Mechanochemical basis of protein degradation by a double-ring AAA+ machine

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Molecular machines containing double or single AAA+ rings power energy-dependent protein degradation and other critical cellular processes, including disaggregation and remodeling of macromolecular complexes. How the mechanical activities of double-ring and single-ring AAA+ enzymes differ is unknown. Using single-molecule optical trapping, we determine how the double-ring ClpA enzyme from Escherichia coli, in complex with the ClpP peptidase, mechanically degrades proteins. We demonstrate that ClpA unfolds some protein substrates substantially faster than does the single-ring ClpX enzyme, which also degrades substrates in collaboration with ClpP. We find that ClpA is a slower polypeptide translocase and that it moves in physical steps that are smaller and more regular than steps taken by ClpX. These direct measurements of protein unfolding and translocation define the core mechanoochemical behavior of a double-ring AAA+ machine and provide insight into the degradation of proteins that unfold via metastable intermediates.

Enzymes of the ATPases associated with various activities (AAA+) family carry out critical mechanical tasks in all cells1,2. For example, AAA+ proteases catalyze ATP-dependent protein degradation to maintain protein homeostasis and quality control in organisms from bacteria to humans. The simplest of these proteolytic machines consist of a self-compartmentalized peptidase, in which the proteolytic active sites are sequestered within a barrel-like structure and a homohexameric, unfolding ring, in which each subunit contains a single AAA+ motor domain. In some ATP-dependent proteases, each subunit of the hexamer contains two AAA+ domains, which are organized into discrete, stacked rings in structures determined by EM and X-ray crystallography. The single or double AAA+ rings of these molecular machines recognize specific degradation-inducing sequences (degrons) in target proteins, exert an unfolding force when loops lining the axial pore grip and pull on the substrate, and then processively translocate the unfolded polypeptide into the associated peptidase for degradation (Fig. 1a). Related single-ring and double-ring AAA+ enzymes carry out diverse protein-remodeling functions. For example, the single-ring katanin and spastin enzymes sever microtubules, whereas double-ring enzymes such as NSF, p97 and ClpB or Hsp104 disassemble SNARE complexes, extract proteins from membrane channels and solubilize protein aggregates, respectively.

ATP hydrolysis by AAA+ proteases provides the energy to power protein degradation, but it is not known how double-ring AAA+ hexamers with a total of 12 ATPase active sites differ in mechanical activity from single-ring hexamers with a total of six active sites. E. coli ClpAP consists of the double-ring ClpA AAA+ enzyme in complex with the ClpP peptidase. The rings formed by the N-terminal and C-terminal AAA+ domains of ClpA are called D1 and D2, respectively. Elimination of ATP hydrolysis in either ring by mutations in the Walker-B ATPase motifs reduces rates of ClpAP degradation, but the D2 ring appears to be more important than D1 for unfolding and degrading more-stable substrates. The single-ring ClpX AAA+ hexamer also degrades proteins in collaboration with ClpP. Does the double-ring structure of ClpA endow it with mechanical properties superior to those of ClpX? If so, does ClpA's having twice the number of active sites for ATP hydrolysis than ClpX also double its power, speed, translocation step size or grip on protein substrates? Here, to answer these questions, we used single-molecule optical trapping to determine how ClpAP mechanically unfolds and translocates multidomain protein substrates and then compared these activities to those determined previously for E. coli ClpXP.

We found that ClpA unfolds most protein domains substantially faster than ClpX does, but it translocates the unfolded polypeptide more slowly, taking individual physical steps that are smaller and more regular on average. We found no evidence that ClpAP can generate more force than ClpXP, supporting a model in which a stronger grip on the substrate is responsible for faster protein unfolding by ClpA. Understanding the mechanoochemical activities of these single-ring and double-ring AAA+ machines provides a foundation for understanding the diverse members of this family of enzymes that mediate protein destruction and remodeling.

