence of ClpP. To minimize this problem, we constructed NTE-DHFR-ClpA$^{\Delta N}$ and DHFR-ClpA$^{\Delta N}$ chimeras (Figure 2-9a). The N- and C-termini of DHFR are close in space, allowing an attached NTE access to the ClpA pore (Figure 2-9a). When NTE-DHFR-ClpA$^{\Delta N}$ or DHFR-ClpA$^{\Delta N}$ was incubated with ClpP, ^{SF}GFP-ssrA, ATP, and MTX, some autodegradation was still observed as assayed by SDS-PAGE (Figure 2-9b). Importantly, however, ^{SF}GFP-ssrA was degraded by ClpP and DHFR-ClpA$^{\Delta N}$ but was not degraded by ClpP and NTE-DHFR-ClpA$^{\Delta N}$ (Figures 2-9b, 2-9c). Thus, the NTE can suppress degradation of an

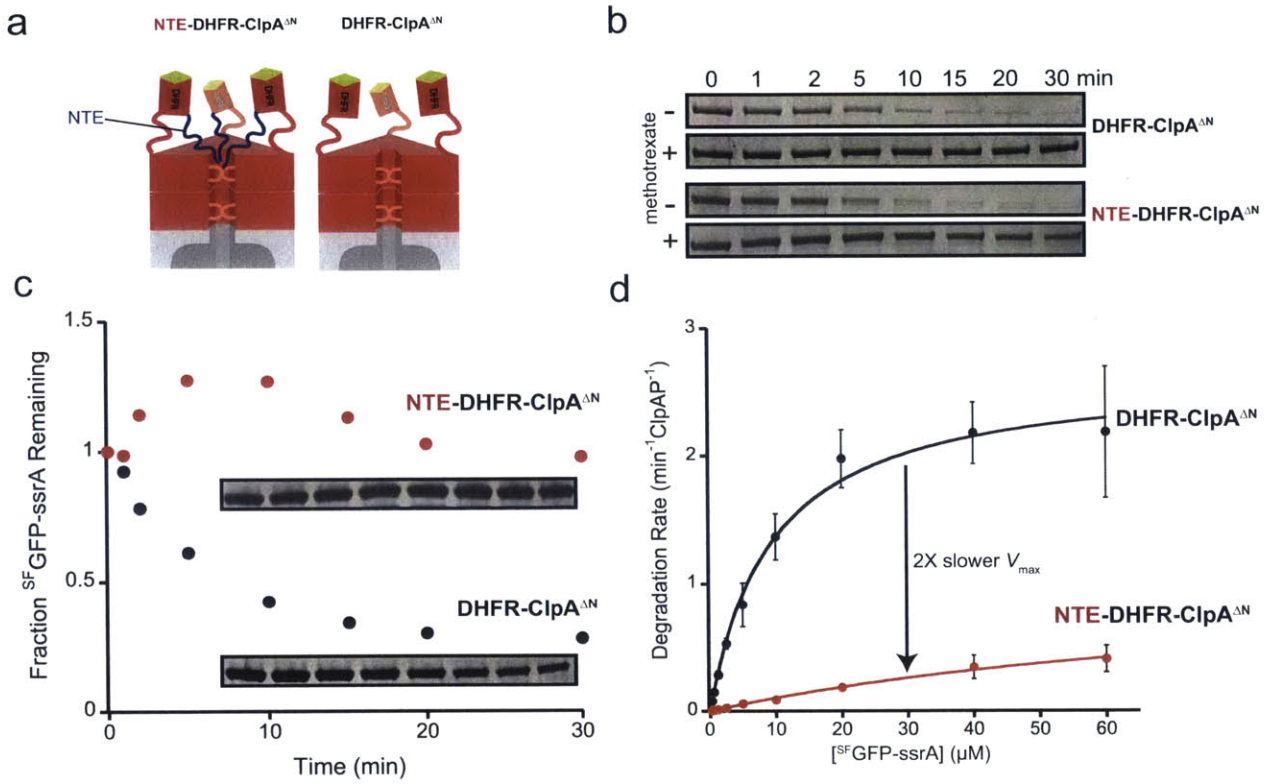

Figure 2-9 (**a**) The NTE is sufficient for inhibition. (**a**) Cartoons NTE-DHFR-ClpA$^{\Delta N}$P (left), or DHFR-ClpA$^{\Delta N}$P (right). (**b**) Autodegradation of NTE-DHFR-ClpA$^{\Delta N}$ or DHFR-ClpA$^{\Delta N}$ in the presence and absence of MTX (10 μM) assayed by SDS-PAGE. Experiments contained a ClpA variant (0.5 μM), ClpP (1 μM), and ^{SF}GFP-ssrA (20 μM) but only the ClpA variant band is shown. (**c**) Quantification kinetics of ^{SF}GFP-ssrA degradation from the plus MTX experiments described in panel **b** by densitometry of the SDS gels (shown as insets). (**d**) Steady-state kinetics of ClpP (0.8 μM) degradation of ^{SF}GFP-ssrA supported by DHFR-ClpA$^{\Delta N}$ or NTE-DHFR-ClpA$^{\Delta N}$ (0.4 μM each) with MTX (10 μM). For DHFR-ClpA$^{\Delta N}$ supported degradation, K_M was 9.5 μM and V_{max} was 2.7 min^{-1} enz^{-1}. For NTE-DHFR-ClpA$^{\Delta N}$ supported degradation, K_M was 94 μM and V_{max} was 1.1 min^{-1} enz^{-1}. Values are averages (n = 3) ± 1 SD.

ssrA-tagged substrate in the absence of both the core domain of ClpS and the N-terminal domain of ClpA. We also assayed the steady-state kinetics of ^{SF}GFP-ssrA degradation by ClpP in the presence of MTX and NTE-DHFR-ClpA$^{\Delta N}$ or DHFR-ClpA$^{\Delta N}$. The fusion enzyme containing the NTE displayed a 10-fold higher K_M and 2.5-fold lower V_{max} for degradation compared to the enzyme lacking the NTE (Figure 2-9d). These results parallel the inhibitory effects of ClpS and support a model in which the NTE is largely responsible for inhibition, with ClpS binding to the ClpA N-terminal domain simply positioning it properly for engagement by ClpA.

