

Stochastic but Highly Coordinated Protein Unfolding and Translocation by the ClpXP Proteolytic Machine

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SUMMARY

ClpXP and other AAA+ proteases recognize, mechanically unfold, and translocate target proteins into a chamber for proteolysis. It is not known whether these remarkable molecular machines operate by a stochastic or sequential mechanism or how power strokes relate to the ATP-hydrolysis cycle. Single-molecule optical trapping allows ClpXP unfolding to be directly visualized and reveals translocation steps of ~1–4 nm in length, but how these activities relate to solution degradation and the physical properties of substrate proteins remains unclear. By studying single-molecule degradation using different multidomain substrates and ClpXP variants, we answer many of these questions and provide evidence for stochastic unfolding and translocation. We also present a mechanochemical model that accounts for single-molecule, biochemical, and structural results for our observation of enzymatic memory in translocation stepping, for the kinetics of translocation steps of different sizes, and for probabilistic but highly coordinated subunit activity within the ClpX ring.

INTRODUCTION

AAA+ proteases (ATPases associated with diverse cellular activities) maintain protein quality control in the cell by converting the energy derived from ATP binding and hydrolysis into work that powers mechanical protein unfolding, translocation, and ultimately degradation (Sauer and Baker, 2011). How these destructive enzymes degrade proteins with widely varying sequences, structures, and stabilities is only beginning to be understood. ClpXP, one of the best-characterized members of

this family of degradation machines, consists of ClpX, a hexameric AAA+ ATPase, and ClpP, a barrel-shaped peptidase (Baker and Sauer, 2012). Degradation is initiated when the ClpX ring binds a substrate via an unstructured degron, such as the *ssrA* tag, and attempts to translocate this peptide through its narrow axial pore. For native substrates, degron translocation by ClpX pulls on the folded portion of the protein, driving mechanical denaturation that allows subsequent translocation steps to spool the unfolded polypeptide into ClpP for degradation.

Single-molecule studies, using optical tweezers to monitor ClpXP unfolding and translocation of multidomain substrates, establish that ClpXP can work against forces of 20 pN or higher, demonstrate that the smallest translocation steps are ~1 nm (approximately four to eight amino acids), and reveal physical steps that are multiples of this value, resulting from kinetic bursts of two, three, or four power strokes (Aubin-Tam et al., 2011; Mail-lard et al., 2011; Sen et al., 2013). Studies of variants containing inactive subunits support a probabilistic mechanism of ATP hydrolysis and mechanical function by ClpXP (Martin et al., 2005), but this model is not firmly established and a related AAA+ protease has been proposed to operate by a sequential mechanism (Smith et al., 2011). At present, it is not known how the physical properties of native and unfolded substrates affect the kinetics of single-molecule ClpXP unfolding and translocation or if these reactions account for solution-degradation rates. Moreover, no current model satisfactorily explains how the ClpX ring generates translocation steps of different sizes, accounts for the kinetics of unfolding and translocation, or explains the linkage between ATP consumption and these mechanical reactions. Any deep understanding of AAA+ proteases and related remodeling machines requires answers to these questions.

Here, we use optical trapping to assay single-molecule ClpXP unfolding and translocation of substrates consisting of domains with varying stabilities and sequences. We find that ClpXP unfolds most domains by a single pathway, with kinetics that depend on the native fold and structural stability. Subsequent translocation or pausing occurs at rates that vary with the

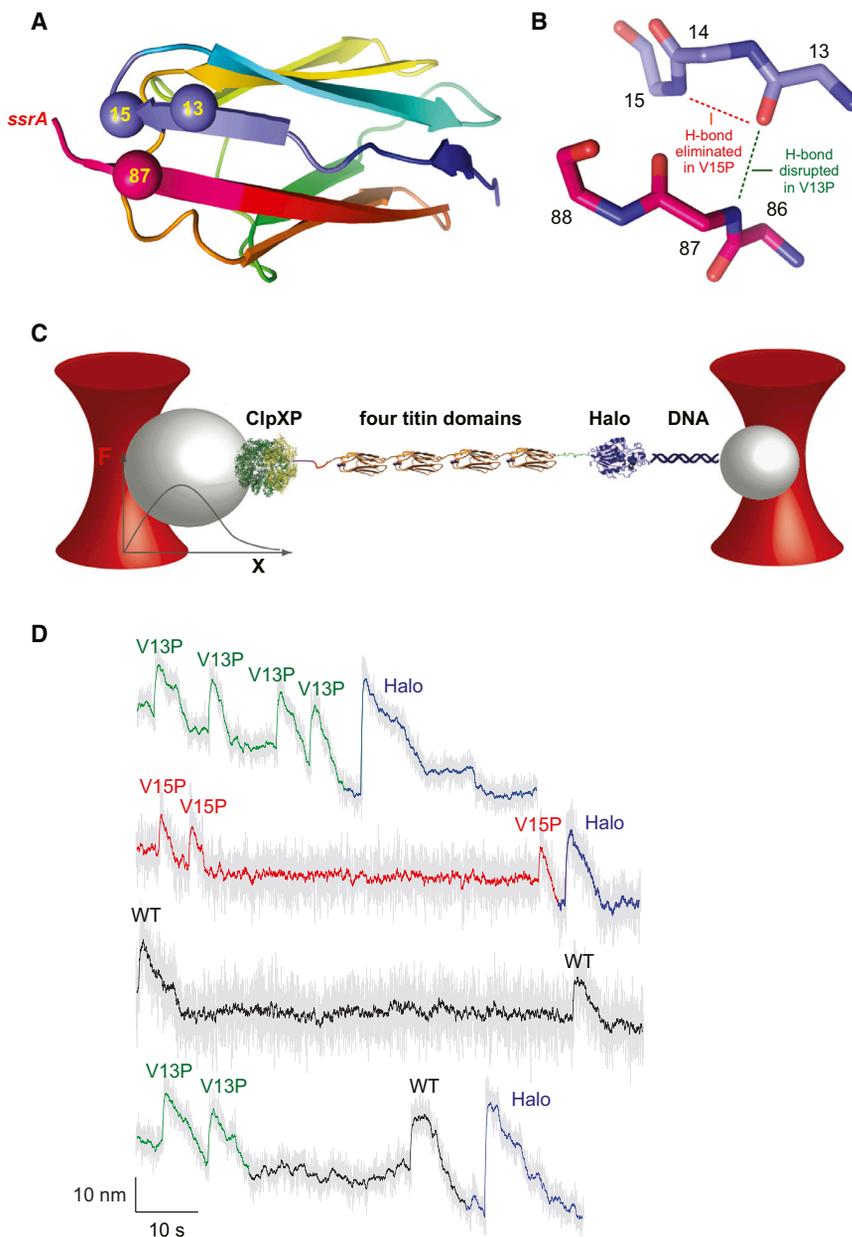


Figure 1. Single-Molecule Unfolding and Translocation of Substrates

(A) Cartoon structure of titin^{I27} (Protein Data Bank code 1TIT), colored from the N terminus (blue) to the C terminus (red). Spheres show α carbons for residues 13, 15, and 87. ClpXP pulling on a C-terminal ssrA tag is resisted by local structure, including β sheet hydrogen bonding between the C-terminal β strand and the β strand with residues 13 and 15.

(B) The V13P and V15P mutations disrupt hydrogen bonds that directly or indirectly stabilize the titin^{I27} domain.

(C) Experimental setup for single-molecule assays of ClpXP unfolding and translocation. ClpXP is attached to one laser-trapped bead and has engaged the ssrA tag of a multidomain substrate consisting of four titin domains and a Halo domain, which is attached to a second laser-trapped bead via a DNA linker.

