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

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

Results

The ClpS NTE Acts in cis During Substrate Delivery. Multiple ClpS-substrate complexes can dock on the N-domains of a single ClpA hexamer (23–25). As established previously, an NTE of at least 14 amino acids is necessary for ClpS to deliver an N-degron substrate (Fig. L1) (23); however, whether the NTE acts in cis to deliver the substrate bound to its own ClpS molecule or in trans to activate delivery of a substrate bound to another ClpS molecule is unknown. The optimal ratio of ClpS to ClpA hexamer in the delivery complex has not been established, but many ratios yield functional complexes (6, 23, 24).

In one experiment (Fig. 2A, Left), ClpS and ClpSΔ13/M40A were mixed with ClpAP, an N-degron dipeptide [to promote formation of an HADC (19)], as well as the *N-degron substrate VLFVQELA-GFP. In this experiment, the functional NTE was provided by WT ClpS, whereas the *N-degron substrate only bound ClpSΔ13/M40A (20). If engagement of the NTE can work in trans, then *N-degron substrate delivery would be observed; however, if engagement of the NTE functions only in cis, then location that remodels the delivery complex to achieve substrate engagement (Fig. 1C) (19).

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

Significance

Adaptor proteins often regulate substrate selection by AAA+ enzymes, but the molecular mechanisms of adaptor-mediated substrate delivery are poorly understood. We find that an unstructured N-terminal extension (NTE) of ClpS, the adaptor that delivers N-degron substrates to the ClpAP protease, enters the ClpA translocation pore during substrate delivery and must be actively engaged for delivery to occur. ClpA engagement of the ClpS NTE promotes delivery of substrate bound to the same adaptor molecule. These results support a model in which ClpA remodels ClpS by translocating its NTE, triggering delivery of the N-degron substrate. Active remodeling of components in delivery complexes by AAA+ unfoldases and proteases is likely a widespread mechanism.


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the absence of a functional NTE in ClpS\(^{\Delta 13/M40A}\) would prevent degradation of the \(^{+}\)N-degron substrate. Upon addition of ATP, the \(^{+}\)N-degron substrate was not efficiently degraded (Fig. 2B, pink trace). Under conditions similar to those of the degradation experiments, fluorescence-anisotropy experiments established that a fluorescent-labeled ClpS\(^{\Delta 13/M40A}\) variant bound ClpA tightly (Fig. 2C). Thus, the lack of efficient degradation of the \(^{+}\)N-degron substrate was not caused by a failure of ClpS\(^{\Delta 13/M40A}\) to bind ClpAP. Rather, these data indicate that the NTE does not function in \textit{trans} to trigger substrate delivery.

To ensure that ClpS\(^{M40A}\) with a functional NTE is able to perform substrate delivery under the conditions of this assay, we mixed it with ClpS\(^{\Delta 13}\) (nonfunctional NTE), ClpAP, \(^{+}\)N-degron peptide, and \(^{+}\)N-degron substrate (Fig. 2A, Right). In this case, the \(^{+}\)N-degron substrate was efficiently degraded (Fig. 2B). Taken together, these experiments show that delivery requires a functional substrate-binding pocket and a functional NTE within the same ClpS molecule.

The ClpS NTE Physically Enters the ClpA Pore. Previous studies suggested a model in which N-degron substrate delivery requires engagement of the ClpS NTE by the ClpA translocation pore (Fig. 1C) (19, 23). To test this model directly, we used Förster resonance energy transfer (FRET) between a donor fluorophore, 5-(2-aminoethylamino)-1-napthalene sulfonate (EDANS), at the entrance of the ClpP proteolytic chamber (ClpP residue 17, adjacent to the bottom of the ClpA pore; ClpP\(^{ED}\)) (25) and an acceptor fluorophore (fluorescein) placed at different positions either along the ClpS NTE or on the surface of the folded domain (Fig. 3A). The calculated Förster radius for the EDANS-fluorescein pair is \(\sim 46\) Å. Based on the dimensions of ClpC, a close relative of ClpA, a distance of \(\sim 100\) Å separates the top of the ClpA pore from the ClpP neck (16). Consequently, robust FRET would only be expected if a fluorescein dye on ClpS were able to enter the ClpA pore.

