The ClpS Adaptor Mediates Staged Delivery of N-End Rule Substrates to the AAA+ ClpAP Protease

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SUMMARY

The ClpS adaptor delivers N-end rule substrates to ClpAP, an energy-dependent AAA+ protease, for degradation. How ClpS binds specific N-end residues is known in atomic detail and clarified here, but the delivery mechanism is poorly understood. We show that substrate binding is enhanced when ClpS binds hexameric ClpA. Reciprocally, N-end rule substrates increase ClpS affinity for ClpA. Enhanced binding requires the N-end residue and a peptide bond of the substrate, as well as multiple aspects of ClpS, including a side chain that contacts the substrate α-amino group and the flexible N-terminal extension (NTE). Finally, enhancement also needs the N domain and AAA+ rings of ClpA, connected by a long linker. The NTE can be engaged by the ClpA translocation pore, but ClpS resists unfolding/degradation. We propose a staged-delivery model that illustrates how intimate contacts between the substrate, adaptor, and protease reprogram specificity and coordinate handoff from the adaptor to the protease.

INTRODUCTION

The N-end rule relates degradation susceptibility to a protein’s N-terminal amino acid (Bachmair et al., 1986; Varshavsky, 2008). In bacteria, four N-terminal residues (Tyr, Phe, Trp, and Leu) serve as primary N-end degrons (Tobias et al., 1991). The ClpS adaptor binds these residues and delivers attached substrates to the AAA+ ClpAP protease for degradation (Erbse et al., 2006; Wang et al., 2007). In eukaryotes, a family of E3 ubiquitin ligases with a small region homologous to ClpS recognizes and covalently modifies N-end rule substrates with polyubiquitin, targeting these modified proteins to the proteasome (Lupas and Koretke, 2003; Tasaki and Kwon, 2007).

ClpAP, one of five degradation machines in Escherichia coli, consists of the ClpP14 protease and the ClpA6 unfoldase. ClpA6 is active as a hexamer composed of two AAA+ rings (D1 and D2) and also carries a family-specific N domain, which is flexibly attached to the D1 ring (Gottesman and Maurizi, 1992; Cranz-Mileva et al., 2008; Effantin et al., 2010). Using the energy of ATP binding and hydrolysis, machinery in the axial pore of ClpA unfolds and translocates protein substrates through this pore and into the ClpP14 chamber (Figure 1A; Hinnerwisch et al., 2005; Kress et al., 2009).

E. coli ClpS has a folded core domain (residues 26–106) and a poorly structured N-terminal extension (NTE; residues 1–25; Figure 1B; Zeth et al., 2002; Guo et al., 2002). Importantly, the NTE is required for delivery of N-end rule substrates, although it is not needed to bind substrates or ClpA, and shows little evolutionary sequence or length conservation (Hou et al., 2008) (see Figure S1 available online). Crystal structures are known for E. coli ClpS bound to the N domain of E. coli ClpA, and for E. coli or Caulobacter crescentus ClpS bound to peptides beginning with Tyr, Phe, Trp, and Leu (Zeth et al., 2002; Guo et al., 2002; Xia et al., 2004; Wang et al., 2008a; Román-Hernández et al., 2009; Schuenemann et al., 2009). In each N-end rule complex, the side chain of the N-end residue is completely buried in a deep hydrophobic pocket, and the α-amino group and first peptide bond make additional contacts with ClpS. Differences in E. coli and C. crescentus ClpS binding to N-end rule peptides have been proposed (Dougan et al., 2010), but we present evidence here for equivalent recognition by these highly homologous adaptors.

ClpS delivery of substrates to ClpAP must overcome several obstacles. For example, ClpS docks with the highly mobile N domain of ClpA, which could easily leave the substrate more than 80 Å from the axial pore of the D1 AAA+ ring, where unfolding/translocation initiates (Cranz-Mileva et al., 2008; Effantin et al., 2010). A similar issue occurs for the proteasome, where many substrates dock with receptors at sites far from the enzyme’s processing center (Striebel et al., 2009). Moreover, some experiments suggest that ClpS and ClpA both recognize the N terminus of N-end rule substrates (Wang et al., 2007). Because the N-terminal side chain is buried in ClpS, substrate handoff to the ClpA pore would need to be actively promoted. However, little is known about the factors that control interactions between N-end rule substrates, ClpS, and ClpA during substrate delivery.

Here, we dissect molecular interactions responsible for assembly of functional delivery complexes. We present evidence for complexes of ClpA6, ClpS, and substrate that differ markedly in stability and delivery activity. The most stable complex requires interactions mediated by the ClpS NTE, a ClpS residue that contacts the substrate N terminus, the substrate N-end.
Figure 1. N-End Rule Substrate Recognition

(A) In bacteria, the ClpS adaptor (light blue) recognizes and binds N-end rule substrates (pink) and delivers them for degradation by the ClpAP protease.