(D) Trajectories for ClpXP unfolding and translocation of multidomain substrates. Unfolding of individual domains increases bead-bead distance (upward movement), whereas translocation decreases bead-bead distance (downward movement). After completed translocation of one domain, there is a variable dwell time before ClpXP unfolds the next domain. The dwell baselines before and after titin-unfolding events are spaced as expected for the end-to-end distance of a native titin domain (4.4 nm) or native titin plus the linker to the Halo domain.

followed by a cascade of coordinated power strokes. This model explains our single-molecule results and also accounts for a wide range of previous biochemical, genetic, and structural results.

RESULTS

Substrate Design and Single-Molecule Degradation

ClpXP degrades ssrA-tagged variants of the titin^{I27} domain at different rates (Kenniston et al., 2003). For example, the

V13P and V15P mutations disrupt or eliminate hydrogen bonds close in space to the C-terminal ssrA tag (Figures 1A and 1B); reduce thermodynamic, kinetic, and mechanical stability; and accelerate ClpXP degradation, with the wild-type (WT) domain being most stable and degradation resistant, V15P having intermediate stability and degradation rates, and V13P being least stable and most rapidly degraded (Li et al., 2000; Kenniston et al., 2003). For single-molecule studies, we constructed Halo-WT-WT-WT-ssrA, Halo-V13P-V13P-V13P-V13P-ssrA, Halo-V15P-V15P-V15P-V15P-ssrA, and Halo-WT-V13P-V13P-V13P-ssrA substrates, in which Halo is an N-terminal HaloTag domain that allows covalent attachment to a biotinylated DNA spacer. For optical-trapping (Figure 1C), multidomain substrates were attached via the Halo domain and DNA spacer to one

sequence of the unfolded substrate. During translocation, ClpXP does not exhibit a sequential pattern of step sizes, supporting a fundamentally stochastic reaction, but a mechanism of enzymatic memory results in short physical steps being more probable after short steps and longer physical steps being more likely after longer steps, allowing the enzyme to run at different speeds. Surprisingly, two ATP-hydrolysis events can drive more than two power strokes, as an engineered ClpX hexamer with just two active subunits also takes ~ 1 –4 nm physical steps. Finally, we show that solution proteolysis is many times slower than predicted from single-molecule results. We discuss the implications of these results for understanding ClpXP structure and biological function and present a mechanochemical model in which initial stochastic ATP hydrolysis in the AAA+ ring can be

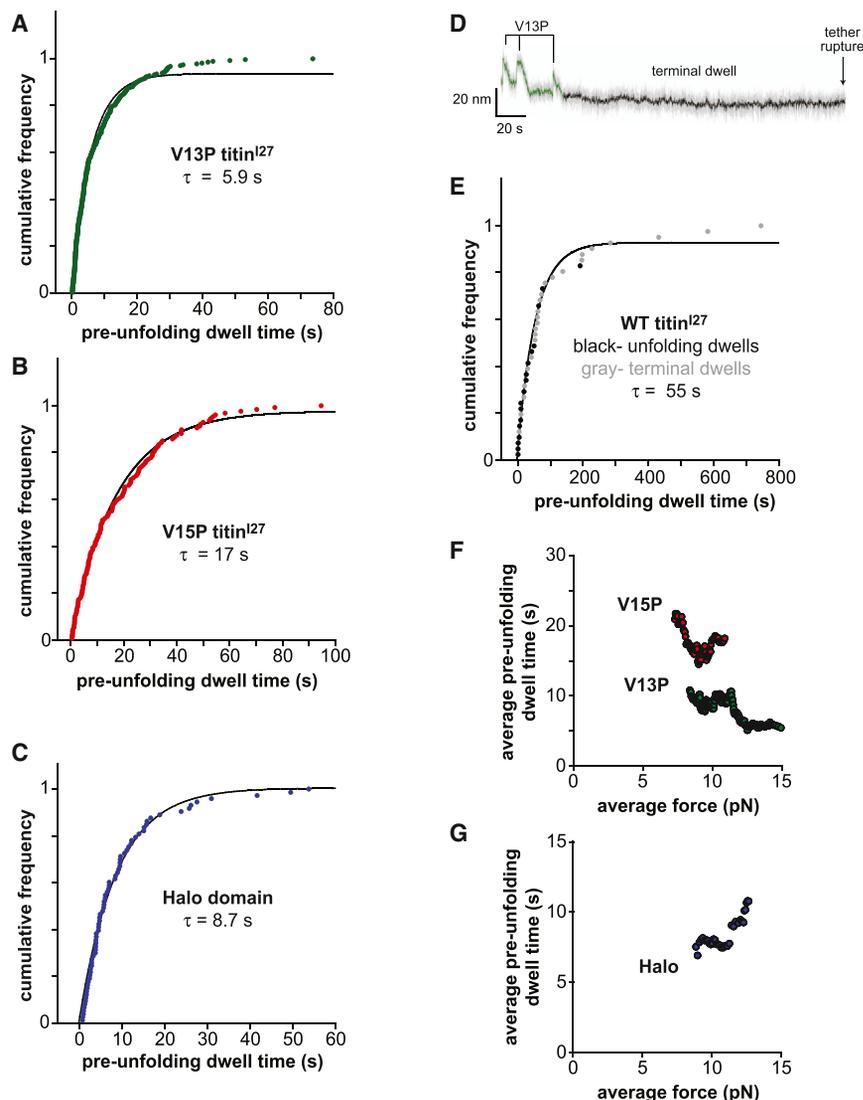


Figure 2. ClpXP Unfolding of Domains in Multidomain Substrates

(A–C) Distributions of preunfolding dwell times for the V13P, V15P, and Halo domains. In each plot, the solid line is a nonlinear-least-squares fit to $y = A*(1 - \exp[-t/\tau_{\text{unf}}])$.

(D) For the Halo-WT-V13P-V13P-V13P-ssrA substrate, long “terminal” dwells were often observed following unfolding and translocation of the V13P titin domains.

(E) ClpXP unfolding of wild-type titin¹²⁷ domains. Black symbols are preunfolding dwells; gray symbols are terminal dwells. The line is a fit to $y = A*(1 - \exp[-t/\tau_{\text{unf}}])$.

(F) Plots of average force versus average pre-unfolding dwell times (calculated over a moving 50-point window) for the V13P and V15P domains. (G) Plot of average force versus average pre-unfolding dwell times (calculated over a moving 40-point window) for the Halo domain. See also Figures S1, S2, and S5.

native domain, there was a preunfolding dwell with little change in bead-to-bead distance.

Preunfolding Dwell Times Depend on Substrate Stability

The preunfolding dwell represents the time that ClpXP pulls on a native protein domain before denaturation occurs. Pre-unfolding dwells for the first unfolding event in each trajectory were not quantified, as recording began after some attempted unfolding, unfolding, or translocation by ClpXP had occurred. For example, the second and fourth traces in Figure 1D contain just three titin unfolding events and one Halo unfolding event. Because there are four titin domains in

streptavidin-coated bead and a biotinylated variant of ClpXP was attached to a second streptavidin-coated bead (Aubin-Tam et al., 2011). In all substrates, the Halo domain was connected to the adjacent titin domain by a 22-residue linker, whereas the remaining titin domains were connected by four-residue linkers.

Optical-trapping measurements under constant force (Aubin-Tam et al., 2011) were used to visualize single-molecule ClpXP unfolding and translocation. Individual traces displayed three signatures of ClpXP mechanical function as shown in Figure 1D. First, abrupt increases in bead-to-bead distance occurred upon unfolding, with the size of the transition being smaller for titin domains than for the Halo domain. Second, bead-to-bead distance decreased following unfolding, as ClpXP translocated the unfolded polypeptide, with the total decrease depending upon the size of the denatured domain and the length of the linker to the next domain. Third, between completed translocation of one unfolded domain and denaturation of the next

the multidomain substrate, one V15P or V13P domain must have been unfolded and translocated before these traces began.