When residue 5 of the ClpS NTE was labeled with fluorescein (ClpS\(^{5-Fl}\)) and incubated with ClpAP\(^{ED}\), N-degron substrate, and ATP\(\gamma\)S (Fig. 3B), FRET was observed between the donor and acceptor dyes. Excitation of the donor fluorophore in ClpP\(^{ED}\) increased acceptor fluorescence (525 nm) and decreased donor fluorescence (475 nm) (Fig. 3B, red trace) compared with the sum of the spectra of each component alone (Fig. 3B, gray trace). If this signal resulted from FRET between the NTE and ClpP\(^{ED}\), then a reduced signal would be expected if the fluorescein were placed at position 17 of the ClpS NTE, a more C-terminal location that should be farther from ClpP. Furthermore, little or no FRET would be predicted if the dye were attached to ClpS residue 96, near the N-degron-binding pocket and far from the NTE (Fig. 3D). This pattern of FRET signals was observed (Fig. 3C), suggesting our hypothesis that the ClpS NTE enters the ClpA axial pore with its N-terminal residues reaching close to the ClpA-ClpP complex junction.

To further test whether FRET between ClpS\(^{5-Fl}\) and ClpP\(^{ED}\) occurs because the NTE is located within the pore rather than on the surface of the enzyme, we repeated the experiment with donor dye at NTE position 5 in the presence of the solution quencher 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4-amino-TEMPPO), which has an anhydrous diameter of \(\sim 10\) Å and thus should not efficiently diffuse into the ClpA pore (26). Indeed, fluorescence of free ClpS\(^{5-Fl}\) was quenched \(\sim 30\%\) by 4-amino-TEMPPO, whereas quenching of the fluorescence of ClpS\(^{5-Fl}\) in complex with ClpAP\(^{ED}\) and substrate was \(\sim 5\%\) (Fig. 3D). Taken together, these results support a model in which the ClpS NTE enters the ClpA pore in the ClpAPS-substrate complex.

Increasing the Length of the ClpS NTE Results in Truncation by ClpP. As an orthogonal method to determine whether the ClpS NTE enters the ClpA pore during N-degron delivery, we constructed an NTE\(_2\)-ClpS variant with a duplicated NTE (Fig. 4A). We reasoned that if the NTE enters the ClpAP pore during N-degron delivery, then the longer NTE\(_2\) sequence would enter the ClpP chamber, where it can be cleaved by the ClpP active sites. Control experiments revealed that NTE\(_2\)-ClpS delivered the N-degron substrate \(\text{YL}F\text{V}O\text{E}\text{L}A\text{-GFP}\) for degradation, albeit somewhat less efficiently than ClpS (Fig. 4B). Importantly, during these time-course delivery experiments, the NTE\(_2\)-ClpS both delivered the...
antiproteases (27), the addition of methotrexate stabilized proteases involves for-

cis enzymes play important roles in the tag of DHFR served as a ClpA degron (19),

![Diagram](image)

**Fig. 2.** The ClpS NTE delivers N-degron substrates in *cis.* (A) Cartoon showing the protein variants for the mixing experiments performed to test *cis vs. trans* activation by the ClpS-NTS. Although present, the N-degron peptide (Phe-Val) is not depicted. (B) Degradation of the *N*-degron substrate (YLFVQELA-GFP) by ClpAP. Only when the full-length functional NTE and *N*-degron-binding pocket were present on the same ClpS molecule was this substrate efficiently degraded (*cis* delivery experiment, blue trace). The mixing experiments contained each of the ClpS variants shown in *A* (1.2 μM each), 1 μM of an N-degron peptide, and 1 μM of *N*-degron substrate. (C) Binding of fluorescein-labeled ClpS<sup>Δ13Δ40A</sup> (1.2 μM) to ClpA<sub>S</sub> in the presence of ATP<sub>S</sub> (2 mM), ClpS (1.2 μM), N-degron peptide (1 μM), and N*-degron peptide (1 μM), as assayed by fluorescence anisotropy (κ<sub>0</sub> = 112 ± 13 Nm<sup>2</sup>).</p>

N-degron substrate and was truncated by ClpP (Fig. 4 B and C). Truncation of the NTE<sub>2</sub>-ClpS depended on the ATP-driven translocation activity of ClpA, given that it did not occur either in the absence of ATP or with the poorly hydrolyzed analog, ATPγS (Fig. 4C). N-terminal sequencing of the smallest truncated ClpS species revealed removal of 19 amino acids of NTE<sub>2</sub>-ClpS, leaving the native ClpS sequence with 9 additional N-terminal residues originating from the NTE duplication (Fig. 4C). Such NTE<sub>2</sub>-ClpS cleavage by ClpP strongly supports the model in which the NTE of WT ClpS is engaged by the ClpA axial pore during delivery of N-degron substrates.