(B) (Top) ClpS has a flexible NTE required for N-end rule substrate delivery and a folded ClpScore domain, which binds N-end rule substrates. The ALKPPS sequence at the NTE-core junction is important for adaptor function. (Bottom) Backbone Ca superposition (rmsd < 0.5 Å) of ClpScore (3O1F, green), a peptide-bound ClpS structure (2W9R, red), and ClpS from a complex with the ClpA N domain (1R6O, blue).

(C) (Left panel) In the rerefined 2WA9 structure, the side chain of Leu22 from an adjacent ClpS subunit was bound in the N-end rule binding pocket, and density (1σ) for Leu22, Lys23, Pro24, and Pro25 was continuous with that for Ser26, Met27, Tyr28, and Lys29, which are part of ClpScore. (Right panel) The rerefined map for the 2WA9 structure contained density (1.5σ) for eight ClpS subunits in the asymmetric unit, arranged head to tail in a ring. The original 2WA9 structure (Schuenemann et al., 2009) had seven ClpS subunits, each with a bound N-end rule peptide.
residue and peptide bond, the AAA+ rings of ClpA, and a sufficiently long linker between the N and D1 domains of ClpA. Efficient substrate delivery also requires NTE residues, which appear to be engaged by the ClpA$_6$ translocation machinery. Our results support a model in which formation of a high-affinity delivery complex (HADC) reduces the mobility of the adaptor-bound substrate complex and positions the substrate’s N terminus close to the pore of the D1 AAA+ ring. This staged delivery mechanism illustrates an attractive general model to explain how substrates/adaptors that initially dock far from a AAA+ protease’s active center may be localized to the site where they are eventually processed.

RESULTS

ClpS Structures with N-End Rule Peptides Are Highly Conserved and Unstrained

E. coli ClpS has been extensively used for studies of function (Dougan et al., 2002; Erbse et al., 2006; Wang et al., 2007; 2008b; Hou et al., 2008; Román-Hernández et al., 2009; Schuenemann et al., 2009). However, a structure of the free protein had not been solved, leaving open the possibility that a conformational change occurs upon substrate and ClpA N-domain binding. Moreover, conflicting structures suggested that the details of N-end rule recognition might differ in potentially important ways between the E. coli and C. crescentus adaptors (Dougan et al., 2010). We crystallized E. coli ClpS$_{\text{core}}$ (residues 26–106) and solved the structure at 1.4 Å resolution (Table 1). The backbone structure was similar to previously reported E. coli ClpS structures bound to the ClpA N domain or to N-end rule peptides (Figure 1B; Zeth et al., 2002; Guo et al., 2002; Xia et al., 2004; Schuenemann et al., 2009). Thus, major changes in the conformation of the ClpS$_{\text{core}}$ domain do not accompany N-domain or N-end rule substrate binding.

Validation of our ClpS$_{\text{core}}$ structure by MolProbity (Davis et al., 2007) revealed excellent geometry (Table 1). By contrast, analysis of the 2WR9, 2WA8, and 2WA9 complexes of E. coli ClpS with N-end rule peptides (Schuenemann et al., 2009) revealed bad rotamers, poor bond angles, Ramachandran outliers, Cβ deviations, and unexpected cis peptide bonds (Table 1), which could arise if N-end rule peptide binding introduced strain or if these structures were incorrect. To resolve these issues and gain deeper insight into substrate recognition by ClpS, we rerefined these complexes, producing structures with no geometric anomalies and substantially improved refinement statistics (Table 1). Thus, binding of N-end rule substrates does not introduce strain within ClpS.

Rerefinement allowed us to identify and correct additional errors. For example, the 2WA9 structure purportedly contained a peptide with an N-terminal Trp side chain, which was interpreted to be poorly ordered although it should have been snugly bound in the ClpS hydrophobic pocket (Schuenemann et al., 2009). By contrast, in our rerefined structure, a well-ordered Leu$^{25}$ side chain from the NTE of a neighboring molecule occupied this pocket, with unambiguous electron density connecting the intervening residues to the adjacent ClpS$_{\text{core}}$ domain (Figure 1C). This same head-to-tail interaction was observed in eight ClpS molecules, which formed a closed ring in the asymmetric unit (Figure 1C). The original 2WA9 structure contained seven subunits in the asymmetric unit, and the NTE density was incorrectly interpreted as an N-end Trp peptide included during crystallization.

