Dynamic and static components power unfolding in topologically closed rings of a AAA+ proteolytic machine

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In the Escherichia coli ClpX protease, a hexameric ClpX ring couples ATP binding and hydrolysis to mechanical protein unfolding and translocation into the ClpP degradation chamber. Rigid-body packing between the small AAA+ domain of each ClpX subunit and the large AAA+ domain of its neighbor stabilizes the hexamer. By connecting the parts of each rigid-body unit with disulfide bonds or linkers, we created covalently closed rings that retained robust activity. A single-residue insertion in the hinge that connects the large and small AAA+ domains forms part of the nucleotide-binding site uncoupled ATP hydrolysis from productive unfolding. We propose that ATP hydrolysis drives changes in the conformation of one hinge and its flanking domains and that the changes are propagated around the AAA+ ring through the topologically constrained set of rigid-body units and hinges to produce coupled ring motions that power substrate unfolding.

Mechanical force is used to drive vectorial work in many cellular processes. In organisms ranging from bacteria to humans, molecular machines of the ATPases associated with various cellular activities (AAA+) family couple energy derived from ATP binding and hydrolysis to the degradation, remodeling or movement of macromolecules1,2. These enzymes work as protein unfoldases, disaggregating machines, DNA and RNA helicases, macromolecular pumps, and microtubule motor and severing proteins. How these machines function is an active area of investigation.

E. coli ClpX is a hexameric AAA+ machine that unfolds protein substrates and translocates them into the degradation chamber of ClpP, a self-compartmentalized peptidase3 (Fig. 1a). Crystal structures of ClpX have been solved in an open helical conformation and as a ring hexamer4,5. Lock-washer and helical conformations of some AAA+ machines are thought to be the relevant species for biological function6–10. For ClpX, present evidence supports a functional role for the ring conformation, but it is unclear whether the ring opens during the conformational fluctuations that power protein unfolding or when multiple polypeptides need to be translocated during the degradation of disulfide-bonded substrates5. Another aspect of understanding ClpX function is determining which structural elements provide the flexibility to adopt the different conformations needed for machine function while maintaining a sufficiently rigid overall structure to apply the forces needed to unfold native proteins. Macromolecular machines typically consist of rigid components that are flexibly linked to allow them to move with respect to each other. For example, the pistons of an internal combustion engine connect to the crankshaft in a way that allows piston movement to rotate the crankshaft, which can then be coupled to many different kinds of mechanical work.

The AAA+ module of ClpX consists of a large domain (residues 65–314), a short hinge (residues 315–318) and a small domain (residues 319–424)1,11,12. In ring hexamers, the small AAA+ domains are on the periphery of the ring, whereas the large AAA+ domains surround an axial pore, which is lined by loops that help the enzyme grip and translocate substrates5,13–17 (Fig. 1b). Although ClpX subunits are identical in sequence, they adopt distinct conformations and roles during the ATPase cycle5,15,16,18. In four loadable subunits, a cleft between the large and small AAA+ domains provides a binding site for ATP or ADP, with nucleotide contacts made by each domain and by the intervening hinge (Fig. 1c). These subunits show a range of nucleotide-binding properties and modest changes in the nucleotide-dependent conformation of the hinge5,18. Two opposed unloadable subunits in the ClpX ring do not bind nucleotide because a radically different hinge conformation rotates the large and small AAA+ domains enough to destroy the binding cleft. Despite inherent asymmetry of the ring, each large AAA+ domain packs against the small AAA+ domain of the neighboring subunit in a nearly invariant manner5 (Fig. 1d,e). A static ring can therefore be viewed as six rigid-body units, each spanning two subunits, connected by six hinges (Fig. 1d).

Here, we ask whether a ClpX hexamer functions as a closed ring and whether the rigid-body packing between subunits is preserved throughout all of the different conformational changes that define the ATP-fueled mechanical cycle. Specifically, we engineered and assayed the functional effects of covalent ties that tightly restrict the rigid-body packing between neighboring large and small domains in hexamers. Notably, mutants constrained in this fashion had a topologically closed ring but mediated robust unfolding and degradation. These results, in combination with the properties of a hinge mutant,

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strongly support a model in which conformational changes that originate in one hinge and its flanking domains as a consequence of ATP binding or hydrolysis are propagated around the AAA+ ring through the topologically constrained set of rigid-body units and hinges, producing coupled ring motions that power substrate unfolding.