**Antagonizing NTE Engagement Inhibits N-Degron Substrate Delivery.** To probe whether entry of the ClpS NTE into the ClpA pore is required for delivery, we constructed a ClpS variant with mouse dihydrofolate reductase (DHFR) attached to the N terminus of the ClpS NTE (H<sub>6</sub>-DHFR-ClpS) (Fig. 5A). In our experiment, the N-terminal H<sub>6</sub> tag of DHFR served as a ClpA degron (19), and the DHFR domain of this substrate was unfolded and degraded by ClpAP, exposing the ClpS NTE (Fig. 5B, Left). As expected based on previous studies of DHFR degradation by AAA+ proteases (27), the addition of methotrexate stabilized DHFR and prevented truncation of the DHFR-ClpS chimera by ClpAP (Fig. 5B, Right).

Importantly, the H<sub>6</sub>-DHFR-ClpS adaptor promoted degradation of the N-degron substrate YLFVQELA-GFP in the absence of, but not in the presence of, methotrexate (Fig. 5C). Interestingly, H<sub>6</sub>-DHFR-ClpS stimulated degradation of YLFVQELA-GFP only after a lag of ~100 s, suggesting that degradation of the DHFR domain is a prerequisite for NTE engagement and subsequent substrate delivery (Fig. 5C). As expected, methotrexate did not inhibit WT ClpS delivery of YLFVQELA-GFP to ClpAP (Fig. 5C). Furthermore, H<sub>6</sub>-DHFR-ClpS assembled normally with ClpAP, ATPγS, and a fluorescent N-degron peptide (YLFVQELA<sup>Δ13</sup>) both in the absence and the presence of methotrexate (Fig. 5D). Thus, the degradation defect caused by blocking entry of the ClpS NTE into the ClpA pore appears to occur at a step after assembly of the initial substrate-adaptor-enzyme ternary complex.

Taken together, our experiments with the H<sub>6</sub>-DHFR-ClpS chimera demonstrate that preventing entry of the ClpS NTE into the ClpA pore inhibits delivery and degradation of ClpS-bound N-degron substrates. These results strongly support a model in which engagement and partial translocation of the ClpS NTE through the ClpA pore is an essential step in the delivery of N-degron substrates.

**Discussion**

Regulation of macromolecular complexes is commonly implemented by the formation of multiple weak binary interactions that synergistically stabilize the complex (19, 28). Stable complexes can serve as checkpoints in a sequential mechanism to enhance specificity, but also can make downstream steps slow or inaccessible if stabilizing interactions must be broken before the next step can occur. AAA+ enzymes play important roles in catalyzing both the remodeling and destabilizing macromolecular complexes, including the examples of severing microtubules and promoting both assembly and critical reaction transitions during RNA splicing (1, 2, 19, 28, 29).

Previous studies have established that adaptor-mediated recognition of several substrates by AAA+ proteases involves formation of a high-affinity complex between the enzyme, substrate, and adaptor (19, 30–35). The delivery complex consisting of ClpAP, N-degron substrate, and the ClpS adaptor is one such example (Fig. 1B) (19). Here we identify features of the interactions between ClpA and ClpS that are critical for releasing substrates from this high-affinity complex and thus enabling the downstream steps of unfolding and degradation. Our FRET and protein processing experiments demonstrate that the NTE enters the ClpA pore during substrate delivery. Importantly, these experiments also show that engagement of the NTE by the ClpA pore is essential for ClpS-mediated degradation of N-degron substrates. Consistent with these observations, previous experiments have established that the ClpS NTE can act as a ClpAP degradation tag when attached to other proteins (19).