RESULTS

Single-molecule degradation by ClpAP

We monitored single-molecule unfolding and translocation by ClpAP by using a dual-laser optical trap in passive force-clamp mode.

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Figure 1 Single-molecule protein degradation by ClpAP. (a) Cartoon representation of ClpAP and ClpXP, which consist of the ClpP peptidase and either the double-ring ClpA or single-ring ClpX unfoldase and translocase, respectively. (b) Experimental setup used in single-molecule measurements. An enzyme–multidomain protein substrate complex is tethered between two laser-trapped streptavidin-coated beads. The use of a biotinylated variant of ClpP (ClpP*) allows for the assembly and attachment of the ClpAP complex to one of the beads. (c) Representative traces of single-molecule protein degradation by ClpAP and ClpXP. Changes in bead-to-bead distance occur as ClpAP unfolds (sharp increases) and translocates ClpXP. Changes in bead-to-bead distance occur as ClpAP unfolds (sharp increases) and translocates ClpXP. Changes in bead-to-bead distance occur as ClpAP unfolds (sharp increases) and translocates ClpXP. 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pulled apart in a stepwise manner, whereas the titin and GFP domains terminate with β-strands embedded in β-sheets that require simultaneous shearing of multiple hydrogen bonds to initiate unfolding.

Unfolding by AAA+ rings appears to occur when mechanical pulling, which is driven by polypeptide translocation, coincides with transient, stochastic destabilization of the substrate. Therefore, the double-ring structure of ClpA might allow it to unfold some domains faster than ClpX does because it translocates faster or takes larger steps during translocation. However, our results support neither of these possibilities. Over a range of experimental forces, ClpAP translocated unfolded polypeptides ~30% more slowly than did ClpXP (Fig. 3a), and we detected no substantial changes in translocation velocity for different denatured substrate domains. During translocation, ClpAP takes physical steps ranging from 1 to 4 nm in length, with an average of ~2 nm (refs. 14–17). By contrast, we found that ClpAP predominantly takes 1-nm translocation steps, a few 2-nm steps and almost no 3-nm or 4-nm steps (Fig. 3b,c). The 2-nm steps did not occur in clusters, and we did not observe a clear pattern of 1-nm and 2-nm steps. For example, the probability of observing a 2-nm step was ~30% at the n = 2, n = 1, n + 1 and n + 2 positions relative to either a 1-nm or 2-nm step. Because translocation occurs in ~1 nm steps or multiples of this value, we conclude that the fundamental ClpAP step size, defined as the mechanical movement coupled to one ATPase cycle, is ~1 nm, which corresponds to translocation of 4–8 amino acids (aa) over the range of forces tested. This value agrees with a recent estimate of the ClpAP kinetic step size of ~5 aa with a single-turnover stopped-flow assay of polypeptide translocation.

To probe the mechanochemical cycle of ClpA, we examined the dwell times preceding each translocation step, which fit well to a sum of two exponentials, with a major population of ~90% (τ = 0.4 ± 0.01 s) and a minor population of ~10% (τ = 2.0 ± 0.1 s) (Fig. 3d). Dwell times preceding 1-nm steps had a similar distribution to dwell times preceding 2-nm steps (Fig. 3d). The prestep dwell for ClpXP were previously found to be distributed nonexponentially with an average of ~0.4–0.6 s (refs. 16,17). Therefore, the slower average translocation velocity of ClpAP compared to ClpXP results primarily from taking translocation steps of shorter length. Furthermore, most ClpAP translocation steps appear to result from a single kinetic pathway, on the basis of stepping dwell-time kinetics described above, consistently with a rate-limiting transition probably involving ATP binding, hydrolysis or product release in one subunit. By contrast, the majority of ClpXP steps involve the coordinated firing of multiple subunits, resulting in kinetic bursts that generate steps of ~2–4 nm (refs. 16,17). The slow component of the ClpAP dwell-time distribution might represent translocation pausing, as observed previously with ClpXP14–17, or might reflect differences between the mechaanochemical cycles of the D1 and D2 ClpA AAA+ rings. For example, slower firing of subunits in D1 compared to D2 is consistent with mutagenic and biochemical studies15. However, our finding that 1-nm and 2-nm steps occur with similar kinetics (Fig. 3d) suggests that it is unlikely that the D1 ring is responsible for short steps and the D2 ring for long steps or vice versa.