RESULTS

Tight subunit-subunit tethering does not affect activity

For the studies described below, we used ClpX<sup>ΔN</sup> variants that contain the complete AAA+ module but lack a family-specific N-terminal domain. ClpX<sup>ΔN</sup> is active in ClpP-mediated degradation of protein substrates bearing an ssrA degradation tag at the C terminus<sup>11,12,15</sup>. The N- and C termini of adjacent ClpX<sup>ΔN</sup> subunits, which span the rigid-body interface between subunits, could be connected by 20-residue peptide tethers (T<sub>20</sub>) with retention of full ATP-dependent unfolding and degradation activity in pseudohexamers composed of single-chain dimers, trimers or hexamers<sup>15,19</sup>. By covalently connecting the small AAA+ domain of one subunit to the large AAA+ domain of the next subunit, these peptide tethers fuse both elements of the rigid-body unit.

To investigate the importance of tether length and limit potential motions between the small and large domains of single rigid-body units, we constructed single-chain trimers with three ClpX<sup>ΔN</sup> subunits connected by tethers of four (T<sub>4</sub>) or zero (T<sub>0</sub>) residues for comparison with the T<sub>20</sub> single-chain trimer. The structural elements in these single-chain trimers included two complete rigid-body units (Fig. 2a). Two single-chain trimers form a pseudohexamer (Fig. 2b). As with the T<sub>20</sub> enzyme<sup>15</sup>, the T<sub>4</sub> and T<sub>0</sub> variants purified predominantly as pseudohexamers during gel-filtration chromatography (Fig. 2c). Irrespective of tether length, each variant had a similar basal ATPase activity (Fig. 2d). Moreover, these ATPase activities were all repressed modestly in the presence of ClpP and were all enhanced ~3-fold in the presence of an unfolded protein substrate (carboxymethylated titin-I<sub>27</sub>-ssrA<sup>20</sup>; Fig. 2d). Notably, each single-chain trimer supported similar rates of ClpP-mediated degradation of a GFP-ssrA substrate over a 40-fold range of substrate concentrations (Fig. 2e), and fitting of these data yielded similar K<sub>m</sub> and V<sub>max</sub> values (Table 1). ClpX unfolding of GFP-ssrA, which is a thermodynamically and kinetically stable protein, is the rate-limiting step in proteolysis<sup>21</sup>. Moreover, ClpXP degradation of this substrate requires highly coordinated unfolding and translocation driven by a high rate of ATP hydrolysis<sup>22,23</sup>. We concluded that the T<sub>4</sub> and T<sub>0</sub> tethers are compatible with all of the different conformational states adopted by ClpX during the machine cycles that drive substrate unfolding and translocation into ClpP.

The N- and C-terminal residues of adjacent ClpX<sup>ΔN</sup> subunits (residues 61–423) are directly fused in the T<sub>0</sub>-linked ClpX<sup>ΔN</sup> single-chain trimer. In the crystal structure of the nucleotide-bound ClpX<sup>ΔN</sup> hexamer (PDB 3HWS)<sup>5</sup>, the last ordered C-terminal position of each subunit (residues 413–415) was ~24 Å from the first ordered N-terminal position of the next subunit (residues 62–63). The ~10 residues missing in the crystal structure would be sufficient to span this gap between subunits in the T<sub>0</sub> ClpX<sup>ΔN</sup> single-chain trimer, but the linkage would be tight and would thus substantially constrain movement of the parts of the rigid-body unit.

Covalently closed ClpX rings are fully functional

As an additional method of limiting movement within the rigid-body units, we searched the crystal structure of the ClpX hexamer to identify pairs of residues where disulfide bonds could covalently connect the small and large domains of adjacent subunits. Two solutions with good geometry were found, one corresponding to the substitution of cysteines for ClpX residues Thr<sub>66</sub> and Pro<sub>388</sub>, and one corresponding to the substitution of cysteines for Glu<sub>109</sub> and Asn<sub>331</sub>. We initially constructed a T<sub>20</sub> single-chain dimer (Fig. 3a). Association of three of these dimers in a pseudohexamer should permit formation of three native disulfide bonds (Fig. 3b). Immediately after purification,
nonreducing SDS-PAGE showed a (T66C P388)–(T66 P388C) species corresponding to the reduced single-chain dimer (~80 kDa), as well as smaller amounts of disulfide-linked tetramers (~160 kDa) and disulfide-linked hexamers (~240 kDa; Fig. 3c, lane 2).