ClpXP unfolding of a protein domain typically requires many ATP-hydrolysis events (Kenniston et al., 2003). If enzymatic unfolding occurs by a single pathway and one rate-limiting kinetic step, then preunfolding dwells should be exponentially distributed. Multiple unfolding pathways with one rate-limiting step would give dwells distributed as a sum of exponentials, whereas multiple kinetic steps with similar time constants would give a gamma distribution of dwell times. For ClpXP unfolding of V13P ($n = 278$ events), V15P ($n = 127$ events), and Halo ($n = 73$ events), the preunfolding dwell distributions fit well to single exponentials ($R^2 \geq 0.987$), with average unfolding times (τ_{unf}) of 5.9, 17, and 8.7 s, respectively (Figures 2A–2C). Only 17 WT unfolding events, some of which may be ClpXP independent (see Extended Results available online), were observed in ~ 200 experiments, indicating that most experiments terminated before WT unfolding. Indeed, some Halo-WT-V13P-V13P-V13P-ssrA

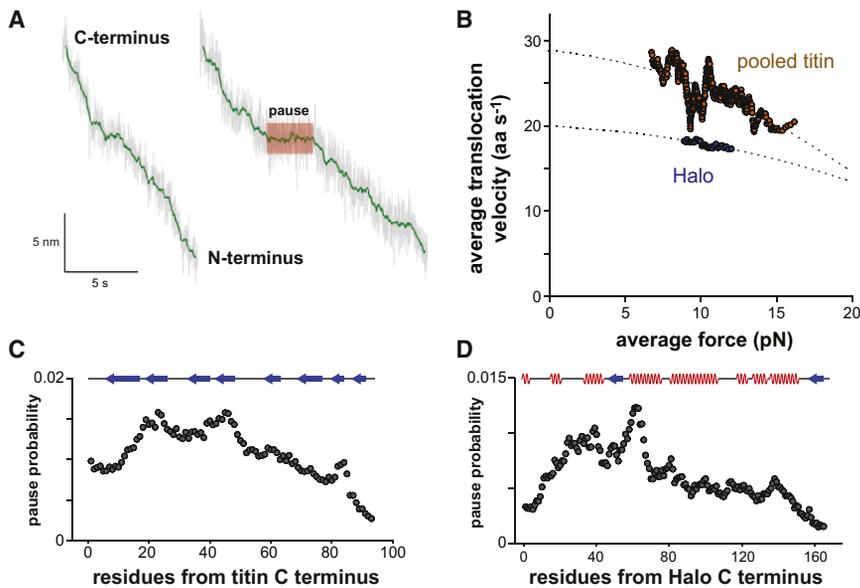


Figure 3. Translocation and Pausing

(A) V13P translocation traces proceeding with approximately constant velocity (left panel) or with a pause (right panel).

(B) Plots of average force versus average translocation velocity were calculated over a moving 50-point window for the V13P and V15P domains and over a 40-point window for the Halo domain. The lines are fits to a single-barrier Boltzmann equation $v = v_0 \cdot (1.05) / (1 + 0.05 \cdot \exp[F \cdot 0.7 / kT])$, where F is the average force and kT is 4.1 pN·nm at room temperature.

(C) Probability of pausing of ClpXP along the length of a titin domain ($n = 25$).

(D) Probability of pausing of ClpXP along the length of a Halo domain ($n = 24$). Secondary structure in the native structure is indicated schematically in (C) and (D) (arrows represent β strands; zigzag lines represent α helices).

traces contained three V13P unfolding events, a long terminal dwell, and rupture of the bead-bead tether before ClpXP could unfold the WT domain (Figure 2D). Including WT preunfolding dwells and these terminal dwells, which represent a lower bound of the preunfolding dwell, gave an exponential distribution with $\tau_{\text{unf}} \sim 55$ s ($n = 41$; Figure 2E). Fitting just the WT preunfolding dwells gave a τ_{unf} about half this value, which was unrealistically small given the distribution of terminal dwells. Rates of ClpXP unfolding in the order V13P > V15P > WT are consistent with the relative stabilities of these domains (Li et al., 2000; Kenniston et al., 2003). Thus, destabilizing mutations proximal to the site of ClpXP pulling result in faster enzymatic denaturation. The exponential distribution of preunfolding dwells for these proteins indicates that one kinetic step is largely rate limiting for ClpXP unfolding, a finding supported by inspection of the randomness of the process (see Extended Results; Figure S1). Models with parallel faster and slower exponential processes improved the residuals of the V13P and V15P fits modestly (Figure S2), consistent with the possibility of two unfolding pathways (see Discussion).

Force has opposing effects, reducing ClpXP activity but also destabilizing domains in the substrate to a degree that depends on the distance to the unfolding transition state (Carrion-Vazquez et al., 1999). We ranked preunfolding dwells by force, calculated averages over a moving window, and plotted average dwell time against average force (Figures 2F and 2G). Unfolding of V13P and V15P was faster at higher force (Figure 2F), suggesting that force destabilizes these titin domains more than it decreases ClpXP activity. By contrast, Halo unfolding was slower at higher force, suggesting that force destabilizes Halo less than it decreases ClpXP activity, a result consistent with the distance to the transition state being smaller for ClpXP unfolding of Halo than the titin domains (Li et al., 2000; Popa et al., 2013). The ratios of ClpXP-dependent to ClpXP-independent unfolding events were ~ 20 , ~ 7 , and ~ 1 for the V13P, V15P, and WT domains, respectively, a trend consistent with distances to the

unfolding transition state determined from atomic-force microscopy experiments for these domains (Li et al., 2000; see Extended Results).

Translocation Velocity and Pausing

ClpXP translocation typically proceeded monotonically, but pauses longer than 2.5 s were occasionally observed (Figure 3A). After subtracting these pauses, we calculated average translocation velocities. The V13P, V15P, and WT velocities were similar, as expected because these sequences differ at only one residue position. For 656 pooled titin translocation traces, the mean velocity was 24 ± 0.4 aa s⁻¹ (4.4 ± 0.1 nm s⁻¹), where the errors are SEM values. For 78 Halo translocation traces, the mean velocity was slower (18 ± 0.8 aa s⁻¹; 3.3 ± 0.1 nm s⁻¹). Thus, the polypeptide sequence has a modest impact on ClpXP translocation velocity, a result consistent with biochemical studies (Barkow et al., 2009). Figure 3B shows average translocation velocities plotted against average force. Fitting these data gave unloaded translocation velocities of 29 aa s⁻¹ for titin domains and 20 aa s⁻¹ for Halo domains.

Pausing occurred with higher probability at some titin and Halo sequences (Figures 3C and 3D) and was less common during translocation of titin (3.7% of events) than Halo (17% of events). Sequence-dependent pausing could occur either because of direct interactions of the translocating polypeptide with ClpXP or because some sequences have a higher probability of forming transient structure that impedes translocation.