Subunit mixing supports nonsequential ATP hydrolysis

Our finding that ClpA takes a mixture of 1-nm and 2-nm steps with no specific sequence or pattern suggests that ATP hydrolysis...
has some stochastic or probabilistic character rather than being strictly sequential (described in Discussion). If a defined pattern of ATP hydrolysis involving all subunits in either ClpA ring were required for mechanical activity, then elimination of ATP hydrolysis in any single subunit should prevent substrate unfolding and translocation. We initially assayed rates of ClpAP degradation of GFP-ssrA\textsuperscript{22} to determine the dependence on the ATP and protein substrate concentrations (Figs. 4a,b). Both $K_m$ and $k_{\text{deg}}$ ($V_{\text{max}}/\text{concentration of enzyme hexamer}$) for degradation of the protein substrate changed as a function of ATP concentration (Fig. 4b), and the second-order rate constant for degradation ($k_{\text{deg}}/K_m$) decreased as the ATPase rate was reduced (Fig. 4c). Next, using saturating concentrations of ATP and GFP-ssrA, we assayed rates of degradation after mixing a fixed concentration of active ClpA with increasing concentrations of ClpA\textsuperscript{E286Q E565Q}, a variant in which mutations in the Walker-B motifs of the D1 and D2 AAA+ domains eliminate robust ATP hydrolysis (Fig. 4d). Assuming unbiased mixing of inactive and active subunits, the results fit best to a model in which two inactive subunits in a hexamer are required to abrogate unfolding and degradation of GFP-ssrA, supporting a model in which strictly sequential ATP hydrolysis in either ring of ClpA is not required for function.

**DISCUSSION**

In combination with previous single-molecule studies of ClpXP proteolysis\textsuperscript{14–17}, our results indicate that ClpA and ClpX use some similar and some different strategies to unfold and translocate substrate proteins into ClpP for degradation. Despite differences between the double-ring architecture of ClpA and the single-ring structure of ClpX, the size of the fundamental translocation step for both enzymes is $\sim 1$ nm. This result suggests that constraints imposed by the structures of different nucleotide states of a single AAA+ ring determine the size of a single power stroke both in single-ring and double-ring enzymes\textsuperscript{5–7,23,24}. However, ClpAP takes many steps in kinetic bursts of $\sim 1$ nm, which result in physical steps as large as 4 nm. ClpAP, by contrast, mostly takes 1-nm steps with a minority of 2-nm steps. Nevertheless, both ClpAP and ClpXP processively degrade multidomain substrates consisting of titin, GFP and Halo, thus suggesting that the ability of ClpXP to take larger physical translocation steps is not an essential factor in its ability to degrade these proteins.

To degrade GFP-ssrA, ClpXP initially extracts and translocates the ssrA-tagged $\beta$-strand at the C terminus, but global unfolding and degradation are unsuccessful when the rate of ATP hydrolysis is below a specific threshold value\textsuperscript{20,25}. Because ClpXP does not take bursts of four highly coordinated 1-nm translocation steps at low ATP concentrations, which also fail to support GFP-ssrA degradation, Bustamante and colleagues proposed that fast 4-nm steps are required to translocate the extracted $\beta$-strand before refolding occurs\textsuperscript{15}. However, this rapid-burst model cannot account for ClpAP unfolding and translocation of GFP, because ClpAP takes essentially no 4-nm steps at saturating ATP in single-molecule experiments and yet degrades GFP-ssrA in solution and in single-molecule experiments faster than does ClpXP. Like ClpXP, ClpAP also loses the ability to degrade GFP-ssrA as its ATPase activity is reduced. We propose that both ClpXP and ClpAP fail to unfold and degrade GFP at low ATP concentrations because ATP-free subunits in the AAA+ rings of these enzymes grip the extracted $\beta$-strand too weakly to prevent substrate release and refolding\textsuperscript{25}. In support of this hypothesis, we found that the $K_m$ for ClpAP degradation of GFP-ssrA increased as the ATP concentration was reduced, as expected if substrate binding becomes weaker at lower ATP occupancies.