After catalyzing oxidation by addition of copper phenanthroline, we observed time-dependent changes in the concentrations of three disulfide-bonded species (Fig. 3c). The apparent molecular weights and kinetics of appearance and disappearance of these species allowed their assignment as disulfide-bonded linear tetramers (one disulfide), disulfide-bonded linear hexamers (two disulfides) and disulfide-bonded circular hexamers (three disulfides), respectively. These assignments were confirmed by following the kinetics of reduction by DTT, which showed that the circular hexamer was progressively reduced to form the linear hexamer, followed by the linear tetramer and finally the linker dimer (Fig. 3d). Notably, after oxidation, 90% to 95% of the protein was present as the disulfide-bonded circular hexamer. A small fraction remained as dimers or linear tetramers or hexamers, presumably because of competing cysteine modifications that prevented complete disulfide bond formation.

We also constructed and purified a T20-linked dimer with the E109C mutation in the first ClpXAN subunit and the N331C mutation substitution in the second subunit (E109C N331C)–(E109 N331C). This protein formed the disulfide bond circular hexamer in an ~90% yield after incubation with copper phenanthroline (Fig. 3e).

In the disulfide-bonded circular hexamers consisting of (T66C P388)–(T66 P388C) or (E109C N331C)–(E109 N331C) single-chain dimers, every subunit was covalently connected to both flanking subunits, either by a disulfide bond that fused a rigid-body unit or by a T20 tether (Fig. 3b). Both classes of disulfide-bonded circular hexamers hydrolyzed ATP at least as well as the reduced enzymes (Table 1). In addition, both disulfide-bonded enzymes supported ClpP-dependent degradation of GFP-ssrA, with steady-state rates and kinetic parameters essentially identical to the pseudohexamer formed by wild-type single-chain dimers (Fig. 3f and Table 1). We concluded that hexameric rings that are topologically closed by covalent linkage can accomplish all of the normal machine functions of ClpX, including ATP hydrolysis, recognition, unfolding, translocation of GFP-ssrA, and association and cooperation with ClpP during protein degradation.

**Table 1 Steady-state kinetic parameters for ClpXP degradation of GFP-ssrA substrates**

| ClpX variant | V_{max} (min^{-1} ClpX_{6}^{-1}) | K_{m} (µM) |
|--------------|---------------------------------|------------|
| ClpXAN-T20 | 1.3 | 1.8 |
| ClpXAN-T4 | 1.3 | 1.4 |
| ClpXAN-T0 | 1.5 | 1.6 |
| ClpXAN-N331C | 1.2 | 1.6 |
| Oxidized ClpXAN(T66C)-T20 | 1.2 | 1.9 |
| ClpXAN(P388C) | 1.2 | 2.7 |
| Oxidized ClpXAN(E109C)-T20 | 1.2 | 2.7 |
| ClpXAN | 1.1 | 1.5 |
| Oxidized T66C P388C ClpXAN | 0.9 | 0.7 |
| Oxidized T66C P388C E109C N331C ClpXAN | 0.27 | 1.8 |
| Oxidized T66C P388C E109C N331C ClpXAN | 0.1 | 0.6 |
| Oxidized T66C P388C E109C N331C ClpXAN | 0.7 | 0.8 |

*For oxidized enzymes, >90% of the protein was present as the covalently closed circular hexamer, as determined by SDS-PAGE. For oxidized enzymes, >75% of the protein was present as the covalently closed circular hexamer, as determined by SDS-PAGE.
the protein into the disulfide-bonded circular hexamer (Fig. 4a), a species with disulfide bonds connecting the two elements of each rigid-body unit. The ~3:1 mixture of disulfide-bonded circular and linear hexamers supported ClpP degradation of GFP-ssrA at rates only slightly lower than those of the ClpX\textsuperscript{Nt} parental enzyme (Fig. 4b and Table 1), supporting a model in which the disulfide bonds that lock each rigid-body unit in the hexameric ring are compatible with ClpX function. By contrast, when we fully reduced the T66C P388C enzyme by incubation with DTT, the $V_{\text{max}}$ for degradation of GFP-ssrA decreased by a factor of ~4 and the $K_m$ increased by a factor of ~20, compared with the oxidized enzyme (Fig. 4b and Table 1). This result suggested that the T66C and/or P388C mutations destabilize the interfaces between the parts of each rigid-body unit, resulting in slower unfolding and degradation, whereas formation of the disulfide bonds stabilizes these interfaces.