In the apo structure, the His$^{66}$ side chain occupied part of the N-end binding pocket. In the rerefinement complexes, the His$^{66}$ ND1 nitrogen hydrogen bonded to the α-amino group of the N-end residue, which required a 180° side-chain flip from the original structures, but the new position fit the electron density well and made better chemical sense (Figure 1D, Figure S2). In the rerefined 2WR9 structure, for example, the unprotonated ND1 nitrogen of His$^{66}$ accepts a hydrogen bond (1.9 Å; 170°) from a peptide -NH$_3$ proton, whereas the proton on the His$^{66}$ NE2 nitrogen donates a hydrogen bond (2.2 Å; 166°) to a side-chain oxygen from Glu$_{94}$ in a neighboring molecule (Figure S2). By contrast, when we added hydrogens to the original 2WR9 structure using REDUCE (Word et al., 1999), the nonpolar HD2 hydrogen of the His$^{66}$ ring clashed with a peptide NH$_3$ proton, a hydrogen bond between the peptide carbonyl oxygen and the proton on the NE2 nitrogen had poor geometry (2.6 Å; 117°), and the close interaction with the Glu$_{94}$ carboxylate involved a nonpolar hydrogen on the His$^{66}$ ring. Importantly, in the rerefinement complexes, contacts between E. coli ClpS and the N-terminal substrate residue were essentially indistinguishable from those observed in complexes of N-end rule peptides with C. crescentus ClpS, including hydrogen bonds with the side chains of Asn$_{34}$ (Figure 1D) and a water molecule that bridges the α-amino group and Asp$_{35}$ side chain (Wang et al., 2008a; Román-Hernández et al., 2009). We conclude that the mechanism of recognition of N-end rule peptides by ClpS is highly conserved.

Enhanced N-End Rule Affinity Requires the ClpS NTE and ClpA$_6$

The NTE is required for substrate delivery (Hou et al., 2008), but its functional role is obscure. We established that the NTE does not affect binary binding to N-end rule substrates, as intact ClpS and ClpS$_{\text{core}}$ bound to a fluorescent N-end rule peptide (LLY/QRoDSKEC-fl) with comparable affinities (~3 μM) in assays monitored by fluorescence anisotropy (Figure 2A). Strikingly, ClpS binding to this peptide was much tighter (~40 nM) in the presence of ClpA and ATP$_7$S, which stabilizes ClpA hexamers (Figure 2B). The final anisotropy value was also higher with ClpA present, as expected for slower tumbling of the larger ClpA-ClpS-peptide complex compared to a ClpS-peptide complex. Importantly, tighter binding was not observed when

(D) In the rerefined 2WR9 structure (right panel), the correct rotamer of the His$^{66}$ side chain makes hydrogen bonds (dashed lines) with the α-NH$_3$ group of the bound N-end peptide and fits nicely into the electron density. In the original 2WR9 structure (Schuenemann et al., 2009; left panel), His$^{66}$ rotamer chosen makes a poor hydrogen bond with the carbonyl oxygen of the first peptide residue and does not fit optimally into the electron density. In both panels, the electron density (1.25σ) is from our rerefined map.
ClpScore was used in place of intact ClpS, when the N domain of ClpA or ΔNClpA were used in place of ClpA, or when ATPγS was omitted (Figures 2A and 2B; data not shown). Thus, N-end rule binding by ClpS is substantially strengthened in an NTE-dependent manner by interactions with the N domain and hexameric ring of ClpA6.

### Table 1. Refinement Statistics

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Numbers in parentheses represent values for the highest-resolution bin. Unit cell and data collection statistics for the rerefined structures are from Schuenemann et al. (2009). R<sub>r</sub> = S<sub>h</sub>S<sub>j</sub> |l(h)−<l(h)>| / S<sub>h</sub>S<sub>j</sub> |<l(h)>>.<l(h)> is the average intensity of all observations of l(h). R<sub>r</sub> = S<sub>h</sub> |F<sub>obs(h)</sub>|− |F<sub>all(h)</sub>| / S<sub>h</sub> |F<sub>obs(h)</sub>|<F<sub>calc(h)</sub>|, calculated over the 93%–95% of the data in the working set. R<sub>r</sub> free equivalent to R<sub>r</sub> except calculated over 5%–7% of the data assigned to the test set.<br><sup>a</sup>Favorable/allowed/disallowed Ramachandran angles, favorable rotamers, Cβ deviations, residues with bad angles, and the clash score (number of steric overlaps ≥ 0.4 Å per 1000 atoms) were calculated using Molprobity (Davis et al., 2007).<br><sup>b</sup>The original 2W9R and 2WA9 structures (Schuenemann et al., 2009) contain two additional C-terminal residues that are not present in the protein sequence. In our rerefined structures, there was no density for these “extra” residues, and the chain terminated with Ala<sup>106</sup> as expected.<br><sup>c</sup>As discussed in the text, it is possible that the polypeptide was cleaved between Ala<sup>31</sup> and Leu<sup>22</sup>.<br><sup>d</sup>The peptide sequence is listed as LLT in the pdb file but as WRSKGEELFTGV in Schuenemann et al. (2009). In our rerefined structure, there was no peptide. Instead, an N-terminal segment of a neighboring ClpS molecule occupied the binding pocket.<br><sup>e</sup>ANISOU records are included in the 2W9R PDB file, although the header reports no TLS groups.