Why is engagement of the ClpS NTE by the ClpA pore critical for transfer of the N-degron of the substrate from ClpS to the ClpA pore? At the simplest level, ATP-dependent translocation of the ClpS NTE through the ClpA pore pulls the folded domain
One speculative model posits that NTE-tugging by ClpA both distorts and inverts ClpS by at least transiently pulling out the β strand proximal to the NTE (β1 strand), which is part of a three-stranded β sheet (Fig. 6 A–C). Pulling this central strand out of the sheet and into the ClpA pore would flip the remaining ΔβClpS structure relative to ClpA (Fig. 6C), positioning the N-degron–binding pocket close to the axial pore for transfer (Fig. 6 B–D). In this model, ΔβClpS remains stably folded but has reduced N-degron affinity, facilitating transfer of the substrate to ClpA. This model also requires that ΔβClpS not be globally denatured and degraded by ClpAP, because it has been established that ClpS is not degraded during substrate delivery (19). There is precedent for this type of β strand extraction by AAA+ unfoldases; for example, we note that ClpXP initially extracts a terminal β strand from a sheet in GFP-ssrA without causing global unfolding (37, 38). Moreover, under some conditions, the extracted β strand appears to slip from the pore of the AAA+ unfoldase, allowing refolding to native GFP (38). For the ClpS-delivery model, we suggest that following transfer of the N-degron, a slipping event could also allow ΔβClpS to refold and thus restore native ClpS. This reaction would reinvert the structure and favor ClpS escape, because its affinity for ClpA is weaker without bound N-degron (19).

A strong prediction of any NTE-tugging model is that an NTE would only promote delivery of a substrate bound to the same ClpS molecule and would not influence delivery of substrates bound to different molecules of ClpS, even if they were bound to the same ClpA hexamer. Our results strongly support this cis-only aspect of ClpS NTE function, demonstrating that only N-degron substrates bound to a ClpS molecule with a functional NTE were degraded by ClpAP. These results support an NTE-pulling model and argue against models in which the NTE serves simply as an allosteric activator of ClpA (23). During substrate transfer, both the ClpS NTE and the N-terminal residues of the N-degron substrate may need to occupy the ClpA pore. We assume that these multiple polypeptide chains can be accommodated in the ClpA pore, given that experiments with the related ClpXP enzyme show that pore engagement of multiple polypeptides is possible (39).

Parallels can be drawn between our active handoff model and other protein-degradation systems. For example, the SspB adaptor delivers ssrA-tagged substrates to the ClpXP protease via the formation of a high-affinity ternary complex that involves interactions among SspB dimers, the N-domain(s) of ClpX, and obtained from the emission of the donor and acceptor proteins (gray line); and observed emission spectra characteristic of FRET obtained in reactions containing ATPγS, ClpSΔβγS, ClpAPβγS, and the N-degron substrate γFAD-titin I27 (red trace). The red arrow pointing up at ~525 nm denotes an increase in fluorescence of the acceptor fluorophore, and the red arrow pointing down at ~475 nm denotes the decreased signal of the donor fluorophore. (C) FRET was also observed when the experiment shown in B was repeated with ClpSΔβγγS as the acceptor molecule (Left, red). In contrast, no FRET was detected when the acceptor molecule was ClpSβγγS (Right, red). (D) ClpSΔβγS fluorescence was insensitive to the fluorescence quencher 4-amino-TEMPO when bound in a complex with ClpAPβS and N-degron substrate.
a segment of the ssrA-degron (5, 32, 33). In this case, the complex is broken, and initiation of substrate degradation proceeds when the ClpX translocation pore engages the ssrA-degron (5, 32, 35, 40). Translocation of this initiation region of the substrate serves to break interactions in the ternary complex, allowing degradation to begin and the adaptor to be recycled. An unstructured initiation region is also required for unfolding and allowing degradation to begin and the adaptor to be recycled. An ssrA-degron (5, 32, 33). In this case, the unstructured region within the substrate and a polyubiquitin tag.