To constrain possible movements of the interfaces within rigid-body units further, we also constructed and purified a ClpX\textsuperscript{Nt} variant containing the T66C, E109C, N331C and P388C mutations, allowing formation of two disulfide bonds within each of the six rigid-body units. The purified enzyme again contained monomers and disulfide-bonded dimers, trimers, tetramers, pentamers and linear hexamers, but oxidation converted >90% of the protein into a circular hexamer (Fig. 4c). The oxidized T66C P388C E109C N331C enzyme supported ClpP degradation of GFP-ssrA with a $V_{\text{max}}$ ~30% of that of ClpX\textsuperscript{Nt} (Fig. 4d and Table 1). We also tested proteolysis of a circularly permuted GFP variant that is easier for ClpXP to unfold\textsuperscript{23}, cp7-GFP-ssrA, which was degraded with a $V_{\text{max}}$ ~70% of that of the ClpX\textsuperscript{Nt} enzyme (Fig. 4d and Table 1). Following reduction of the T66C P388C E109C N331C protein, by contrast, the rate of ATP hydrolysis was reduced by a factor of ~15 (Table 1), and the protein did not support ClpP-mediated degradation of GFP-ssrA or cp7-GFP-ssrA (data not shown).

**ClpP enhances unfolding by covalently closed ClpX hexamers**

The protein-unfolding activity of ClpX is enhanced in the ClpXP complex\textsuperscript{19,21}. This observation might be explained if ClpP binding stabilizes the rigid-body interactions between ClpX subunits and prevents opening of the ClpX ring. However, if this were the only mechanism by which ClpP affected unfolding, ClpP binding would not be expected to enhance the unfolding activity of ClpX variants in which

Figure 4 Closed hexamers with disulfide bonds across all rigid-body subunit interfaces. (a) Nonreducing SDS-PAGE showing end-point disulfide bond formation of T66C P388C ClpX\textsuperscript{Nt} before (No) and after (Yes) oxidation with copper phenanthroline. (b) Michaelis-Menten plots of GFP-ssrA degradation by ClpX\textsuperscript{Nt}, oxidized T66C P388C ClpX\textsuperscript{Nt}, or reduced T66C P388C ClpX\textsuperscript{Nt} pseudohexamer and ClpP\textsubscript{14}. (c) Nonreducing SDS-PAGE showing end-point disulfide bond formation of T66C P388C E109C N331C ClpX\textsuperscript{Nt} before (No) and after (Yes) oxidation with copper phenanthroline. (d) Michaelis-Menten plots of GFP-ssrA or cp7-GFP-ssrA degradation by ClpX\textsuperscript{Nt} or oxidized T66C P388C E109C N331C ClpX\textsuperscript{Nt} pseudohexamer and ClpP\textsubscript{14}. Bands in a and c were visualized by staining with Coomassie blue, and the leftmost lanes show the positions of molecular weight standards. Symbols in b and d represent averages of three independent measurements. Table 1 lists $K_m$ and $V_{\text{max}}$ values.
the subunit-subunit interfaces were already stabilized by disulfide bonding. To test this possibility, we devised an assay to monitor protein unfolding by ClpX in the absence of ClpP. The fluorescent protein Kaede undergoes photocleavage at a single site after exposure to UV light, changing the fluorescence properties of the native protein. We appended an ssrA tag to Kaede and found that photocleavage prevented refolding after denaturation, allowing unfolding of cleaved Kaede-ssrA by ClpX variants to be monitored by tracking the loss of native fluorescence (Fig. 5a).