Why does ClpAP unfold GFP and the V13P and V15P titin domains substantially faster than ClpXP does? One possibility is that the ClpA N domain enhances substrate unfolding compared to the ClpX\textsuperscript{AN} variant used in single-molecule and biochemical studies. However, Weber-Ban and colleagues showed that in the presence of ClpP, ClpA missing its N domain degrades GFP-ssrA as fast as does full-length ClpA\textsuperscript{26}. Because ClpX\textsuperscript{AN} also degrades ssrA-tagged substrates at the same rate as does full-length ClpX in the presence of ClpP\textsuperscript{27}, differences in the mechanochemical behavior of these two enzymes are likely to arise from differences in machine activity and not from the presence or absence of the N domains.

There is no evidence that ClpA applies more force than ClpX, because results presented here and previously show that both machines translocate at a relatively constant speed over a wide range of applied loads and perform $\sim 5 kT$ of work when taking $\sim 1$-nm steps against forces of $\sim 20$ pN (refs. 14–17). We propose that the superior unfolding ability of ClpA may result because it can grip the substrate more tightly during unfolding. The axial-pore loops in both the D1 and D2 rings of ClpA appear to interact with substrate\textsuperscript{18}, thus roughly doubling the number of interactions that could be made in comparison with the single AAA+ ring of ClpX. This larger interaction surface for ClpA could allow tighter gripping of the substrate and could increase the probability that the power stroke will result in substrate unfolding rather than in futile slipping of the substrate relative to the enzyme. In a tug-of-war, for example, a team that could grip the rope more tightly would have a substantial advantage over equally strong but less sure-handed opponents.

Hexameric ClpX rings with only one or two ATPase-active subunits retain the ability to unfold and translocate substrates, thus supporting a model in which ATP hydrolysis in the ClpX AAA+ ring occurs by a mechanism that is fundamentally stochastic or probabilistic\textsuperscript{17,27}. A model in which coordinated translocation bursts depend upon probabilistic ATP hydrolysis in different ClpX subunits could also explain why this enzyme takes physical steps of different sizes without a repeating pattern\textsuperscript{17}. However, strictly sequential models in which pairs of subunits in a hexamer hydrolyze ATP in a cyclic pattern have been proposed for other single-ring AAA+ proteases\textsuperscript{29}. Because ClpAP efficiently degrades some substrates when ATP hydrolysis in either the D1 or the D2 rings is eliminated\textsuperscript{13}, it is clear that each ClpA ring can hydrolyze ATP to produce mechanical work independently of ATP hydrolysis in the other ring. Our findings that ClpA takes 1-nm and 2-nm physical translocation steps in random patterns and that it can tolerate at least one ATPase-defective subunit in both the D1 and D2 rings without compromising GFP-ssrA degradation also suggest that strictly sequential ATP hydrolysis in either ring is unlikely to be an essential mechanistic feature. Nevertheless, whether and/or how subunits in a single ClpA ring or subunits in different rings coordinate their activities to allow unfolding of difficult substrates remains to be determined.