Stochastic Steps of Different Size and Kinetic Complexity Contribute to Translocation

Using a chi-square algorithm (Kerssemakers et al., 2006), we resolved individual physical steps in a subset of translocation traces with good signal to noise (for examples, see Figure 4A). As reported (Aubin-Tam et al., 2011; Maillard et al., 2011; Sen et al., 2013), the smallest physical steps were ~ 1 nm, but many steps were 2-fold, 3-fold, and 4-fold larger (Figures 4A

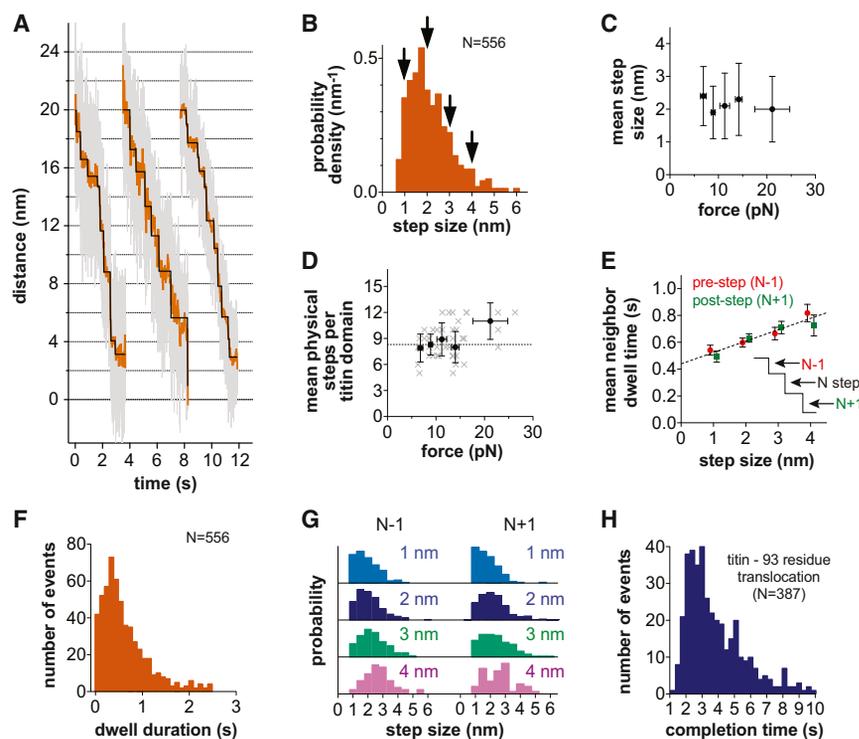


Figure 4. Physical Steps during Titin Translocation

(A) Representative stepping in ClpXP translocation trajectories. Raw data were decimated to 500 Hz (gray) or 50 Hz (orange). Chi-square fits to the 50 Hz data are shown in black.

(B) Distribution of physical steps sizes during titin translocation.

(C) Mean physical step size during titin translocation as a function of force. X and Y error bars are ± 1 SD ($n = 70$ –221).

(D) Mean number of physical steps required to translocate an 89-residue titin domain and four-residue linker as a function of force (black squares). X and Y error bars are ± 1 SD ($n = 6$ –20). Gray X's are step numbers from individual translocation trajectories.

(E) Mean dwell times \pm SEM ($n = 45$ –236) before (red) or after (green) physical steps of 1–4 nm (pre- and poststep values are offset slightly on the x axis for clarity).

(F) Distribution of dwell times preceding steps of all sizes during titin translocation.

(G) Occurrence of steps of different size either before ($n - 1$) or after ($n + 1$) physical steps of 1–4 nm. (H) Distribution of times required to complete translocation of 89-residue titin domains and subsequent four-residue linkers after subtracting pauses.

See also Figures S3, S4, S6, S7, and S8.

and 4B). Force had little effect on the average step length (~ 2 nm; Figure 4C), and complete translocation of each titin domain (~ 90 residues) required an average of approximately eight physical steps (Figure 4D).

During titin translocation, the dwell times both preceding and following a physical step increased with the size of the step (Figure 4E). The dwell times for pooled steps of all sizes (Figure 4F) and for individual steps of different sizes (Figure S3) were distributed nonexponentially, suggesting that multiple kinetic steps contribute to each physical translocation step. Importantly, there was no strong sequential pattern of step sizes (Figure 4G). In the trajectories shown in Figure 4A, for example, the order of steps was 1-2-1-1-1-2-3-3-1-1 for the leftmost trace, 3-2-2-2-3-4 for the center trace, and 1-1-1-1-1-2-2-3-2-2-1-1 for the rightmost trace. Despite the absence of a clear pattern, 1 nm steps had a higher probability of being preceded or followed by another 1 nm step compared to longer steps, and steps of 2–4 nm also tended to be preceded and followed by longer steps (Figure 4G). These results support a stochastic mechanism of subunit firing with some degree of motor memory. ClpXP translocation of the Halo domain also showed a distribution of steps ranging from ~ 1 to 4 nm (Figure S4).

To investigate mechanism independently of the detection of individual steps, we calculated times from the beginning to the end of translocation of V13P and V15P domains followed by the four-residue linker (93 total residues; $n = 387$) and subtracted any pauses. The histogram of completion times showed multiple peaks (Figure 4H), supporting populations of faster- and slower-moving enzymes, a finding consistent with our observation that ClpXP has an increased probability of taking short steps after short steps and vice versa.

Unfolding, Translocation, and Pausing by a Hobbled ClpX Motor

To determine the effects of eliminating ATP hydrolysis in multiple ClpX subunits, we used a variant containing two subunits with ATPase-defective R370K sensor-II mutations (R), two wild-type subunits (W), and two subunits with ATPase-defective E185Q Walker-B mutations (E) in the order RWERWE. The ATPase defective subunits in this ClpX variant, which supports degradation of *ssrA*-tagged V13P, V15P, and WT titin substrates at 15%–30% of wild-type ClpXP rates, can still bind and release nucleotide (Joshi et al., 2004; Hersch et al., 2005; Martin et al., 2005). In optical tweezer experiments, we observed RWERWE ClpXP unfolding and translocation of V13P domains in Halo-V13P-V13P-V13P-V13P-*ssrA* (Figure 5A) at forces up to 10.4 pN, whereas the wild-type enzyme was active at forces as high as 26 pN. An exponential fit of preunfolding dwell times for RWERWE ClpXP gave a τ_{unf} of 50 s ($n = 19$; Figure 5B), corresponding to ~ 8 -fold slower unfolding than by ClpXP with six active subunits. In experiments using Halo-V15P-V15P-V15P-V15P-*ssrA* or Halo-WT-WT-WT-WT-*ssrA*, we detected no RWERWE ClpXP unfolding. Thus, preventing ATP hydrolysis in four ClpX subunits dramatically slows the rate of unfolding of V13P, the least stable of the three titin-domain variants tested, and makes enzymatic unfolding of the V15P and WT domains too slow to detect under the forces used for optical trapping.

For V13P translocation by RWERWE ClpXP, the average translocation velocity after removing pauses was 5.7 ± 0.5 aa s^{-1} , a rate ~ 4 -fold slower than ClpXP. Pauses defined as dwells longer than 7.5 s were present in $\sim 45\%$ of RWERWE traces, whereas pauses defined as dwells longer than 2.5 s were present in fewer than 4% of wild-type ClpXP traces. Thus, a ClpX ring