ClpX\textsuperscript{SN} unfolded the cleaved Kaede-ssrA substrate ~250% more rapidly in the presence of ClpP than in its absence (Fig. 5b). However, ClpP also enhanced the rate at which the singly (T66C P388C) and doubly (T66C P388C E109C N331C) disulfide-bonded variants of ClpX\textsuperscript{SN} unfolded Kaede-ssrA (Fig. 5b). These results suggest that ClpP enhancement of ClpX unfolding is not mediated solely by stabilization of the closed ClpX ring.

**Translocation of multiple polypeptides by closed ClpX rings**

Concurrent translocation of two or three polypeptide chains through the axial pore of ClpX is required when ClpXP initiates degradation at internal sites or degrades disulfide-bonded substrates\textsuperscript{25-27} (Fig. 5c). Compared with normal translocation, this activity almost certainly requires substantial pore expansion, which might be accomplished by ClpX-ring opening. If so, covalently constraining the subunit-subunit interfaces would prevent degradation of disulfide-bonded substrates. To test this possibility, we assayed degradation of a disulfide-bonded variant of the N11C Arc-ssrA dimer by SDS-PAGE\textsuperscript{25}. Notably, ClpX\textsuperscript{SN} and an enzyme mixture with >90% disulfide-bonded circular hexamers of T66C P388C E109C N331C ClpX\textsuperscript{SN} supported ClpP-mediated degradation of this cross-linked substrate equally well (Fig. 5d).
We concluded that ClpX unfolding and translocation of multiple polypeptide chains does not require ring opening or other major distortions of the rigid-body packing units that form the major interface between subunits.

Hinge mutations uncouple ATP hydrolysis from unfolding

Our results indicated that the rigid-body units of the ClpX hexamer remain intact during ATP hydrolysis and the coupled processes of substrate unfolding and translocation, leaving the hinges between domains as the likely source of the nucleotide-dependent rearrangements that allow the ring to change conformation. In E. coli ClpX, the hinge sequence is Asn315-Glu316-Leu317-Ser318, and only Leu317, which contacts the nucleotide base, shows strong sequence conservation in orthologs. However, the length of the ClpX hinge is conserved, suggesting that changes in hinge length could be detrimental to function (Fig. 6a).

To test this hypothesis, we deleted Asn315 in the hinge (Δ315N) or duplicated Asn315 (+315N), purified the variants and assayed their activities. Asn315 was chosen as the hinge residue to mutate because it shows the greatest conformation variation in backbone dihedral angles in different ClpX subunits. Both mutants purified as hexamers (data not shown) and retained substantial ATP-hydrolysis activity in the presence and absence of substrate (Fig. 6b). In protein degradation assays with ClpP, Δ315N ClpX had ~10% of the wild-type $V_{\text{max}}$ in degrading GFP-ssrA (Fig. 6c) and ~70% of wild-type activity in degrading cp7-GFP (Fig. 6d). Notably, however, the +315N variant showed very low levels of degradation of GFP-ssrA or the less stable cp7-GFP substrate (Fig. 6d). In principle, this severely decreased degradation activity of the +315N enzyme could arise from defects in substrate recognition, unfolding, translocation and/or interaction with ClpP. We found, however, that +315N ClpXAN–ClpP degraded carboxymethylated titin-I27-ssrA at ~20% of the rate of ClpXAN–ClpP (Fig. 6e). Proteolysis of this unfolded protein requires recognition and translocation of the substrate into ClpP by ATP-dependent coupling (Fig. 6d). Thus, inserting one residue in the ClpX hinge did not prevent ATP hydrolysis, substrate binding or ClpP recognition, but it severely decreased protein-unfolding activity and also slowed translocation. These results support a critical role for the hinge in allowing ClpX to carry out these mechanical processes.

DISCUSSION

Cellular machines use an energy source, typically NTP hydrolysis or a proton gradient, to power a sequence of conformational changes that can be coupled to mechanical work. Thus, one challenge in deciphering a molecular mechanism is determining which macromolecular conformations participate in the functional machine cycle. Crystal structures provide a snapshot of accessible three-dimensional conformations, but biochemical studies are needed to determine whether these structures are functionally relevant. In the case of ClpX, crystal structures show that subunits can interact to form open structures with helical symmetry or closed hexameric rings that are fundamentally asymmetric. Here, we have engineered ClpX variants in which the hexameric ring is topologically closed. These rings hydrolyze ATP, recognize protein substrates, unfold and translocate these molecules (even when translocation of multiple polypeptide chains is required) and interact productively with ClpP to carry out energy-dependent protein degradation. In combination, these studies provide strong evidence that open-ring ClpX structures are not required for basic machine function or for protein unfolding and processive translocation and degradation, which can require hundreds of cycles of ATP hydrolysis and coupled conformational changes in the ring.