2.3.6 NTE length and the mechanism of inhibition

Previous studies established that ClpS bearing an NTE of nine residues ($ClpS^{\Delta N16}$) inhibits degradation of GFP-ssrA as well as wild-type ClpS, whereas deletion of one additional residue ($ClpS^{\Delta N17}$) results in essentially no inhibition (Hou et al., 2008). These experiments were performed under conditions where weakened substrate recognition was the major cause of ClpS inhibition. Using a set of ClpS variants with truncated NTEs of different lengths (Hou et al., 2008), we determined V_{max} values for ClpAPS* degradation of ^{SF}GFP-ssrA. As the length of the NTE increased from nine to 14 residues, there was an almost linear decrease in V_{max} (Figure 2-10). These results support a model in which inhibition of substrate recognition and inhibition of substrate unfolding and translocation via changes in the ATP-hydrolysis rate are mediated by slightly different parts of the ClpS NTE.

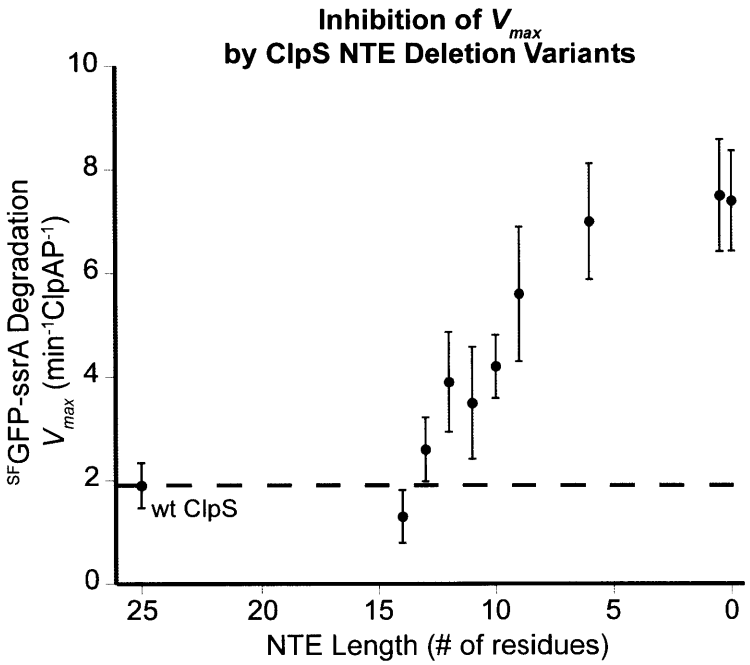

Figure 2-10. Effect of NTE length on V_{max} for degradation. ClpAP (0.4 μM $ClpA_6$, 0.8 μM $ClpP_{14}$) degradation of different concentrations of ^{SF}GFP-ssrA were determined by Michaelis-Menten analysis of experiments performed in the presence of NTE truncation variants of ClpS (2.4 μM). Values are averages (n = 3) ± 1 SD.

2.4 Discussion

AAA+ adaptors are typically described as facilitators of substrate recognition by their partner proteases (Baker and Sauer, 2006; Dougan et al., 2002a; Kirstein et al., 2009; Sauer and Baker, 2011). Although this paradigm holds for many adaptors, ClpS represents an interesting exception. In fact, ClpS appears to be unique, as it acts as an efficient stimulator of recognition of one class of substrates while strongly inhibiting degradation of other substrate classes (Dougan et al., 2002b; Hou et al., 2008). Most previous studies have focused on elucidating how ClpS acts as an enhancer of N-degron substrate degradation (Erbse et al., 2006; Hou et al., 2008; Rivera-Rivera et al., 2014; Roman-Hernandez et al., 2009; 2011; Stein et al., 2016; Wang et al., 2007; 2008a; 2008b). Our current work reveals new mechanistic aspects of the strategy that ClpS employs to modulate substrate degradation by ClpAP. We find that, in addition to modulating substrate recognition, ClpS affects catalytic steps of the ClpAP degradation cycle. Based on these results, our current view of how ClpS regulates ClpAP activity is depicted in the model shown in Figure 2-11. This model describes two general mechanisms that ClpS employs to reprogram the ClpAP protease. In the absence of ClpS, ClpAP preferentially degrades ssrA-tagged substrates compared to N-degron substrates (Hou et al., 2008). When ClpS binds the N-terminal domain of ClpA, it positions its unstructured NTE for recognition and engagement by ClpA, thus acting as a substrate mimic. As ClpA attempts to unfold and translocate ClpS, which