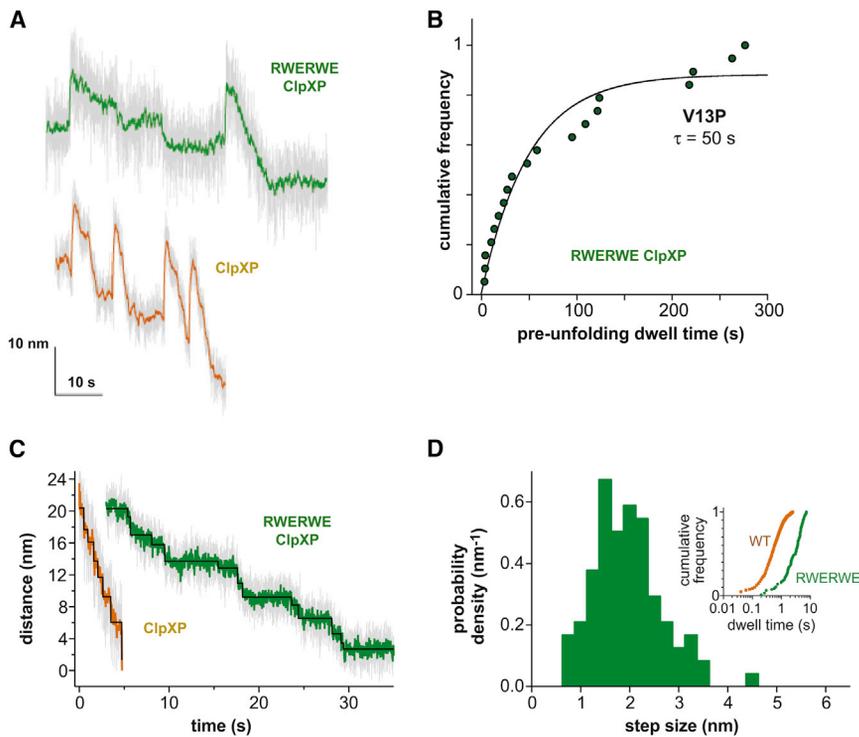


Figure 5. Unfolding and Translocation by RWERWE ClpXP

(A) V13P unfolding and translocation traces for RWERWE ClpXP (top) and ClpXP with six active subunits (bottom).

(B) Distributions of RWERWE ClpXP preunfolding dwell times for the V13P domain. The line is a nonlinear-least-squares fit to $y = A \cdot (1 - \exp[-t/\tau_{\text{unf}}])$. (C) Representative stepping in titin V13P translocation by ClpXP (orange) and RWERWE ClpXP (green). Decimation and fits (black) are described in Figure 4A.

(D) Distribution of RWERWE physical step sizes. Inset: cumulative frequency distributions of dwell times preceding steps for ClpXP (orange) or RWERWE ClpXP (green). See also Figure S8.

with just two active subunits pauses more frequently and for longer times than a ring with six active subunits. The dwells between RWERWE ClpXP translocation steps were substantially longer than between ClpXP translocation steps (Figures 5C and 5D). Strikingly, however, individual physical steps in RWERWE ClpXP translocation traces also ranged from ~ 1 to 4 nm (Figures 5C and 5D). We conclude that large physical steps do not require ATP hydrolysis in more than two ClpX subunits.

Commitment Is a Slow Step in Solution Degradation

Previous studies show that ClpP proteolysis is not a slow step in degradation (Thompson and Maurizi, 1994; Kenniston et al., 2003). How well do average times of unfolding (τ_{unf}) and translocation (τ_{trans}) determined in single-molecule experiments predict average degradation times determined at substrate saturation ($\tau_{\text{deg}} = 1/V_{\text{max}}$) in solution? If the average commitment time (τ_c) is defined to satisfy the equation $\tau_c + \tau_{\text{unf}} + \tau_{\text{trans}} = \tau_{\text{deg}}$, then $\tau_{\text{unf}} + \tau_{\text{trans}} \approx \tau_{\text{deg}}$ only when τ_c is small compared to $\tau_{\text{unf}} + \tau_{\text{trans}}$. For six substrates of varying stability, a plot of $(\tau_{\text{unf}} + \tau_{\text{trans}})$ against τ_{deg} gave a linear correlation with a slope of ~ 0.25 (Figure 6A), indicating that solution degradation is approximately four times slower than expected from single-molecule unfolding and translocation. Although differences in conditions between solution and single-molecule experiments could account for some variation (see Figure 6A legend), this result suggests that τ_c is the slow step in solution degradation or that $\sim 75\%$ of ClpXP enzymes are inactive, as calculation of V_{max} assumes 100% activity. To distinguish between these possibilities, we monitored single-turnover binding and unfolding of GFP-ssrA by a 20-fold molar excess of ClpXP (5- to 20-fold excess over K_M) at a series of temperatures and fit the exponential trajectories to determine

τ values (Figure 6B). We also performed steady-state degradation at each temperature to determine τ_{deg} (Figure 6C), K_M for protein substrate (Figure 6D), and measured rates of ATP hydrolysis in the presence of saturating GFP-ssrA (Figure 6E). To calculate fractional activity, we added the time expected for GFP translocation to the single-turnover τ values for binding/unfolding and divided this time by τ_{deg} . The fractional ClpXP activity was ~ 0.4 at 15°C and increased to ~ 0.9 at 37°C (Figure 6F). The latter result indicates that ClpXP is $\sim 90\%$ active, a result consistent with previous studies (Hersch et al., 2005; Shin et al., 2009). Lower “activity” at lower temperatures may be a consequence of more ClpXP enzymes assuming a conformation that does not support substrate binding or activity.

The time required for ClpXP unfolding of pre-engaged GFP in solution is ~ 6 s at 30°C (Martin et al., 2008a). Subtracting this time from the 34 s required to bind and unfold GFP in our single-turnover experiment at 30°C yields a τ_c of 28 s, which is ~ 4.5 -fold longer than the pre-engaged unfolding time. As τ_{deg} is substantially longer than $\tau_{\text{unf}} + \tau_{\text{trans}}$, even for substrate proteins with marginal stability (Figure 6A), τ_c represents much of the time required for ClpXP degradation and appears to increase in proportion to substrate stability. For ClpXP degradation of wild-type titin¹²⁷ substrates, cycles of binding, attempted engagement and/or unfolding, and substrate release contribute to the time needed for degradation (Kenniston et al., 2005). The linearity of the Figure 6A plot suggests that similar cycles of substrate binding and release contribute to the degradation time required for many substrates.

DISCUSSION

Domain Stability and ClpXP Unfolding

Matouschek and colleagues first reported that the local stability of structural elements adjacent to the degradation tag determined resistance to enzymatic unfolding (Lee et al., 2001). Our results support their model, as we find that mutations that