ClpS<sub>binds</sub> ClpA<sub>ΔN</sub> More Tightly in the Presence of N-End Rule Peptides

We constructed ClpS<sup>+</sup> (C73V; C101S; E96C), which contains one surface-exposed cysteine and was fully active in multiple functional assays (data not shown), labeled it with fluorescein (ClpS<sup>F</sup>), and measured fluorescence anisotropy in the presence...
of increasing concentrations of ClpA<sub>6</sub> (stabilized by ATP·γ·S). The fitted K<sub>app</sub> value was ~180 nM (Figure 3A). Next, we assayed binding in the presence of excess LLYVRQDSKEC, a Phe-Val dipeptide, or Trp<sub>-CONH</sub>$_2$, all of which have good N-end rule side chains. In each case, ClpA<sub>6</sub> bound ClpS<sup>−</sup> with at least 9-fold more tightly than observed with a MLYVRQDSKEC peptide or in the absence of ligand (Figure 3A). Although binding was too tight to calculate accurate K<sub>app</sub> values, Trp<sub>-CONH</sub>$_2$ enhanced ClpA<sub>6</sub> affinity for ClpS<sup>−</sup> as well as the longer N-end rule peptides, indicating that the N-terminal residue and a few nearby atoms play the dominant role in enhanced binding.

If there are no ligand-induced conformational changes in ClpS, as the structures argue, then how does Trp<sub>-CONH</sub>$_2$ enhance ClpA<sub>6</sub> binding to ClpS? Because the side chain of Trp<sub>-CONH</sub>$_2$ would be buried in ClpS, binding stimulation might involve contacts between ClpA<sub>6</sub> and exposed main-chain atoms of Trp<sub>-CONH</sub>$_2$, or contacts between ClpA<sub>6</sub> and ClpS side chains whose conformations are stabilized by contacts with Trp<sub>-CONH</sub>$_2$. Therefore, we tested if His<sup>66</sup> in ClpS might participate in binding enhancement, as this residue contacts the substrate N terminus (Figure 1D), the H66A mutation increased K<sub>M</sub> and reduced V<sub>max</sub> for ClpAP degradation of N-end rule substrates (Wang et al., 2008a), and His<sup>66</sup> adopted different conformations in the apo and peptide-bound structures of E. coli ClpS (Figure 3B). Importantly, we found that peptide-free<sup>1666</sup>ClpS<sup>−</sup> bound ClpA<sub>6</sub> nearly as well as ClpS<sup><sub>−</sub></sub>, but peptide-bound<sup>1666</sup>ClpS<sup>−</sup> bound ClpA<sub>6</sub> ~9-fold more weakly than the parent (Figure 3C). Thus, this mutant does not form the high-affinity complex. Although the H66A mutation reduces N-end substrate affinity (Figure 3D), the experiment in Figure 3C was performed using a peptide concentration 35-fold higher than K<sub>app</sub> for<sup>1666</sup>ClpS·ClpA<sub>6</sub> binding, ensuring that most mutant ClpS molecules were peptide bound. We conclude that the His<sup>66</sup> side chain stabilizes a high-affinity complex of ClpA<sub>6</sub>, ClpS, and substrate.

De Donatis et al. (2010) reported that NTE deletion reduced binary ClpA<sub>6</sub> affinity. We found that NTE deletion in a ClpS<sup>−</sup> variant reduced binary ClpA<sub>6</sub> affinity ~10-fold and also reduced the anisotropy observed at binding saturation (Figure S3). These results show that the NTE helps stabilize the ClpS·ClpA<sub>6</sub> complex, both in the presence and absence of substrate, and suggest that the NTE-mediated interaction reduces the segmental mobility of ClpA<sub>6</sub>-bound ClpS.

**ClpS·ClpA<sub>6</sub> Collaboration Requires Mobility of the ClpA N Domain**

ClpS<sup>core</sup> docks with the ClpA N domain, which is linked to the D1 ring by a 26-residue tether. Using a ClpA variant with a four-residue tether (<sup>4</sup>ClpA; Cranz-Mileva et al., 2008) allowed us to probe the importance of linker length. In the absence of substrate, ClpS<sup>−</sup> bound ClpA<sub>6</sub> and <sup>Δ</sup>ClpA<sub>6</sub> equally well (Figure 3E), suggesting that the shorter linker does not prevent formation of the NTE-mediated contacts with ClpA<sub>6</sub>. Importantly, however, when binding was measured in the presence of N-end rule peptide, <sup>Δ</sup>ClpA<sub>6</sub> showed much weaker binding (Figure 3E). Thus, an N-D1 linker of sufficient length is important in forming stable ternary complexes with ClpS and N-end rule substrates. Analysis of the steady-state kinetics of ClpAPS degradation of YLFVQELA·GFP revealed a 7-fold weaker K<sub>M</sub> and 3-fold lower V<sub>max</sub> when <sup>Δ</sup>ClpA<sub>6</sub> was substituted for wild-type ClpA<sub>6</sub> (Figure 3F). Thus, the longer linker is also required for efficient substrate delivery to ClpAP. Using a different substrate, Cranz-Mileva et al. (2008) also found defects in degradation using <sup>Δ</sup>ClpA<sub>6</sub>, albeit significantly smaller than those shown in Figure 3F.