Rigid-body subunit contacts limit functional conformations

Except for differences in loops and side chain conformations, the three-dimensional structures of the large and small AAA+ domains of ClpX show little variation in different crystal structures and are also highly conserved in structures of the paralogous HslU, ClpA, FtsH, Lon and ClpC enzymes. Moreover, although crystallographic ClpX rings are asymmetric, the small AAA+ domain of every subunit packs against the large AAA+ domain of the neighboring subunit in a very similar rigid-body manner (ref. 5 and Fig. 1d,e). Indeed, we found that different covalent constraints across this subunit-subunit interface were compatible with ClpX function, demonstrating that the enzyme could adopt all of the different conformational states that comprise the functional reaction cycle without breaking or substantial distortion of the crystallographically observed rigid-body interfaces between subunits. These observations strongly support a model in which the rigid-body units undergo very little motion as ATP binding and hydrolysis drive ClpX through the different conformations needed to unfold and translocate protein substrates.

The hinge connecting ClpX domains has an important role

If the six rigid-body units in an E. coli ClpX ring remain mainly structurally invariant, as our results indicate, then conformational changes in the hinge (residues 315–318) that links the large and small AAA+ domains of each subunit (Fig. 6a) are likely to mediate the nucleotide-dependent ring motions required for generating the mechanical work of protein unfolding and translocation. Indeed, we found that inserting a single residue in the ClpX hinge uncoupled ATP hydrolysis from robust protein unfolding. ATP and ADP bind in clefts between the large and small AAA+ domains of the four loadable ClpX subunits (ref. 5 and Fig. 6a), with nucleotide contacts made by both AAA+ domains and one hinge side chain (Leu317 in E. coli ClpX). Hence, nucleotide binding, hydrolysis and dissociation could easily result in changes in the orientations of the large domain, hinge and small domain in a single subunit. These structural changes would then be propagated to the immediate flanking subunits through the rigid-body units, which would alter the next set of subunits, and so on. Moreover, because the ClpX ring remains topologically closed during function, the backbone torsion angles of the hinge residues in every subunit would be constrained to combinations compatible with ring closure. ATP hydrolysis in a single ClpX subunit is able to power protein unfolding and translocation. Topological closure and the conservation of rigid-body packing units would allow nucleotide-dependent changes initiated by ATP binding or hydrolysis in a single subunit to change the conformation of the entire ring in a concerted fashion.

Force generation requires a stable subunit interface

ClpX mutants, such as L381K, D382K and Y385A, with substitutions in the rigid-body interface between neighboring subunits are competent to hydrolyze ATP and bind ssrA-tagged substrates but cannot unfold protein substrates unless ClpP is also present. These results suggest that ClpP binding helps to stabilize the rigid-body interfaces between subunits in a ClpX ring and imply that the integrity of these interfaces is required for the efficient generation of mechanical force, perhaps by preventing substrate slipping during translocation-induced unfolding. Here, we found that the reduced form of the T66C P388C ClpXAN variant supported ClpP-mediated degradation of GFP-ssrA with a maximum velocity decreased by a factor of ~4 compared to the same enzyme after formation of disulfide bonds across the rigid-body subunit interfaces. Because protein unfolding is the rate-limiting step in GFP-ssrA degradation
by ClpXP\textsuperscript{21}, the T66C and/or P388C mutations must reduce the unfolding activity of the reduced enzyme, most likely by destabilizing the rigid-body interfaces between subunits. The reduced variant containing the T66C, E109C, N331C and P388C mutations was inactive in protein unfolding and degradation, but these activities were restored when the rigid-body interface between subunits was stabilized by disulfide bond formation.