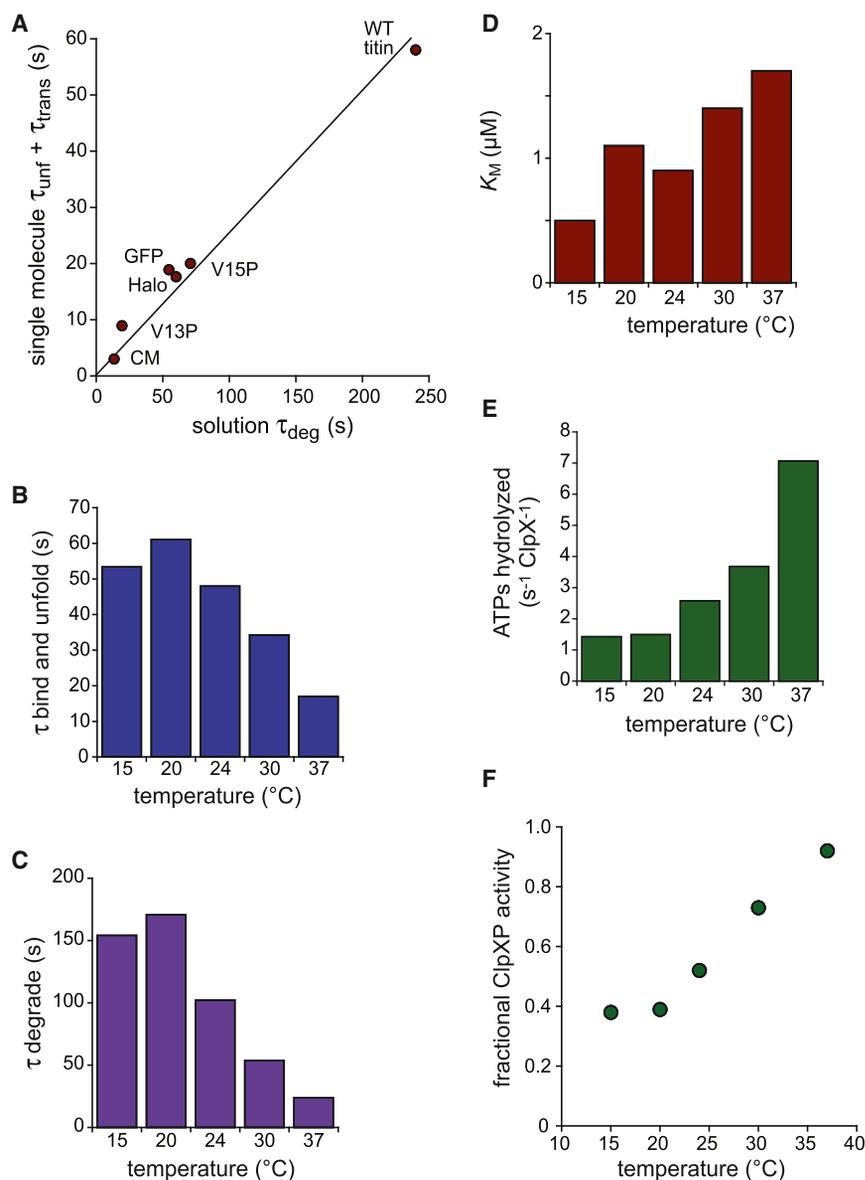


Figure 6. Solution Degradation Times Are Poorly Predicted by Single-Molecule Unfolding and Translocation Times

(A) Plot of average times required for solution degradation (τ_{deg}) of titin-ssrA (WT), V15P-titin-ssrA (V15P), V13P-titin-ssrA (V13P), carboxymethylated titin-ssrA (CM), GFP-ssrA (GFP), and Halo-ssrA (Halo) versus $\tau_{unf} + \tau_{trans}$ times from single-molecule experiments (Kim et al., 2000; Kenniston et al., 2003; Sen et al., 2013; this work). Times for titin and GFP degradation were determined at 30°C, whereas single-molecule experiments and Halo-ssrA degradation were performed at room temperature. Degradation is slower at lower temperatures (see C), which would increase the discrepancy between the solution and single-molecule results. τ_{unf} values were determined under load and could be different at zero force, but V13P and V15P τ_{unf} values (Figure 2E) would not increase 4-fold and the Halo τ_{unf} value appears to decrease (Figure 2F).

(B) τ values for single-turnover binding and unfolding of GFP-ssrA (0.5 μM) by ClpXP (10 μM ClpX^{AN}; 20 μM ClpP) at different temperatures.

(C) τ_{deg} values ($1/V_{max}$) at different temperatures determined from Michaelis-Menten plots of steady-state rates of degradation of different concentrations of GFP-ssrA by ClpX^{AN} (0.3 μM) and ClpP (0.9 μM).

(D) K_M values for GFP-ssrA degradation at different temperature (conditions as in C).

(E) Rates of ClpXP ATP hydrolysis at different temperatures by ClpX^{AN} (0.3 μM) in the presence of ClpP (0.9 μM) and GFP-ssrA (20 μM).

(F) Fractional activity of ClpXP at different temperatures calculated as $(\tau_c + \tau_{unf} + 5 s)/\tau_{deg}$, where the $\tau_c + \tau_{unf}$ value is taken from (B) and 5 s is the estimated time for translocation of GFP-ssrA.

that helices are inherently easier to unfold than strands in β sheets.

To a first approximation, the preunfolding dwell times for the V13P, V15P, and Halo domains were exponentially distributed, supporting one major unfolding

decrease stability by altering hydrogen bonds to the C-terminal β strand of titin also decrease the average preunfolding dwell time in single-molecule ClpXP experiments. However, rates of ClpXP degradation are not always correlated with global stability. For example, ClpXP degrades an ssrA-tagged variant of a hyperstable RNase-H ($\Delta G_u \approx 12$ kcal/mol) faster than it degrades V13P-titin²⁷-ssrA ($\Delta G_u \approx 3$ kcal/mol; Kenniston et al., 2003, 2004). In RNase-H-ssrA, ClpXP initially pulls against a C-terminal helix as opposed to pulling against a β strand in titin. Lee et al. (2001) speculated that AAA+ proteases might be able to unfold an α helix, which can be pulled apart by stepwise unzipping, more easily than a strand in a β sheet, which requires simultaneous shearing of multiple hydrogen bonds (Figure S5). In the absence of force, our results suggest that ClpXP unfolds the Halo domain, which has a C-terminal helix, substantially faster than any of the titin domains, supporting the possibility

pathway and a single rate-limiting kinetic step. Nevertheless, unfolding times were substantially longer than times required for even a burst of power strokes (~ 0.6 s based on the ATPase rate and translocation dwells), as expected if unfolding requires coincidence between a power stroke and transient stochastic thermal destabilization. Because most protein domains fold cooperatively, ClpXP disruption of even a small number of stabilizing native interactions could result in rapid global unfolding of the remaining structural elements in the domain. At a second level, ClpXP unfolding of V13P fit better to exponential processes acting on less-stable and more-stable populations of similar size (Figure S2), with enough events ($n = 262$) to make sampling error unlikely. This result is consistent with the existence of two unfolding pathways, which could depend upon which parts of the V13P domain are stochastically destabilized. For example, the N-terminal portion of V13P might be transiently frayed in

the more-stable population and the C-terminal region transiently frayed in the less-stable population.

A Model for Unfolding, Different Physical Step Sizes, and Motor Memory

We find that a substantial number of physical translocation steps occur in multiples of ~ 1 nm, in agreement with previous results (Aubin-Tam et al., 2011; Maillard et al., 2011; Sen et al., 2013). Based on structures of ClpX rings, conformational changes larger than 1 nm seem unlikely, and it is commonly assumed that an ~ 1 nm step involves hydrolysis of one ATP and one power stroke (Glynn et al., 2009; Aubin-Tam et al., 2011; Maillard et al., 2011; Sen et al., 2013; Stinson et al., 2013). Thus, bursts involving multiple power strokes are likely to drive larger physical steps. For wild-type ClpXP, each power stroke could result either directly or indirectly from hydrolysis of one ATP, as a ClpX hexamer binds a maximum of four ATPs (Hersch et al., 2005). Despite having just two catalytically active subunits, however, RWERWE ClpXP also takes physical steps ranging from ~ 1 to 4 nm, raising the possibility that a single ATP-hydrolysis event can generate more than one power stroke. For example, an initial power stroke might be generated by ATP hydrolysis and ADP/P_i release in one subunit and a subsequent power stroke by ATP dissociation from an inactive subunit in RWERWE ClpX (see below).

Any model of ClpXP function needs to be consistent with structural and biochemical results. For example, subunits in the ClpX hexamer display structural and biochemical asymmetry, suggesting a large number of different states and nucleotide-bound ring configurations (Baker and Sauer, 2012). Moreover, based on equilibrium and kinetic studies, two subunits in a ClpX hexamer do not appear to bind ATP, two bind ATP weakly, and two bind ATP strongly (Hersch et al., 2005; Stinson et al., 2013). ATP binding to subunits with weak affinity drives conformational changes required for the ClpX ring to hydrolyze ATP and perform mechanical work (Stinson et al., 2013). ATP hydrolysis and coupled mechanical work by ClpX rings cannot depend on a strictly sequential mechanism, as variants with numerous ATPase-inactive subunits still unfold and degrade protein substrates in solution (Martin et al., 2005) and in the single-molecule RWERWE studies here. Moreover, a strictly sequential mechanism should generate a clear sequence of translocation step sizes, which we do not observe. Finally, a model should account for the fact that ATP hydrolysis is substantially slower during ClpXP unfolding of native substrates than during translocation (Kenniston et al., 2003).