**Defining NTE Lengths Required for Efficient ClpS Delivery and Formation of High-Affinity Complexes**

To probe the importance of NTE length, we purified ClpS mutants with N-terminal truncations from 3 to 20 amino acids and assayed their ability to slow ATP hydrolysis by ClpA and to deliver YLFVQELA·GFP for ClpAP degradation (Figure 4A). Strikingly, a precipitous decline in delivery and loss of ability to suppress ATPase rates occurred over a very narrow truncation range defined by the Met-Leu<sup>13</sup> and Met-Ala<sup>14</sup> variants. To investigate this delivery defect in greater detail, we determined
the steady-state kinetics of ClpAP degradation of different concentrations of YLFVQELA-GFP in the presence of ClpS or the variants starting with Met-Leu\(^{13}\) and Met-Ala\(^{14}\) (Figure 4B). The Met-Leu\(^{13}\) mutant delivered the substrate with a 1.3-fold reduction in \(V_{\text{max}}\) compared to ClpS. By contrast, delivery by the Met--Ala\(^{14}\) variant displayed a 7.5-fold decrease in \(V_{\text{max}}\). These results demonstrate that the ClpS NTE must have a critical minimum length to promote efficient delivery to ClpAP.

We also determined \(K_{\text{app}}\) values for binding of several ClpS-NTE variants to a fluorescent N-end rule peptide in the presence of ClpA\(^6\)ATP\(^{g}\)S (Figure 4C). Mutants beginning at Asp\(^{20}\) or earlier formed relatively stable substrate complexes with ClpA\(^6\) (\(K_{\text{app}} = 560 \text{ nM}\)) versus \(K_{\text{app}} = 42 ± 6 \text{ nM}\) for wild-type). (E) An N-end rule peptide (LLYVQRDSKEC; 20 \(\mu\)M) enhanced binding of ClpS\(^{16}\) to ClpA\(^6\) but not to ClpA\(^{26}\), which has shorter linker between the N and D1 domains (Cranz-Mileva et al., 2008). (F) Michaelis-Menten plots showed that substituting ClpA\(^{26}\) (four-residue linker) for ClpA\(^{26}\) (26-residue linker) decreased \(K_M\) and \(V_{\text{max}}\) for ClpAP degradation (100 \(\mu\)M ClpA\(^6\) or ClpA\(^{26}\); 200 \(\mu\)M ClpP, 600 \(\mu\)M ClpS) of the N-end rule substrate YLFVQELA-GFP. Reported \(K_D\), \(K_M\), and \(K_{\text{app}}\) values are averages (n ≥ 2) with errors calculated as \(\sqrt{K_{\text{app}}/K_{\text{app}}^2/n}\).

**FeBABE Mapping Places the ClpS NTE Near the ClpA Pore**

To probe regions of contact between the NTE and ClpA, we attached FeBABE to residue 12 of ClpS\(^{16}\) (Figure 5A). In the presence of ascorbic acid and hydrogen peroxide, the Fe\(^{3+}\) atom in the NTE-bound FeBABE generates free radicals which can cleave regions of ClpA in close proximity. ClpS was mixed with ClpA containing a C-terminal FLAG tag in the presence of ATP\(^{g}\)S, ADP, or no nucleotide. After 30 min, cleavage was initiated, allowed to proceed for 30 s, quenched, and the products analyzed by SDS-PAGE. In presence of ATP\(^{g}\)S, FeBABE
cleavage resulted in two fragments of ~50 kDa and two of ~29 kDa either in the absence (Figure 5A) or presence (data not shown) of N-end rule peptides. No specific cleavage products were observed without nucleotide or with ADP, suggesting that cleavage requires ATP/S-dependent formation of ClpAP·ClpS complexes (Figure 5A).

Western blots using anti-FLAG antibody indicated that the larger FeBABE cleavage products corresponded to the C-terminal portion of ClpA and the smaller bands were N-terminal segments (data not shown). Edman sequencing of these products was unsuccessful. However, based on molecular-weight standards and fragments produced by cleavage before cysteines in the Y259C, K265C, and K268C variants of ClpA, FeBABE cleavage appeared to occur near ClpA residue 260 (Figure S4). Residues 259–268 are located near the axial pore of the D1 AAA+ ring of ClpA. The FeBABE-ClpS linkage would allow the reagent to reach regions of ClpA within ~12 Å of the site of NTE attachment. Mapping these potential contacts on a model of the ClpA hexamer suggested that the ClpS NTE could physically contact the central pore of ClpA and/or the top of the D1 ring (Figure 5B).