The proteasome recognizes the ubiquitin tag and initiates degradation by the proteasome (41). Proteins are targeted to the proteasome by a two-part degradation signal consisting of a disordered region within the substrate and a polyubiquitin tag. The proteasome recognizes the ubiquitin tag and initiates unfolding at the unstructured region within the substrate. Once the proteasome has engaged its substrate, the polyubiquitin tag is cleaved off by deubiquitination enzymes, allowing recycling of ubiquitin. For ClpXP-SspB degradation of ssrA-tagged proteins and degradation of substrates by the proteasome, disassembly of the proteasome is broken, and initiation of substrate degradation proceeds when the ClpX translocation pore engages the ssrA-degron (5, 32, 35, 40). Translocation of this initiation region of the substrate serves to break interactions in the ternary complex, allowing degradation to begin and the adaptor to be recycled. An unstructured initiation region is also required for unfolding and allowing degradation to begin and the adaptor to be recycled. An ssrA-degron (5, 32, 33). In this case, the unstructured region within the substrate and a polyubiquitin tag.

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6 μM values proteins: Have engine, will work. were labeled Genome Res (100 nM), ClpP proteases: ATP-fueled machines of protein degradation. ATPase: assembly of SspB dimers with ssrA-tagged proteins and K (200 nM), ClpP Annu Rev Biochem peptides were synthesized by standard Fmoc technology.

were labeled by fluorescein maleimide as ± We thank A. Olivares, B. Stinson, J. Kardon, B. Stein, 1 SD. Formation of ClpS truncation products was monitored by SDS/PAGE and Western blot analysis as described previously (23).

Fluorescent Labeling. Peptides were labeled with fluorescein maleimide as described previously (20). Labeled ClpS variants and ClpA were labeled with fluorescein maleimide and EDANS maleimide, respectively, as described previously (19). In brief, ClpS variants (50 μM) and ClpP containing a single cysteine were incubated with 50 mM DTT in 100 mM TrisCl (pH 8) for 1.5 h at 4 °C, then buffer-exchanged into 100 mM NaPO₄ (pH 8) and 1 mM EDTA. The variants were then singly labeled by the addition of 0.3 mg/mL fluorescein maleimide or EDANS maleimide (Thermo Scientific) for 2 h at room temperature in the dark. Excess reagent was removed by size-exclusion chromatography, and the modified protein was stored in 10 mM HEPES (pH 7.5), 200 mM KCl, and 1 mM DTT.

FRET Experiments. FRET experiments were performed using a Photon Technology International fluorimeter. ClpA (200 nM), ClpS (200 nM), ClpP variants (200 nM), N-degron substrate VLYVQRDEKEC (500 nM) (44), ATP₈ (2 mM), and ADP-Quencher (10 mM), when necessary, were incubated for 10 min at 30 °C in reaction buffer (50 mM HEPES [pH 7.5], 300 mM NaCl, 20 mM MgCl₂, 0.5 mM DTT, and 10% glycerol) at 30 °C before obtaining a spectrum. Samples were excited at 336 nm, and emission scans were obtained from 400 to 600 nm.

Degradation Assays and Western Blot Analysis. ClpAP degradation assays were performed as described previously (44). In brief, ClpA (100 nM), ClpP₄ (200 nM), and ClpS variants (1 μM) were preincubated in reaction buffer (50 mM HEPES [pH 7.5], 300 mM NaCl, 20 mM MgCl₂, 0.5 mM DTT, and 10% glycerol) with VLYVQRDEKEC or VLYVQRDEKEC (1 μM) and methotrexate (10 μM, Sigma-Aldrich), when necessary, for 3 min at 30 °C, followed by the addition of ATP regeneration mix (8 mM ATP, 50 mg/mL creatine kinase, and 5 mM creatine phosphate) or ATP₈ (2 mM) to initiate the assay. GFP degradation was assayed by loss of fluorescence. Reported kinetic parameters were averages (n ≥3) ± 1 SD. Formation of ClpS truncation products was monitored by SDS/PAGE and Western blot analysis as described previously (23). In brief, samples were separated by SDS/PAGE, followed by an anti-ClpP Western blot. For cistrans experiments, degradation assays were conducted under the same conditions but with 1.2 μM ClpS or ClpS variants and 1 μM Phe-Val dipeptide.

Binding Assays. Binding assays, monitored by fluorescence anisotropy, were performed using a Photon Technology International fluorimeter. Data were fitted using a quadratic equation for tight binding. Reported Kᵦ values are averages (n ≥3), with errors calculated as SQRT[(Kᵦ – Kᵦ₀²)/n].

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