The models depicted in Figures 7A and 7B meet the criteria described above and provide a quantitative framework for understanding ClpXP unfolding and translocation. ClpX rings are designated as active (X) or inactive (iX) with the number of bound ATPs specified by a trailing number. Thus, X4 is an active ring with four ATPs and iX2 is an inactive ring with two ATPs. X3 and X4 rings are active. In agreement with biochemical studies (Stinson et al., 2013), all other rings are inactive and must bind additional ATP and/or change conformation to become an active X4 or X3 ring. When a natively folded protein domain cannot enter the axial channel of an X4 or X3 ring, ATP hydrolysis and product release result either in a futile power stroke or in a power

stroke that causes unfolding (Figure 7A). For simulations with the kinetic constants shown, the rates of these processes, conformational changes, and ATP-binding steps result in single-exponential unfolding kinetics ($R^2 = 0.999$) and a τ_{unf} of 6 s, a value close to τ_{unf} for V13P titin. Decreasing just the rate constant for unfolding in the model produces longer preunfolding dwells, as we observe for the Halo, V15P titin, and WT titin domains. The model also predicts hydrolysis of an average of approximately five ATPs for each V13P domain that ClpXP unfolds and higher ATP consumption in proportion to the increased unfolding times for more stable domains, as observed experimentally (Kenniston et al., 2003).

Once unfolding is successful, additional cycles of ATP hydrolysis drive translocation of the polypeptide chain as diagrammed in Figure 7B. Again, only X4 and X3 rings are active. From the X4 ring, physical steps of 1–4 nm are taken, depending upon which ATP-bound subunit hydrolyzes ATP or fires first. For example, initial firing of low-affinity subunit a results in a 1 nm step, initial firing of low-affinity subunit b results in a 2 nm step, and so on (Figure 7C). From the X3 ring, firing of the b subunit results in a 1 nm step, whereas firing of the c and d subunits result in steps of 2 and 3 nm, respectively. For steps of 2, 3, or 4 nm, we assume that two, three, or four ATPs are hydrolyzed and/or released in rapid succession, generating a burst of power strokes that are not experimentally resolved. Simulations using the rate constants in Figure 7B produce step-dwell distributions (Figure S4) and step-size distributions close to the experimental distributions (Figure S6). Step memory, which depends on the rates at which the X3 ring takes additional steps or recycles to X4, was also recapitulated (Figure S7) but to a smaller extent than observed. In simulated data, for example, 38% of all 1 nm steps were followed by a second 1 nm step, whereas this value was 41% in the experimental data (controls for the accuracy of the step-finding algorithm are presented in Figure S8). In the absence of memory, only 29% of the next steps would also be 1 nm. Sen et al. (2013) reported almost complete loss of 4 nm steps at ATP concentrations near K_M . At low ATP concentrations, our model predicts that the population of X3 rings would increase substantially compared to X4 rings, reducing the average step size and fraction of 4 nm steps. The ATPase rate in our model is effectively determined by the slow conformational rearrangements needed to generate active X4 and X3 rings (2.2 s^{-1} for translocation; 0.5 s^{-1} for unfolding), predicting ~ 4 -fold faster ATP hydrolysis during translocation than unfolding, as is experimentally observed during ClpXP degradation of native and denatured titin substrates (Kenniston et al., 2003). Thus, our model accounts for a broad range of experimental results. We were unable to match the experimental results using models in which ClpX conformational changes precede rather than follow ATP binding or in which X4 rings are the only active species.

Stochastic and Coordinated ATP Hydrolysis

In our model, initial ATP hydrolysis in the X4 or X3 rings is probabilistic, as first proposed based on studies of ClpX rings with mixtures of active and inactive subunits (Martin et al., 2005). Contrary to arguments by Smith et al. (2011), a probabilistic or stochastic model does not imply that subunits act independently. Indeed, Martin et al. (2005) found that ATP-hydrolysis

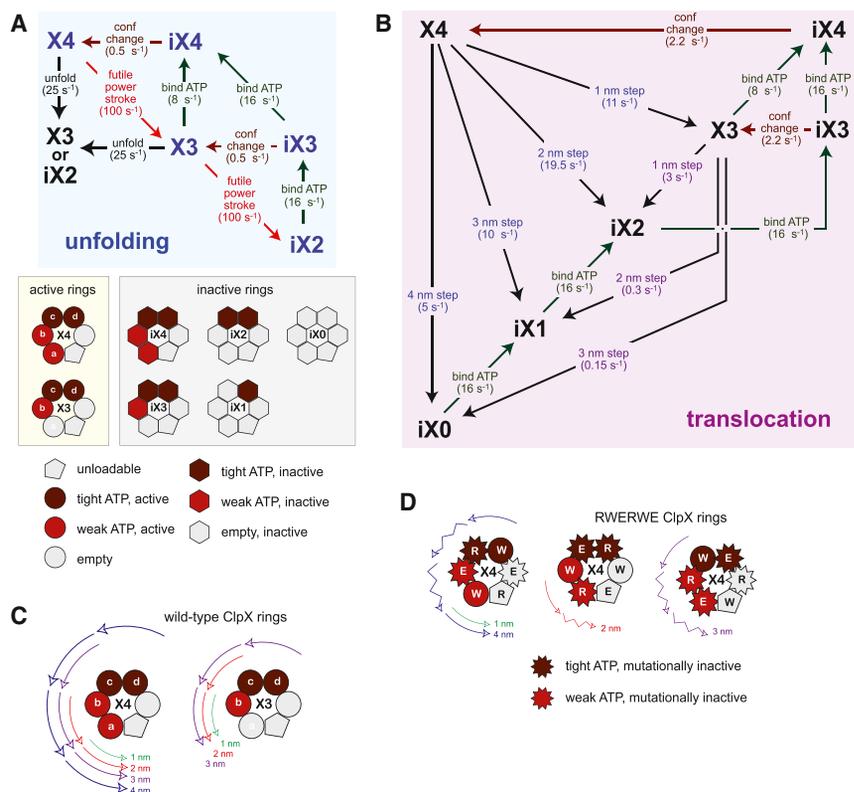


Figure 7. Mechanochemical Models for ClpXP Function

X4 and X3 rings are hydrolytically and mechanically active. iX3, iX2, iX1, and iX0 rings are inactive. Numbers after the X are bound ATPs. In the cartoons of the ClpX hexamer, dark red subunits bind ATP tightly, red subunits bind ATP weakly, and light gray subunits do not bind ATP.

(A) Unfolding model. ATP hydrolysis in the X4 or X3 rings results in an unfolding power stroke, which allows translocation to begin, or in a futile power stroke. ATP-binding reactions are represented by green arrows and conformational changes by dark red arrows. For simplicity, ATP binding to iX0 or iX1 rings is not shown in this panel, ATP-dissociation reactions are not included, and different configurations of nucleotide-bound subunits in the X3 ring are not considered. Pseudo-first-order rate constants for ATP-association reactions are for saturating concentrations of ATP. The mechanical stability of a native protein determines the rate of the unfolding reaction; other rates are determined by the properties of ClpXP. The rate constants in parentheses give exponential unfolding kinetics ($\tau_{\text{unf}} \sim 6$ s).

(B) Translocation model. Depending on which ATP-bound subunit in the X4 or X3 rings hydrolyzes ATP first, physical translocation steps of 1, 2, 3, or 4 nm are taken (black arrows). A physical step of N nm is associated with N hydrolysis/release events. Numbers in parentheses are rate constants that were adjusted to provide a reasonable fit to experimental data.