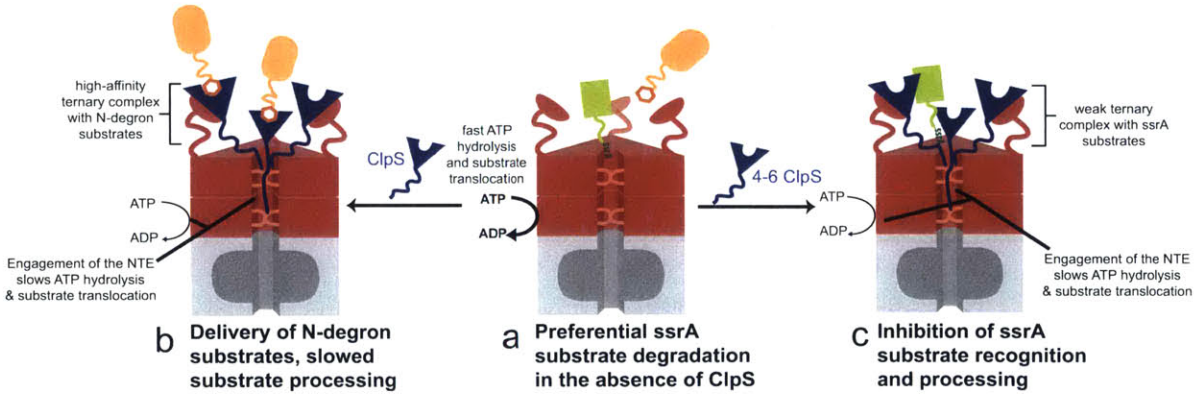

Figure 2-11 Model for reprogramming of ClpAP by ClpS. (**a**) In the absence of ClpS, ClpAP preferentially degrades ssrA-tagged substrates (green) relative to N-degron substrates (orange). (**b** and **c**) When the ClpS core binds the ClpA N-terminal domain, it positions the NTE for engagement by ClpA. NTE interactions with the translocation machinery suppress the rate of ATP hydrolysis by ClpA, slowing degradation. ClpS weakens recognition of ssrA-tagged substrates (c), while enhancing recognition of N-degron substrates (b).

resists degradation, ATP hydrolysis is slowed, delivery of N-degron substrates is enhanced markedly, but degradation of these substrates is slowed because of the reduced ATPase rate. The ClpAPS complex both binds and degrades ssrA-tagged substrates more slowly than ClpAP does. Thus, as in cases of kinetic proofreading (Blossey and Schiessel, 2008; Hopfield, 1974; Swain and Siggia, 2002; Yamane and Hopfield, 1977), ClpAPS efficiency is sacrificed to obtain higher specificity.

Our experiments reveal that ClpS is more than a simple binding switch for ClpAP substrate preference. Kinetic analysis of ^{SF}GFP-ssrA substrate degradation, as well as solution binding assays using λ-ssrA, demonstrate that ClpS weakens but does not prevent the binding of ssrA-tagged substrates to ClpA. This aspect of inhibition, along with the observation that inhibition of ssrA-tagged substrate recognition progressively increases as more ClpS molecules bind to the ClpA hexamer (Hou et al., 2008), suggests that ClpS can tune substrate recognition. For example, an increased ratio of ClpS to ClpAP in the cell could temporarily favor degradation of N-degron substrates without completely halting the degradation of ssrA-tagged substrates. In *E. coli*, the ClpS:$ClpA_6$ ratio is ~6:1 during exponential growth but shifts to ~2:1 during stationary phase, resulting in an increased capacity for degradation of ssrA-tagged proteins and other non-N-degron substrates (Farrell et al., 2005; Hou et al., 2008).

A second striking aspect of ClpS inhibition is the slowing of substrate processing. We found that ClpS decreases the maximal degradation rate of ssrA-tagged substrates. Importantly, this and previous studies show that the maximal degradation rate of N-degron substrates is also slower in the presence of ClpS (Wang et al., 2007). The ability of ClpS to suppress the rate of ATP hydrolysis by ClpAP ~2-fold (Roman-Hernandez et al., 2011) is probably responsible for the general slowing of substrate unfolding and translocation. Indeed, when we adjusted the ClpAP ATPase rate to 50% of the maximal rate by changing the ATP concentration, ^{SF}GFP-ssrA was also degraded at ~50% of the normal rate. Additionally, we demonstrated that inhibition of the degradation V_{max} and suppression of the ATPase rate (Roman-Hernandez et al., 2011) had very similar dependencies on the length of the ClpS NTE. This collection of evidence strongly argues that ClpS slows substrate processing by suppressing the ATPase rate of ClpAP. PinA, an adaptor that non-competitively inhibits substrate degradation by the Lon protease, also suppresses Lon

ATPase activity (Hilliard et al., 1998). Interestingly, adaptors like SspB, MecA and α-SNAP – which enhance substrate recognition by ClpX, ClpC and NSF, respectively – stimulate ATP hydrolysis of their partner AAA+ enzymes (Barnard et al., 1997; Schlothauer et al., 2003; Wah et al., 2003). Thus, modulation of ATP-hydrolysis rates seems to be a general strategy that adaptors employ to regulate their cognate AAA+ partners.

Slowing of the substrate translocation rate by ClpS is consistent with the idea that conformational changes derived from the cycle of ATP binding, hydrolysis, and product release cause axial pore loop movements that drive translocation (Martin et al., 2008b; Stinson et al., 2013). Efficient inhibition of V_{max} for degradation of ssrA-tagged proteins, ATPase rate suppression, and N-degron substrate delivery (Roman-Hernandez et al., 2011) all require an NTE of at least 14 residues. Furthermore, saturating ClpS suppresses ATP hydrolysis and the degradation of an unfolded substrate ~2-fold. Together, these observations suggest that bound ClpS slows translocation of all ClpA-engaged substrates.