The NTE Is a Degradation Signal, but ClpS Resists ClpAP Proteolysis

ClpAP does not degrade ClpS (Dougan et al., 2002). Nevertheless, because the NTE makes contacts near the ClpA pore, we hypothesized that it might be engaged by the translocation/unfolding machinery. To test this model, we appended the mature NTE of *E. coli* ClpS (residues 2–26) to the N terminus of GFP and assayed degradation. Untagged GFP is not degraded (Figure 5C), with a $K_M$ of 16 μM and $V_{max}$ similar to other GFP substrates. These results support a model in which the ClpA pore can engage the ClpS NTE, but the ClpS C-domain resists proteolysis. To confirm that ClpS-core is refractory to degradation, we purified H$_6$-SUMO-NTE-ClpS-core, H$_6$-SUMO-ClpS-core, and YLFVQELA-GFP-NTE-ClpS-core fusion proteins and assayed ClpAP proteolysis. In each case, partial proteolysis was observed (Figures 5D and 5E), but Edman degradation of the resistant fragment demonstrated

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**Figure 4. A Minimal NTE Length Is Required for ClpS Function**

(A) ClpS variants (1 μM) with N-terminal truncations were assayed for delivery of YLFVQELA-GFP (1 μM) for ClpAP degradation (gray curve) and for effects on ClpAP ATP hydrolysis (blue curve) using 100 nM ClpA$_6$ and 270 nM ClpP$_{14}$ for both assays. Data points represent averages ($n = 3$) ± 1 SD. Each ClpS variant is named by the first wild-type residue in the construct. Those marked with an asterisk contain an additional N-terminal methionine and are therefore one residue longer than the labels indicate; these mutants were expressed as SUMO-fusion proteins and cleaved in vitro (see the Experimental Procedures) or were expressed as standard nonfusión proteins but retained the initiator Met (verified by mass spectrometry). The T4 and W7 variants were also marked * have an additional N-terminal methionine. Apparent affinity constants were 43 nM (wild-type ClpS), 100 nM (*L13), 130 nM (*A14), 83 nM (*V18), 130 nM (*D20), 290 nM (*L22), and 1500 nM (*S26).

(B) Michaelis-Menten plots of YLFVQELA-GFP degradation by ClpAP and ClpS or variants (100 nM ClpA$_6$; 200 nM ClpP$_{14}$; 600 nM ClpS or variants). Wild-type ClpS and *L13 ClpS (beginning Met$^{12}$Leu$^{13}$) supported roughly similar steady-state degradation kinetics, but delivery by *A14 ClpS (beginning Met$^{13}$Ala$^{14}$) resulted in a substantial decrease in $V_{max}$. Thus, the NTE must have a critical minimal length to support efficient substrate delivery. The solid lines are a global fit to a model in which the ClpS-substrate complex binds ClpA in an initial bimolecular step ($K_1 = 1.1$ μM) and then is engaged for degradation in a second unimolecular step ($K_2$), which depends on NTE length. In this model, apparent $V_{max} = F_{suicide} \cdot K_D/(1 + K_D)$ and apparent $K_D = K_P \cdot K_{pr}/(1 + K_{pr})$. For the fits shown, the $K_D$ value was 2.1 min$^{-1}$ and the $K_P$ values were 0.37 (wild-type ClpS), 0.74 (*L13 ClpS), and 9.2 (*A14 ClpS).

(C) Binding to an N-end rule peptide (LLYQVRDSKEC-fl; 150 nM) by complexes of ClpA with ClpS variants (1 ClpS per ClpA$_6$) showed that ClpS junction residues are important for formation of the HADC. Variants marked * have an additional N-terminal methionine. Apparent affinity constants were 43 nM (wild-type ClpS), 100 nM (*L13), 130 nM (*A14), 83 nM (*V18), 130 nM (*D20), 290 nM (*L22), and 1500 nM (*S26).
that ClpScore remained intact, as did an N-terminal tail of 45–50 amino acids before the core domain (Figure 5F). Tails of this length result when AAA+ proteases are unable to unfold a very stable domain in the midst of a multidomain substrate (Lee et al., 2001; Koodathingal et al., 2009), strongly supporting a model in which ClpAP cannot unfold or degrade the ClpScore...
**Assembly of the high affinity delivery complex (HADC)**

(A) Low affinity ternary complex

(B) High affinity delivery complex

Model for ClpA-driven disassembly of HADC and substrate delivery

(C) ATP dependent ClpA “pulling” on ClpS

(D) Substrate transfer and ClpS release

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**DISCUSSION**

The work presented here elucidates important new aspects of the molecular mechanism of ClpS delivery and ClpAP degradation of N-end rule substrates. Our current view of these processes is shown in the model of Figure 6, which begins with formation of a low-affinity ternary complex (LATC; Figure 6A), proceeds to a HADC (Figure 6B), and ends with active substrate handoff from ClpS to ClpA (Figures 6C and 6D). As illustrated in Figure 6A, uncoupled sets of binary contacts between ClpS and the N-end rule substrate and between ClpS core and the ClpA N domain stabilize the LATC. Formation of the HADC involves additional interactions mediated by junction residues of the NTE of ClpS, by His66 of ClpS, by the N-end residue of the substrate, and by the D1 ring of ClpA (Figure 6B). The ClpS substrate portion of the complex is highly mobile in the LATC, because of the flexible tethering of the N domain to the D1 ring of ClpA, but is constrained in the HADC by additional contacts with the D1 ring.