Bacteria and organelles contain multiple AAA+ proteases, suggesting that these enzymes have distinct biological roles. In *E. coli*, adaptor proteins ensure that ClpXP rather than ClpAP degrades most incomplete translation products bearing the tmRNA-derived ssrA tag\textsuperscript{30–33}. In this regard, the faster translocation activity of ClpXP could allow faster degradation of these partial proteins, which are unlikely to be stably folded. ClpAP, by contrast, probably unfolds and degrades many endogenous native proteins faster than does ClpXP, as observed for the model substrates studied here and previously\textsuperscript{30,34}. In terms of the average time required for single-molecule degradation of GFP, for example, ClpAP unfolding accounts for $\sim 20\%$ and translocation for $\sim 80\%$, but these values for ClpXP are $\sim 60\%$ for unfolding and $\sim 40\%$ for translocation.
for translocation. Such enzymatic 'tuning' of specific substrates to specific AAA+ proteases has been previously documented\(^{24,35}\), but it is not unique to these molecular machines. For example, the myosin superfamily of actin-based molecular motors performs a wide variety of biological functions yet shares a common mechanochemical cycle\(^{36}\). Specific variations in the ATPase cycles of different myosin motors provide unique adaptations allowing these enzymes to either processively move cargo, behave as anchor proteins or work in large arrays for muscle contraction. Our results suggest that double-ring AAA+ enzymes will be able to remodel key substrates substantially faster or at a lower energetic cost than their single-ring counterparts.

METHODS

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

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AUTHOR CONTRIBUTIONS

A.O.O. performed and analyzed optical-trapping experiments, performed biochemical experiments and constructed and purified ClpA variants. A.R.N. constructed, purified and assembled the biotinylated, mixed-ring ClpP biochemistry for single-molecule studies. O.I. constructed and purified the multidomain titin-GFP substrate. All authors contributed to writing the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Online Methods

Proteins. AC variants of E. coli ClpA and ClpA<sup>E286Q</sup><sup>ES65Q</sup>, a single-chain ClpX<sup>ΔN</sup> hexamer (ClpX<sup>ΔN</sup>), and ssrA-tagged protein substrates were cloned, expressed and purified as described<sup>14,17,39</sup>. Deletion of the nine C-terminal amino acids of ClpA (ΔC9) prevents autodegradation but does not otherwise affect ClpA activity<sup>39</sup>. One of the titin<sup>27</sup> domains in the substrate containing GFP was unfolded by mutation of both of its cysteines to aspartic acids<sup>40</sup>. For optical trapping, we used a tetradecameric ClpP variant consisting of one heptameric ring of wild-type E. coli ClpP with a C-terminal EENLYFQSH<sub>6</sub> sequence (TEV protease site underlined; ClpP–TEV-His) and one heptameric ring of the M5A ClpP variant<sup>41</sup> with a C-terminal GLNDIFEAKIEWHH<sub>6</sub> sequence (biotin-acceptor peptide sequence underlined; ClpP<sup>M5A</sup>–bAP-His). Both ClpP variants were expressed and purified on Ni-NTA resin. ClpP<sup>M5A</sup>–bAP-His was exogenously biotinylated with purified BirA enzyme, and the His<sub>6</sub>-tag of ClpP–TEV-His was removed by cleavage with TEV protease. An excess of this enzyme was mixed with ClpP<sup>M5A</sup>–bAP-His, dialyzed at 4 °C against buffer containing 150 mM ammonium sulfate to allow exchange of heptameric rings<sup>42</sup> and dialyzed at room temperature into buffer containing 150 mM KCl. Tetradecamers containing His<sub>6</sub> tags were purified by Ni-NTA chromatography and stored at ~80 °C. This procedure generates a majority of ClpP<sup>ΔC9</sup>/ClpP<sup>M5A</sup>–bAP-His<sub>6</sub> (ClpP<sup>ΔC9</sup>) and some ClpP<sup>M5A</sup>–bAP-His<sub>6</sub>/ClpP<sup>M5A</sup>–bAP-His<sub>6</sub> enzymes, but the latter species does not bind ClpA or ClpX tightly because of the double M5A mutation.