We observe more than 2-fold inhibition of V_{max} for degradation of a natively folded ssrA-tagged substrate by ClpS, suggesting additional effects on substrate engagement and/or unfolding. The recent use of single-molecule optical-trapping methods to study ClpXP and ClpAP provides an opportunity to probe these steps. In particular, these experiments reveal distinct phases of degradation reactions. For example, by measuring pre-unfolding dwell times in the presence of ClpS (Olivares et al., 2016), single-molecule studies may clarify how unfolding is influenced by ClpS and if the magnitude of the effect depends on the stability of the native structure. This approach could also characterize more directly how ClpS slows substrate translocation, by allowing direct observation of step size, stepping frequency, back sliding, and pre-step pausing.

Previous studies have highlighted the role of the ClpS NTE for both the mechanisms of delivery and inhibition (De Donatis et al., 2010; Dougan et al., 2002b; Hou et al., 2008; Rivera-Rivera et al., 2014; Roman-Hernandez et al., 2011). The ClpS NTE, which lacks sequence conservation among orthologs, must be at least 14 residues long for efficient N-degron delivery, suppression of ClpA ATP hydrolysis (Roman-Hernandez et al., 2011), and efficient inhibition of V_{max} for degradation of ssrA-tagged substrates. Importantly, we find that the NTE must be actively

engaged by ClpA for inhibition, as is the case for N-degron substrate delivery (Rivera-Rivera et al., 2014). By fusing the NTE to ClpA, we established that the NTE is sufficient for raising K_M and decreasing V_{max} for degradation of ssrA-tagged substrates. Notably, an NTE-DHFR protein is a poor inhibitor *in trans* (data not shown), suggesting that the ClpS core plays two functions: (*i*) binding the ClpA N-terminal domain with tight affinity, and (*ii*) positioning the ClpS NTE for engagement by ClpA. Importantly, the ClpS core resists unfolding and degradation by ClpAP (Rivera-Rivera et al., 2014). Thus, we propose that ClpS acts as a substrate mimic. In fact, the NTE has been shown to act as a degradation signal when appended at the N-terminus of GFP (Roman-Hernandez et al., 2011), and mutations at the junction of the NTE and ClpS core can render ClpS susceptible to ClpAP degradation with the NTE functioning as the degron (Izarys Rivera-Rivera, personal communication).

The model described here may extend to other AAA+ adaptors. For example, the *Caulobacter crescentus* CpdR adaptor binds to the N-terminal domain of ClpX, enhancing degradation without interacting with specific substrates, and also recruits additional co-adaptors (Lau et al., 2015) by protein-protein interactions, which in turn deliver new substrates. If CpdR simulates the ATPase activity of ClpX, then activation of ClpX by CpdR could occur in an opposite but analogous manner to ClpS inhibition. CpdR passively inhibits recognition of some substrates in a manor that also has parallels with ClpS. Namely, both ClpS and CpdR can control access to the enzyme N-terminal domains, which are needed for efficient recognition of some substrates. Thus, regulation of ClpXP substrate preference may involve a multi-part mechanism in which degradation is globally stimulated but recognition of substrates that interact with the N-domain is temporarily inhibited to favor delivery of other substrates that use co-adaptors recruited by CpdR.

2.5 Methods

Strains and plasmids H_6-SUMO- $\lambda(1\text{-}93)^{A21C}$-ssrA was generated using the QuickChange Site-Directed Mutagenesis Kit protocol (Agilent). The cloned construct was inserted into a pET23b vector at the C-terminus of H_6-SUMO. To generate the NTE-DHFR-$ClpA^{\Delta N1\text{-}168}$ chimera, residues 1-26 of the ClpS NTE, followed by mouse dihydrofolate reductase (DHFR) were fused to the N-terminus of $ClpA^{\Delta N1\text{-}168}$ in a pET9a vector using standard cloning techniques. To generate DHFR-$ClpA^{\Delta N1\text{-}168}$, residues 1-26 of the ClpS NTE were deleted from NTE-DHFR-$ClpA^{\Delta N1\text{-}168}$.

Protein expression and purification. All proteins were expressed in *E. coli* strain BL21 (DE3) pLysS that had been transformed with appropriate plasmid vectors. ^{35}S-labelled $titin^{I27}$-ssrA was expressed and purified as described (Kenniston et al., 2003; Stein et al., 2016). Cysteines in ^{35}S-$titin^{I27}$-ssrA were carboxymethylated by incubation for 2 h with a 200-fold molar excess of iodoacetic acid in the presence of 5 M GuHCl (pH 8.9) at 25 °C. ClpA, NTE-DHFR-$ClpA^{\Delta N1\text{-}168}$, and DHFR-$ClpA^{\Delta N1\text{-}168}$ were purified as described (Hou et al., 2008). Briefly, after cell lysis, the cleared lysate was brought to 40% (w/v) saturated ammonium sulfate and centrifuged. The pellet was resuspended in S-Sepharose buffer (25 mM HEPES, pH 7.5, 2 mM DTT, 0.1 mM EDTA, 10% (v/v) glycerol) and centrifuged again. The supernatant was loaded onto an S-Sepharose column (GE Healthcare) and the protein was eluted in a gradient from 0.2 to 1 M KCl in S-Sepharose buffer. Peak fractions were combined and dialyzed into 50 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 0.3M NaCl, 10% (v/v) glycerol and 0.5 mM DTT. ClpP and ClpS were purified as described (Kim et al., 2000; Roman-Hernandez et al., 2011). After expression, H_6-SUMO-ClpS was purified by Ni-NTA affinity chromatography (Qiagen) and then cleaved with Ulp1 protease. A second round of Ni-NTA chromatography removed the H_6-SUMO fragment. ClpS was purified by gel filtration on a Superdex 75 column (GE Healthcare). ClpS was concentrated and stored in 20 mM HEPES (pH 7.5), 150 mM KCl, 1 mM DTT, and 10% glycerol (v/v). H_6-DHFR-ClpS was a gift from Izarys Rivera-Rivera (MIT). ClpS NTE deletions variants were a gift from Jennifer Hou (MIT).