The properties of the LATC are based on previous studies of the interaction of ClpS with substrates or the N domain of ClpA. Our current work supports the existence of the HADC and defines many of its properties. For example, we find that degrons containing just the N-end residue and peptide bond enhance ClpS affinity for ClpA and are bound far more tightly by ClpS and ClpA together than by either individual protein. Moreover, high-affinity binding requires multiple regions of ClpS (including the junction region of the NTE and His66, which contacts the N-end residue of the substrate), as well as the AAA+ body of ClpA and a suitably long linker between the ClpA D1 ring and N domain. Consistent with the Figures 6A and 6B models, we find that the mobility of the ClpS portion of complexes with ClpA is higher when the NTE is absent. Work presented here and previously (Hou et al., 2008; Wang et al., 2008a; Schuenemann et al., 2009) shows that mutation of His66 or deletion of the NTE severely compromises ClpS delivery of N-degron substrates to ClpAP, indicating that formation of the HADC is a critical step in substrate delivery.

It is not currently known what parts of the AAA+ body of ClpA make contacts with the junction residues of the NTE or with His66 and the N-end residue in the HADC, but our studies set the stage for future experiments to define these interactions in greater molecular detail. FeBABE cleavage experiments do show that residues near the center of the NTE can contact the D1 AAA+ ring of ClpA, and given the size of ClpS, other contacts with the AAA+ body of ClpA would likely also be restricted to the D1 ring. For example, a residue in the D1 ring of ClpA could contact the His66 of ClpS and stabilize its interaction with the N-end residue, and facilitates transfer of the N-degron of the substrate to a site in the ClpA pore.

In addition to its role in delivery of N-degron substrates, ClpS binding prevents recognition and degradation of other types of substrates by ClpAP (Dougan et al., 2002). In the absence of N-end rule substrates, it would be counterproductive if ClpS bound ClpA too tightly, as this would preclude degradation of...
other substrates. However, we find that ClpS binds ClpA6 ∼10-fold more tightly when N-degron substrates are present, providing an elegant solution to this problem. Substrate-dependent affinity enhancement would help to ensure the formation of a ClpAPS complex when N-end rule substrates were available but also keep ClpAP largely free to perform other functions when these substrates were absent.

Our working model for substrate delivery culminates with engagement of the substrate N-degron by the ClpA pore (Figure 6). A key feature of this model is binding of a portion of the ClpS NTE in the ClpA pore (Figure 6B), allowing the translocation/unfolding machinery to pull on ClpS and facilitate transfer of the N-degron from ClpS to ClpA (Figures 6C and 6D). Although aspects of the transfer model are speculative, it accounts for many experimental observations. For example, we found that a truncated ClpS variant beginning at NTE-residue 13 mediated efficient substrate degradation, whereas deleting one additional NTE residue dramatically reduced delivery. The presence of the N-degron from ClpS to ClpA (Figures 6C and 6D). Although aspects of the transfer model are speculative, it accounts for many experimental observations. For example, we found that a truncated ClpS variant beginning at NTE-residue 13 mediated efficient substrate degradation, whereas deleting one additional NTE residue dramatically reduced delivery. The presence of the N-degron from ClpS to ClpA (Figures 6C and 6D).

Engagement of the NTE by the ClpA pore is also supported by our finding that appending the ClpS NTE to GFP, a protein which is not normally degraded, results in efficient ClpAP degradation. Despite NTE engagement, our results also show that the folded portion of ClpS resists ClpAP degradation. In combination, these results account for our observation that delivery-competent NTE truncations result in lower ClpA ATPase rates than delivery-incompetent truncations. For example, AAA+ unfoldases hydrolyze ATP more slowly during attempts to unfold a protein (Kenniston et al., 2003; Wolfgang and Weber-Ban, 2008), and the lower ATPase rates seen using delivery-competent NTE truncations are therefore consistent with failed ClpA attempts to unfold ClpS.

How could ClpA tugging on ClpS facilitate handoff of N-end rule substrates? Given that the NTE is distant from the ClpS substrate-binding pocket, an attractive model is that translocation-mediated pulling on the NTE deforms ClpS*trans, facilitating transfer of the N-end degron to a site in the ClpA pore (Figure 6C). This model requires independent recognition of the N-degron by ClpA, which is supported by the observation that ClpAP alone can recognize and degrade N-end rule substrates, albeit with relatively low $K_m$S compared to values obtained with ClpS (Wang et al., 2007). Moreover, in resisting unfolding, ClpS could slip from the grasp of ClpA, as observed for other difficult-to-unfold proteins (Kenniston et al., 2005), clearing the pore as a prelude to substrate degradation (Figure 6D). Experiments with the related ClpXP enzyme also reveal that multiple polypeptide chains can simultaneously occupy the pore (Burton et al., 2001; Bolon et al., 2004).