Single-molecule optical trapping. Complexes of ClpAP and multidomain substrates containing an N-terminal Halo domain covalently linked to biotinylated double-stranded DNA conjugated to a HaloTag ligand (Promega) were tethered between two beads trapped by 1,064-nm lasers in passive force-clamp mode as described<sup>14,17</sup>. Briefly, biotin-DNA–linked substrates were attached to 1-µm streptavidin-coated polystyrene beads (Spherotech) that were loosely tethered to the surface of a glass cover slip via a DNA-linked glass-binding peptide aptamer<sup>43</sup>. Biotinylated ClpP was attached to a 1.26-µm streptavidin-coated polystyrene bead in the presence of ClpA and saturating ATP (4.5 mM). Free enzymes were removed by centrifugation immediately before use. A weakly laser-trapped ClpAP bead was brought near a surface-tethered substrate bead. Upon substrate recognition by ClpAP, a stiff laser trap was used to rupture the aptamer-glass attachment of the substrate bead and result in a ClpAP–substrate complex tethered between two laser-trapped beads (Fig. 1b). As reported for ClpXP degradation of multidomain substrates<sup>14,17</sup>, no traces contained all substrate domains, presumably because ClpAP had unfolded and translocated C-terminal titin domains before measurements began. All experiments were performed at room temperature (18–20 °C), with 4.5 mM Mg<sup>2+</sup>–ATP and ATP-regeneration and oxygen-scavenging systems<sup>14</sup> in PD–T buffer (25 mM HEPES, pH 7.6, 100 mM KCl, 10 mM MgCl<sub>2</sub>, 10% glycerol, 0.1% Tween–20, and 1 mM Tris(2-carboxyethyl)phosphine) supplemented with 1 mg/mL bovine serum albumin.

Data acquisition was carried out as described<sup>14</sup>. Custom MATLAB scripts were used to calculate interbead distances, to measure the magnitude of unfolding distances, and to measure the preunfolding dwell time between the end of one translocation event and the next unfolding event. Translocation events in each trace were fit with a linear equation to determine the average translocation speed.

Finding steps in translocation traces. Data were collected at a 3-kHz sampling frequency and decimated to 50 Hz, and individual physical steps in translocation traces were determined as described<sup>17</sup>. Briefly, to find steps in the decimated data, we used a MATLAB implementation of the chi-squared minimization method<sup>37</sup> provided by J. Kerssemakers (TU Delft). The chi-squared method requires input of the number of steps to fit within a given trace, which we estimated by taking the pairwise distribution of decimated data. Steps smaller than 0.75 nm and backward steps or slips were combined, and the dwell time preceding a combined step was added to the dwell time of the following step.

Biochemical assays. GFP-His<sub>6</sub>–ssrA degradation was assayed by following the loss of GFP fluorescence (excitation, 470 nm; emission, 540 nm). Steady-state ATP hydrolysis was monitored by following the loss of NADH absorbance at 340 nm (ref. 44). Final concentrations were 147 nM ClpA hexamer or 125 nM ClpX<sup>ΔC9</sup>, 400 nM ClpP tetradecamer, and different concentrations of GFP-His<sub>6</sub>–ssrA. Varying concentrations of Mg<sup>2+</sup>–ATP with an ATP-regeneration system (20 U/mL pyruvate kinase, 20 U/mL lactate dehydrogenase, 7.5 mM phospho(enol)pyruvate and 0.2 mM NADH) were added to give the final concentrations listed in Figure 4a,b. For experiments in which active and inactive subunits were mixed, the ATP-regeneration system was present, and final concentrations were 132 nM ClpA hexamer, 1.3 µM ClpP tetradecamer, 20 µM GFP-His<sub>6</sub>–ssrA, and 5 mM Mg<sup>2+</sup>–ATP. Varying concentrations of inactive ClpA were added to give the final ratios shown in Figure 4d. Biochemical experiments were performed at ~24 °C in PD–T buffer.

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