After expression, H_6-SUMO-λ-ssrA fusion protein was purified by Ni-NTA chromatography

(Qiagen) in the presence of 8 M urea. Urea was removed and the protein was cleaved with Ulp1 protease. A second round of Ni-NTA chromatography removed the H_6-SUMO fragment. λ-ssrA was concentrated and stored in 10 mM Tris (pH 8), 100 mM NaCl, 1 mM DTT, and 10% glycerol. ^{SF}GFP-ssrA and CFP-GFP-ssrA were purified as described (Stinson et al., 2013) YLFVQ-GFP was a gift from Benjamin Stein (MIT).

Fluorescent Labeling. λ-ssrA or ClpS variants containing a single cysteine were labeled with fluorescein as described (Roman-Hernandez et al., 2011). Briefly, λ-ssrA or ClpS (25 μM) was incubated with 50 mM DTT in 100 mM TrisCl (pH 8) for 1 h at 4 °C, and then buffer-exchanged into 100 mM Na_2PO_4 (pH 8) and 1 mM EDTA. λ-ssrA was labeled with 0.3 mg/mL fluorescein-5-maleimide (Invitrogen) for 2 h at room temperature in the dark. Excess fluorescein maleimide was removed by size-exclusion chromatography. Fluorescently labeled λ-ssrA was stored in 20 mM Tris (pH 8), 150 mM NaCl, 1 mM DTT, and 10% glycerol. Fluorescently labeled ClpS was stored in 10 mM HEPES (pH 7.5), 200 mM KCl, and 1 mM DTT.

Biochemical assays. ClpAP and ClpAPS degradation assays were performed as described (Roman-Hernandez et al., 2011). Briefly, $ClpA_6$ (0.4 μM), $ClpP_{14}$ (0.8 μM), and ClpS or variants (2.4 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 (v/v) with substrate for 10 min at 30 °C before adding 16 mM ATP and a regeneration system (200 mg/ml creatine kinase, 20 mM creatine phosphate) to initiate degradation. For the YLFVQ-GFP degradation experiments, 0.2 μM $ClpA_6$, 0.4 μM $ClpP_{14}$, and 1 μM ClpS were used. Initial rates of degradation of different concentrations of ^{SF}GFP-ssrA or YLFVQ-GFP were assayed by loss of fluorescence (420 nm excitation; 540 nm emission), and data were fitted to the Michaelis-Menten equation to obtain K_M and V_{max}. ATP-hydrolysis rates were monitored using a coupled assay by following loss of NADH absorbance at 340 nm as described (Burton et al., 2001) under similar conditions used for the protein degradation assays. Reported values of kinetic parameters were averages (n = 3) ± 1 SD.

Solution binding. Binding assays were monitored by fluorescence anisotropy using a Photon Technology International Fluorimeter. λ^{*fl}-ssrA (0.15 μM) was incubated with different concentrations of ClpA and 2 mM ATPγS in the presence or absence of ClpS until equilibrium

was reached. Similarly, $ClpS^{*fl}$ (0.2 μM) was incubated with different concentrations of ClpA in the presence of 30 μM λ-ssrA until equilibrium was reached. Data were fitted to a hyperbolic binding isotherm using a non-linear least-squares algorithm. For anisotropy degradation assays, λ^{*fl}-ssrA was incubated with ClpAP and 2 mM ATPγS in the presence or absence of ClpS. Degradation was initiated by the addition of ATP and the regeneration mix.

Simulations. To simulate the decrease in GFP fluorescence during degradation of CFP-GFP-$titin^{I27}$-ssrA (CGT), we used a two-step CGT→CG→CU model. The first step has a rate constant (k_1) for pseudo first-order binding of the substrate by excess ClpAP and degradation of most of the $titin^{I27}$ domain to generate CG, which retains native CFP and GFP fluorescence. The second step has a rate constant (k_2) for unfolding of the GFP domain to generate CU, which retains native CFP fluorescence. Values of k_1 and k_2 for ClpAP and ClpAPS degradation were determined by fitting the decrease in GFP fluorescence using KinTek Explorer (Johnson, 2009), constraining k_2 for ClpAPS to $0.5 \cdot k_2$ for ClpAP. To model the decrease in CFP fluorescence, a four-step CGT→CG→CU→C→U mechanism was used, with k_1 and k_2 defined as above, k_3 representing translocation of the unfolded GFP domain, and k_4 representing unfolding of the CFP domain. To simulate the CFP data using Tenua (bililite.com), we increased CFP fluorescence by a factor of 1.7 upon unfolding of GFP, used k_1 and k_2 from the GFP fitting, set $k_4 = k_2$, and varied k_3 (with the value for ClpAPS constrained to $0.5 \cdot k_3$ for ClpAP) by trial-and-error until the experimental and modeled trajectories for ClpAP and ClpAPS degradation were similar.