There are parallels between the Figure 6 model and the delivery of ssrA-tagged substrates to the ClpXP protease by the SspB adaptor. For example, one region of SspB binds the N domain of ClpX, another part of SspB binds to a segment of the ssrA degron, a different part of this degron binds to the ClpX pore, and each binary interaction is substantially weaker than the overall ternary interaction (Levchenko et al., 2003; Wah et al., 2003; Bolon et al., 2004; Martin et al., 2008).

Because the ssrA tag of the substrate is positioned in the pore of the ClpX AAA+ ring in the high-affinity complex, ATP-fueled translocation allows tag contacts with the adaptor to be broken at the same time that degradation is initiated. Assembly of increasingly stable macromolecular complexes frequently drives biological recognition. This mechanism provides directionality by proceeding downhill to a thermodynamic minimum but also results in an energy well from which spontaneous escape is difficult, creating a problem if the high-affinity complex is not the final product. For example, recombination catalyzed by MuA transposase is driven by increasingly stable protein-DNA complexes, which eventually must be disassembled in an ATP-dependent process by ClpX (Burton and Baker, 2005).

As shown here and previously, adaptor-mediated delivery of substrates to AAA+ proteases also involves a progression from low-affinity to high-affinity complexes. This type of assembly has several advantages. From a kinetic perspective, splitting the overall pathway into discrete bimolecular and unimolecular steps speeds assembly. For example, ClpS with bound N-degron substrate could initially dock with any of the six N domains of ClpA6. Moreover, these N domains are highly mobile, further increasing the chances for productive collisions. Subsequent assembly steps would then be unimolecular, allowing the use of relatively weak interactions to position the substrate/adaptor near the translocation machinery of ClpA6.

We propose that adaptors for AAA+ proteases will fall into two general categories. In one category, exemplified by SspB, enzymatic pulling on the substrate disrupts the HADC and initiates degradation. In the second category, exemplified by ClpS, enzymatic tugging on the adaptor destabilizes the HADC, allowing substrate transfer and degradation. Many adaptors that function by a ClpS-type mechanism are likely to be degradation resistant. For example, Rad23 facilitates interactions between ubiquitinated substrates and the proteasome and is refractory to degradation (Heessens et al., 2005; Fishbain et al., 2011). However, a ClpS-type mechanism could also work if the adaptor were degraded. Indeed, the MecA adaptor is degraded by ClpCP during substrate delivery (Turgay et al., 1998).

**EXPERIMENTAL PROCEDURES**

**Proteins and Peptides**

Mutants were generated by the QuikChange method (Stratagene) or PCR. ClpS, ClpS mutants, and substrates were initially fused to the C terminus of H$_2$-Sumo in pET23b (Novagen). Following expression, fusion proteins were purified by Ni-NTA chromatography (QIAGEN) and cleaved with Ulp1 protease. The cleaved H$_2$-Sumo fragment was removed by passage through Ni-NTA, and the protein of interest was purified by gel filtration on Superdex 75 (GE Healthcare) and/or ion-exchange chromatography on MonoQ. ClpS variants were concentrated and stored in 20 mM Hepes (pH 7.5), 150 mM KCl, 1 mM DTT, and 10% glycerol. ClpA and ClpP were purified as described (Hou et al., 2008). Trp-CONH$_2$ and Phe-Val (FV) were purchased (Sigma). All remaining peptides were synthesized by standard Fmoc techniques using an Apex 396 solid-phase instrument.

**Crystallization**

Crystals of E. coli ClpS$_{106-136}$ were obtained after 3 weeks at 20°C in hanging drops containing 0.5 μl of protein solution (7.5 mg/ml) and 1 μl of reservoir solution (0.2 M ammonium formate, 20% PEG 3350). Crystals were frozen without
additional cryoprotection, and X-ray diffraction data were collected on a Rigaku Micromax 007-HF rotating anode equipped with Varimax-HR mirrors, an RAXIS-IV detector, and an Oxford cryosystem. Data were processed using HKL-2000 (Otwinowski and Minor, 1997). Initial phases were obtained by molecular replacement using PHASER (Storoni et al., 2004) with E. coli ClpS 2W9R as a search model. The final structure was obtained by iterative model building using COOT (Emsley and Cowtan, 2004) and refinement using PHENIX (Adams et al., 2002), and had excellent geometry and refinement statistics (Table 1). Rerefinement of the 2W9R, 2WA8, and 2WA9 structures, using COOT and PHENIX, also produced structures with excellent geometry and substantially improved refinement statistics (Table 1).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four figures and can be found with this article online at doi:10.1016/j.molcel.2011.06.009.

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**ACCESSION NUMBERS**

Coordinates for the apo E. coli ClpS crystal structure (3Q1F) and the refefined cocrytal structures (3O2B, 3O2H, and 3O2O) have been deposited with the Protein Data Bank.