The work presented here shows that ClpX functions as a closed hexameric ring, with critical subunit-subunit contacts established by rigid-body packing between the large and small AAA+ domains of neighboring subunits. Both the integrity of these rigid-body interfaces between subunits and the hinge sequences that link the six rigid-body units of the AAA+ ring have prominent functional roles in coupling ATP binding and hydrolysis to protein unfolding. It will be interesting to determine if these properties are shared by the AAA+-unfolding rings of other ATP-dependent proteases and disassembly chaperones, including Lon, FtsH, ClpAP, ClpCP, PAN–20S, the 26S proteasome, ClpB and Hsp104.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.E.G. and A.R.N. conducted all experiments. S.E.G., A.R.N., T.A.B. and R.T.S. contributed to experimental design, data interpretation and writing of the manuscript.

COMPETING FINANCIAL INTERESTS

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ONLINE METHODS

ClpXΔN, ClpP14 and Kaede-ssrA proteins. Covalently linked E. coli ClpXΔN dimers or trimers consisted of ClpX residues 61–423 connected by linkers of 20 residues (T20, ASGAGSEGGSSEGGSAT), four residues (T4, ASGS) or no residues (T0), and contained an N-terminal H6 tag. Genes encoding ClpXΔN variants were constructed using PCR mutagenesis in a pACYC vector (Novagen); the encoded proteins were expressed in E. coli BLR (DE3) cells and were purified as described. E. coli ClpP14 and GFP-ssrA were purified as described. PCR mutagenesis was used to append an ssrA tag to the C terminus of Trachyphyllia geoffroyi Kaede (P. Schwille). The Kaede-ssrA protein was expressed in E. coli BLR (DE3) cells and purified by Ni2+-NTA affinity chromatography (Qiagen) followed by size-exclusion chromatography on Superdex S-200 (GE Healthcare) in 25 mM HEPES-KOH, pH 7.5, 100 mM KCl, 10% (v/v) glycerol, 10 mM MgCl2, 1 mM DTT. Photoconversion of Kaede-ssrA was induced by exposure to 365 nm light for 2 h and monitored by absorbance at 540 nm.

Oxidations and reductions. Residue positions for engineered disulfides were identified using the program Disulfide by Design by searching the major subunit-subunit interface of the nucleotide-bound ClpX hexameric structure (PDB 3HWS). Before disulfide formation, ClpX proteins were buffer-exchanged into 50 mM Tris HCl, pH 7.5, 300 mM KCl, 10% (v/v) glycerol and incubated with 4 mM ATPγS, 4 mM MgCl2 and 10 µM ssrA peptide. Oxidation was initiated by addition of 20 µM copper phenanthroline and incubation at 25 °C. For time-course reactions, samples were removed and quenched by addition of 100 mM EDTA and 100 mM iodoacetic acid to alkylate unmodified cysteines. Samples were analyzed by nonreducing SDS-PAGE containing 6 M urea. For use in enzymatic assays, oxidation reactions were quenched by addition of 50 mM EDTA and the oxidized ClpX variants were purified by gel filtration on a Superpose 6 column (GE Healthcare). Time course reductions of disulfide-bonded proteins were carried out by incubation with 10 mM DTT at 37 °C. For enzymatic assays, reduction was carried out by incubation with 50 mM TCEP at 30 °C for 2 h followed by buffer exchange.

Assays. ATPase assays were conducted as previously described. Degradation of GFP-ssrA, cp7-GFP-ssrA and Kaede-ssrA was monitored by changes in fluorescence or by SDS-PAGE and carried out at 30 °C in 15 µl buffer (50 mM HEPES, pH 7.5, 100 mM KCl, 10% (v/v) glycerol, 10 mM MgCl2) containing 0.3 µM ClpX, 0.9 µM ClpP14 and an ATP regeneration system (5 mM ATP, 16 mM creatine phosphate and 0.032 mg ml−1 creatine kinase). SspB dimers were purified and pre-incubated at equimolar concentrations with Kaede-ssrA to prevent formation of unfolding-resistant oligomers. N11C′Arc-ssrA dimers were purified as described, and formation of the Cys11-Cys11′ disulfide bond was confirmed by electrophoresis on nonreducing versus reducing SDS-PAGE. Degradation of 10 µM disulfide-bonded N11C′Arc-ssrA dimers was done at 30 °C and monitored by nonreducing SDS-PAGE. Degradation of 20 µM carboxymethylated titin-I27-ssrA was done at 30 °C and monitored by reducing SDS-PAGE.

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