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Chapter 3

Perspectives and Future Directions

3.1 Introduction and Overview

The ClpS adaptor binds to the N-terminal domain of the ClpAP AAA+ protease and delivers N-degron substrates while inhibiting degradation of ssrA substrates. The work presented here demonstrates that the ClpS adaptor employs a previously unknown mechanism(s) to reprogram its AAA+ protease partner. Kinetics and binding data reveal that ClpS forms a ternary complex with ClpA and ssrA substrates, thereby weakening ssrA binding affinity and slowing both N-degron and ssrA substrate processing. Furthermore, we identified substrate translocation as a step of the degradation cycle that is sensitive to ClpS inhibition. Analysis of ClpS mutants and ClpS-ClpA chimeras revealed that the ClpS NTE is sufficient for the mechanisms of inhibition, and that the NTE needs to be longer for efficient inhibition of substrate processing (14-residues long) than what was previously observed for inhibition of substrate recognition (9-residues long). Notably, this longer length required for inhibition of substrate processing is the same as that required for suppression of ClpAP ATPase rate. This is of particular interest, as our biochemical assays demonstrate that suppression of ATPase rate can slow substrate processing. This result is also consistent with the current working model for substrate processing by AAA+ unfoldases, in which conformational changes derived from ATP binding, hydrolysis and release drive translocation via axial pore loop movements. Thus, I propose that, by suppressing the ATPase rate of ClpAP, ClpS slows translocation of any substrate that is engaged. My current hyphothesis is that ClpS achieves this suppression by acting as a substrate mimic when ClpA engages the NTE and tries to unfold the highly stable ClpS core.

3.2 ClpS As a Substrate Mimic

The ClpS adaptor can be viewed as a pseudo-substrate. ClpS binds the N-domain of ClpA and positions its unstructured NTE for engagement by the ClpA translocation pore. The ClpS NTE, which has been shown to target GFP for degradation when appended to its N-terminus (Roman-Hernandez et al., 2011), is analogous to an unstructured degron. Interestingly, the identity of the NTE residues is not critical for ClpS function (Hou et al., 2008). Thus, this system is an interesting exception to the fundamental idea that specific sequences are required for recognition by AAA+ unfoldases. It is, however important to note that ClpA is known to recognize and engage unstructured polypeptides with little specific sequence preference This "loose"

recognition mode may be very helpful for ClpAP's function in degrading unfolded and damaged proteins (Hoskins et al., 2000). Interestingly, the ubiquitin-proteasome system can serve as an analogous example, as the proteasome binds ubiquitin but engages unstructured initiation sites that lack defined sequence motifs for efficient degradation (Finley et al., 2016).

Consistent with the idea that ClpS mimics substrate interactions with ClpAP, the tightly-folded core domain, which resists degradation (Rivera-Rivera et al., 2014; Roman-Hernandez et al., 2011), becomes susceptible to ClpAP degradation when mutations are introduced at the junction between the NTE and the folded core (Izarys Rivera-Rivera, personal communication). It is thought that the sequences near the ClpS core may cause ClpA to slip as it tries to unfold the highly-stable ClpS core structure. Interestingly, another feature of ClpS that parallels ClpAP degradation substrates is that engagement of ClpS by the translocation machinery results in decreased rate of ATP hydrolysis. This behavior has also been observed when ClpX tries to unfold hyper-stable structures (Burton et al., 2001; Kenniston et al., 2003). Thus, ClpS takes advantage of general features of the ClpAP degradation pathway to provide the means and mechanisms of inhibiting recognition and mechanical work to favor N-end rule substrate degradation over all other substrates. From an evolutionary perspective, it is interesting that cells can develop a regulatory mechanism through a pathway that is already in place (in this case, ClpAP substrate degradation), instead of having to develop additional allosteric mechanisms.

3.3 Comparisons Between Delivery and Inhibition

We show that while the ClpS delivery and inhibition mechanisms are clearly different, they share important features. In particular, the NTE plays a critical role in both mechanisms. First, the ClpS NTE, which is sufficient for inhibition but not delivery, needs to be engaged by the ClpA translocation pore for both delivery and inhibition. Moreover, we find that the same NTE length is required for inhibition of ssrA substrate processing and efficient substrate delivery. Thus, ClpS is able to use a similar set of interactions (NTE contacts with the translocation machinery) to regulate steps downstream of substrate recognition, even as the steps in specific substrate recognition of ssrA- versus N-degron are clearly distinct.

However, there are also several clear differences in the requirements for inhibition and delivery. A long linker between the N-domain and D1 domain of ClpA is critical for delivery (Roman-Hernandez et al., 2011) but dispensable for inhibition. Furthermore, a His at ClpS residue 66 in the ClpS N-degron binding pocket that is critical for N-degron substrate delivery but dispensable for inhibition. In fact, we find that specific contacts between the ClpS core and the AAA+ domains of ClpA are not necessary for inhibition of ssrA substrate degradation. We propose that the ClpS core instead plays a role in binding the N-domain of ClpA with of sufficiently tight affinity and properly positioning the NTE for engagement by the ClpA pore. Furthermore, the ClpS core resists degradation, allowing ClpS to perform its functions and be recycled for another round of regulation. These differences are in alignment with the different roles performed by ClpS. For example, for delivery, high specificity is desired, and is achieved by degron-adaptor contacts in the ClpS binding pocket. Additional interactions between ClpS and the N-domain of ClpA (i.e. ClpS His66 and ClpA N-domain linker) are likely to play a key role in transferring the substrate from the ClpS binding pocket to the ClpA translocation pore (Roman-Hernandez et al., 2011). Inhibition, on the other hand, is a lower specificity process, and its mechanism is therefore general enough to affect a wider range of ClpAP substrates.

3.4 Role of the D2 Ring of ClpA

Each ClpA subunit in a hexameric ring consists of an N-terminal domain followed by two AAA modules, termed D1 and D2. Previous studies established that the D2 domain is responsible for most of the ATPase and degradation activity, and that it is particularly required for processing substrates with high local stability (Kress et al., 2009). Notably, the D2 bottom AAA module is larger than D1, and is more homologous to the single AAA+ ring of ClpX, which is this enzyme's entire motor. The idea that the NTE requires a particular length for inhibition and delivery suggests that it needs to reach a specific binding site in the translocation pore. Previous structural analyses of ClpA and ClpC (a close relative of ClpA) estimated that the height of these double-ring hexamers is ~120 Å (Guo, 2002a; Wang et al., 2011). Based on the ClpC structure, I estimate that the height of the D1 domain in the hexameric structure of ClpC measures ~40 Å. Assuming an average length between each amino acid in an unstructured polypeptide of ~3.6 Å, a 14-residue NTE would be ~50 Å and a 9-residue NTE (which is too short for V_{max} inhibition) would be ~32 Å. Thus, it is conceivable that a 14-residue NTE is long enough to reach the D2

AAA domain whereas a 9-residue NTE is too short to reach D2. Furthermore, the same longer length is required for suppression of the ClpAP ATPase rate, which we establish is very likely a key part of the mechanism of inhibition. Thus, I hypothesize that the ClpS NTE needs to be engaged by the D2 ring of ClpA for efficient delivery and inhibition. For example, engagement of the ClpS NTE by D2 may bring the ClpS core into the entrance of the pore, a location that may be optimal for N-degron substrate transfer and for inhibition of engagement of any substrate that is not being delivered by ClpS. Slowing of the ClpAP machinery may be critical for ClpS to temporarily tune substrate preference by transferring an N-degron substrate bound to its pocket without displacing other substrates. For example, slowing axial loop movements may allow ClpA to have the "grip" on the NTE that it needs to be able to remodel the ClpS core for substrate release and transfer (Rivera-Rivera et al., 2014). Thus, because the D2 ring is responsible for most of the ATPase activity, it is very likely that ClpS needs to make critical contacts in or near D2 to slow ATPase activity. Moreover, it has been observed that ClpS leads to a 2-fold increase in ATPase rate of a variant lacking the D2 AAA+ domain of ClpA (Guo, 2002b), suggesting that contacts with D1 are unlikely to be responsible for the ClpS inhibitory activity.

Interestingly, unlike ClpA, other double-ring AAA+ proteins may primarily rely on the D1 ring. For example, the eukaryotic NSF AAA+ protein is a double-ring ATPase that disassembles SNARE complexes, allowing them to be recycled for more rounds of intracellular membrane fusion. Although the D2 ring is essential for hexamer formation and SNARE disassembly, D1 is the more active ATPase. These data, and similar results from characterization of other two-ring AAA+ motors are yet to give a clear, consistent picture of the key activities and functions of the two rings. Significant additional work on the mechanisms of the two ring enzymes is needed. For ClpAP, however I hypothesize that the double ring architecture provides a long axial pore, which provides space in the pore for interaction and tight griping of distinct substrate classes, as well as perhaps different interaction regions for its regulatory protein(s) to provide both activation of recognition of some proteins and suppression of catalysis and/or recognition of other proteins.

3.5 Are Multiple NTEs Engaged Simultaneously?

Six ClpS molar equivalents per ClpAP complex are required for maximal inhibition of ssrA substrate degradation, and lower stoichiometric ratios allow degradation of both N-degron substrates and ssrA substrates. For example, with 3 μM ClpS and 1 μM ClpAP, we observe ~50% of the maximal inhibition observed with 6 μM ClpS. Furthermore, we show that ClpS variants lacking the appropriate NTE length do not contribute to inhibition and that the NTE is sufficient for inhibition. Given that the NTE needs to be engaged by ClpA in order to inhibit (as blocking pore entry of the NTE by a stably folded protein prevents inhibition), these data suggest that the ClpA translocation pore simultaneously engages multiple NTEs. Simultaneous engagement of polypeptide chains covalently attached via disulfide linkages has been observed in experiments with ClpXP (Bolon et al., 2004; Burton et al., 2001) and the eukaryotic proteasome (Lee et al., 2002).

One way to address this number of NTEs question is to quantify single-turnover truncation of a ClpS variant with an NTE that is long enough to reach down to the ClpP proteolytic sites. Fortunately, a ClpS variant with a duplicated NTE (NTE_2-ClpS), has been characterized and used on studies testing engagement of the NTE by ClpA (Rivera-Rivera et al., 2014). When NTE_2-ClpS is incubated with ClpAP and ATP, cleavage of part of the NTE generates a truncated NTE_2-ClpS that can be resolved from NTE_2-ClpS in an electrophoresis gel. Comparing the amount of NTE_2-ClpS cleaved in a single round of degradation at different ClpS:ClpAP stoichiometric ratios (*i.e.* six ClpS:ClpAP versus one ClpS:ClpAP) would indicate if multiple NTEs are engaged simultaneously or if only one NTE is engaged per round of degradation.

3.6 Physiological Relevance of Inhibition

Protein degradation can be very expensive to the cell. For example, as Olivares and colleagues note, ClpXP degradation of the model substrate titin^{i27} consumes ~600 ATPs, whereas synthesis of a titin domain has an energetic cost that is comparable to the hydrolysis of ~400 ATPs (Olivares et al., 2016). Thus, ClpXP uses more energy to degrade a titin^{i27} domain than it is required for the synthesis of this protein. Notably, most of the ATP spent during degradation is consumed as the enzyme engages and attempts to unfold the substrate (Cordova et al., 2014;

Kenniston et al., 2003). Thus, it would be reasonable to assume that cells have evolved mechanisms to regulate how much ATP is invested in protein degradation. One example of this would be the *B.subtilis* MecA adaptor. MecA is required for ClpC assembly and ATPase activation but gets degraded along with the substrate it delivers. At low concentrations of MecA, ClpC falls apart, thereby shutting off ATP hydrolysis. In this system, MecA is thought to stay in complex with its cognate substrate throughout the degradation cycle. It remains to be seen if ClpS also stays bound to ClpAP for multiple rounds of substrate engagement and processing. The strategy that ClpS employs to regulate substrate degradation may seem like another example of regulating of ATP expenditure. This mechanism would ensure that ATP hydrolysis is maintained at a lower level than ClpAP's basal ATPase rate (which could be up to 1,200 ATP $min^{-1}ClpAP^{-1}$ *in vitro* (Roman-Hernandez et al., 2011). But the idea that ClpS acts as a "hyperstable substrate mimic" suggests otherwise. Whilst the rate of ATP hydrolysis decreases when ClpX tries to unfold substrates with high structural stability, more ATP molecules are spent in the process of trying to unfold these substrates (Kenniston et al., 2003). For example, Kenniston and colleagues observed that unfolding of wildtype $titin^{I27}$ by ClpX required more than 500 cycles of ATP hydrolysis, whereas unfolding of the native V13P variant, which has a less stable folded structure in the C-terminal region near the entry location for ClpXP, required fewer than 20 ATP cycles. Therefore, the ClpAPS system likely invests more ATP than ClpAP to ensure that N-degron substrate degradation is favored when needed. Thus, I propose that the biological role of inhibition must be based on the need to eliminate N-degron substrates at a specific time or under specific growth conditions. In fact, cells have multiple mechanisms to direct ClpAP to N-degron substrates; the ClpX adaptor SspB, while promoting degradation of ssrA-substrates by ClpXP inhibits degradation of these same substrates by ClpAP (Flynn 2001). Also, many α-proteobacteria evolved a more selective version of ClpS (ClpS2), whose expression increases during stationary phase (Stein et al., 2016). ClpS2, which both delivers some N-degron substrates and inhibits other substrates highlights the importance of having mechanisms fine-tune substrate recognition under certain conditions.

3.6.1 Physiological Relevance of N-degron Degradation in *E. coli*

The eukaryotic N-degron pathway has been implicated in many eukaryotic processes, such as meiosis, apoptosis, G-protein signaling, DNA repair, cell division, neurodegeneration and others

(Brower et al., 2013; Liu et al., 2016; Piatkov et al., 2012; 2014; Varshavsky, 2011). However, it is still unclear how N-degron substrates are generated. Notably, a general theme emerging from eukaryotic N-degron pathways is the degradation of peptides that result from cleavage by other proteases as part of these diverse processes. Indeed, destabilizing N-terminal residues (N-degrons) do not occur naturally after protein synthesis. In bacteria, almost all proteins are synthesized with formylmethionine at their N-terminus, which is a stabilizing residue in the N-end rule pathway. Methionine-aminopeptidase, the enzyme responsible for co-translationally removing this initiator Met, only recognizes substrates bearing small residues (Val being the largest) at Position 2 (Varshavsky, 2011). Therefore, N-degron substrates are likely generated as a result of post-translational cleavage events that reveal new N-terminal sequences that would otherwise be internal sequences.

The bacterial N-degron pathway has been extensively studied using model substrates (Erbse et al., 2006; Hou et al., 2008; Roman-Hernandez et al., 2009; Stein et al., 2016; Wang et al., 2007; 2008b). However, the physiological role of this pathway remains poorly understood and thus represents an area of opportunity for more research. Two bacterial N-degron substrates have been identified to date (Ninnis et al., 2009; Schmidt et al., 2009). One of them, *E. coli* putrescine amino transferase (PATase), catalyzes the aminotransferase reaction from putrescine to 2-oxoglutarate to generate L-glutamate and 4-aminobutanal as part of the L-arginine synthetic pathway; this enzyme therefore contributes to levels of poly-amines in the cell (Schmidt et al., 2009). Accumulation of poly-amines can be detrimental to the cell by inhibiting protein synthesis. PATase is post-translationally modified by leucyl/phenylalanyl- tRNA-protein transferase (LFTR) to generate an N-degron, which is recognized by ClpS and degraded by ClpAP. PATase is the only substrate with this N-degron modification that has been reported (Sauer and Baker, 2011). The stress response protein Dps (DNA protection during starvation) is the second proposed *E. coli* N-degron substrate, based on the presence of a destabilizing residue (Leu at position 6) that is at the N-terminus of a prevalent truncated variant of Dps which binds very well to ClpS. However, the physiological relevance of ClpAP degradation or the post-translational modifications that would generate the Dps N-degron are not clear, and ClpXP efficiently degrades this substrate *in vivo*.

Recent studies on *Arabidopsis thaliana* chloroplasts identified a novel Clp adaptor, ClpF, which together with ClpS1 delivers glutamyl tRNA reductase 1 (GluTR) to the ClpC-ClpP/R degradation machinery. Notably, ClpF, and not ClpS1, interacts with GluTR. ClpF and ClpS1 mutually enhance their interaction with the ClpC-ClpP/R machinery. This exciting new finding, suggests that ClpS may act as one subunit of a bipartite adaptor system (Nishimura et al., 2015). Thus, in this system, degradation of other, non-N-end rule substrates could potentially be enhanced or inhibited by ClpS. Thus, although our understanding of ClpS, and other adaptors, has moved dramatically forward in the last ~5-10 years, it seems likely that ClpS, and its interactors, still hold secrets regarding the multiple and sometimes complex means that have evolved to regulate/control protein turnover.

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