(C) In the cartoons shown, initial ATP hydrolysis in subunits of X4 or X3 rings (labeled d, c, b, or a) result in very-fast ATP hydrolysis/release events that generate power strokes (arrows) in the ATP-bound counterclockwise subunits, generating physical translocation steps of 4, 3, 2, or 1 nm, respectively.

(D) As shown on the left, if wild-type (W) subunits occupy the d and a positions in X4 rings of RWERWE ClpX, then translocation steps of 1 nm (subunit a fires first) or 4 nm (subunit d fires first) are taken. When subunit d fires first, ATP is released from the counterclockwise inactive c (R) and b (E) subunits to generate power strokes (crooked arrows). If wild-type (W) subunits occupy the b or c positions in the X4 ring (center and right, respectively), then initial hydrolysis in these subunits results in steps of 2 or 3 nm, respectively, again with ATP release from counterclockwise inactive subunits generating power strokes (crooked arrows). See also Figures S3, S6, and S7.

activity was not strictly proportional to the number of ATPase active subunits and provided strong evidence that directional communication between neighboring subunits regulates ATP hydrolysis and mechanical activity. In crystal structures of hexameric ClpX rings, the nucleotide-binding pockets in each subunit that can bind ATP (loadable subunits) vary slightly (Glynn et al., 2009; Stinson et al., 2013), providing a basis for differential nucleotide affinities and for different probabilities of which subunit fires first. It is also possible, however, that interactions with the protein substrate determine which subunit fires first (Martin et al., 2005). For example, GYVG loops in the axial pore of ClpX are known to contact the *ssrA* tag and translocating substrates and to influence ATP-hydrolysis rates. Thus, an ATP-bound subunit whose pore loop was in direct contact with a translocating polypeptide or the *ssrA* tag might have a higher probability of firing first (Martin et al., 2008b, 2008c), and the highly variable chemical and conformational heterogeneity of an unfolded polypeptide chain could determine the stochastic nature of initial firing.

Following stochastic firing of a specific subunit in the ClpX ring, whether and how many additional subunits fire rapidly will depend on subunit-subunit communication. Although the details of such communication remain to be deciphered, we suggest

one possibility. For example, firing of a given subunit might cause ATP-bound counterclockwise subunits to fire or release nucleotide, so that initial firing in the a, b, c, or d subunits in X4 would result in hydrolysis/release of one, two, three, or four ATPs and translocation steps of 1, 2, 3, or 4 nm and initial firing of the b, c, or d subunits in X3 would result in hydrolysis/release of one, two, or three ATPs and physical steps of 1, 2, or 3 nm (Figure 7C). Thus, a physical translocation step that began with a stochastic ATP-hydrolysis event could be followed by coordinated hydrolysis/release events, which could be programmed sequentially or stochastically. The choice of counterclockwise versus clockwise propagation in the model is arbitrary.

With minor modifications, this model can also explain how RWERWE ClpXP could take steps of ~ 3 nm or larger using only two hydrolytically active wild-type subunits in the ClpX ring. As shown in Figure 7D, multiple configurations of an X4 ring are possible for RWERWE ClpX. When W subunits occupy the a and d positions (Figure 7D, left), initial firing of the a subunit could generate a 1 nm step, whereas initial firing of the d subunit could yield a 4 nm step, with ATP release from inactive subunits generating some power strokes. By contrast, RWERWE X4 configurations with wild-type subunits at the b or c positions (Figure 7D, center and right) could result in 2 or 3 nm steps. Is

it energetically feasible for ATP release to generate a power stroke? The highest force at which we recorded RWERWE ClpXP activity was ~ 10 pN. To drive an ~ 1 nm movement against this force requires ~ 1.5 kcal/mol (~ 2.5 kT) of energy. At the 2 mM ATP concentrations used for our experiments, a conformational change in the ATP-binding pocket that weakened affinity to ~ 30 mM would allow ATP dissociation to generate a favorable free-energy change of ~ 1.6 kcal/mol ($\Delta G = -RT \ln [30 \text{ mM}/2 \text{ mM}]$), making it plausible that ATP release drives a power stroke. ATP-loadable and unloadable subunits in the ClpX ring interconvert during function (Stinson et al., 2013), and thus, the affinity of a given subunit for ATP could become substantially weaker as a consequence of structural changes in neighboring subunits. Why are the dwell times between physical steps in RWERWE ClpXP translocation so much longer than in wild-type ClpXP translocation? The simplest possibility is that the presence of catalytically inactive R or E subunits at ring positions poised to fire requires a slow ring-resetting reaction.

Alternative Models

Although the models in Figure 7 explain our single-molecule results and are consistent with a wide range of observations, related models may do so equally well. For example, we model the active ClpX ring with five loadable subunits and one unloadable subunit (Stinson et al., 2013), but other ratios of loadable to unloadable subunits could work equally well. Similarly, we assume that only four ATPs bind to the ClpX ring based on biochemical results (Hersch et al., 2005), but the results could also be fit if ATP bound to each loadable subunit. The modeled arrangement of high-affinity and low-affinity subunits in the ClpX ring is also speculative.

In a very different model proposed by Sen et al. (2013), the number of ATPs bound to the ClpX ring solely determines the size of the subsequent physical step, which always ends with a nucleotide-free ClpX ring. Thus, they suggest that 4 nm, 3 nm, and 2 nm steps are taken if four, three, or two ATPs are initially bound to the ClpX ring, respectively. Their model excludes the possibility of 1 nm steps. In conflict with biochemical experiments (Stinson et al., 2013), the Sen model requires ClpX rings with ATP bound only to two high-affinity subunits to be active. It also fails to account for the motor memory we observe or to explain why a broad mixture of physical step sizes is observed at saturating concentrations of ATP. Sen et al. (2013) propose that P_i release is the force-sensitive step coupled to each power stroke, rather than ATP hydrolysis, ADP release, or ATP binding. In our view, the chemical step responsible for power strokes remains in question, as the P_i -release model depends upon untested assumptions and fails to account for our finding that RWERWE ClpXP can take steps of ~ 3 nm or longer.

Conformational Switching

Conformational switching between ATP-loadable and unloadable subunits in the ClpX ring, with concomitant changes in the identities of the subunits that bind ATP with high and low affinity, appears to be required for robust mechanical activity (Stinson et al., 2013). How can this requirement be rationalized in terms of the models shown in Figure 7? One possibility is that confor-

mational switching is directly involved in force generation for every power stroke. Another possibility is that the products of ATP hydrolysis are not properly ejected after some unfolding attempts or translocation steps. Loadable-unloadable conformational switching might eject these products and redefine the ATP affinities of individual subunits to reset the ClpX ring and allow resumption of translocation or unfolding attempts (Stinson et al., 2013). Pausing during ClpXP translocation may arise in this manner, with the greater pausing propensity of RWERWE ClpXP resulting from the presence of catalytically inactive subunits, which increase the probability that a translocation step finishes with a ring conformation that must be reset before activity resumes.

Importance of Large and Small Step Sizes

What role do large physical translocation steps play in ClpXP degradation? As a single 4 nm step takes $\sim 35\%$ as much time as four 1 nm steps, bigger physical steps may simply allow faster translocation and thus faster degradation. We note, however, that translocation may represent a small fraction of the time required for degradation of many proteins. Another possibility is that a kinetic burst of power strokes is better able to unfold certain proteins, for example those with larger distances to the unfolding transition state. If large translocation steps are beneficial, then why has ClpXP evolved to take small steps as well? Small steps may allow ClpX to maintain a tighter grip on the substrate because more subunits are ATP bound (Nager et al., 2011), allowing more efficient transfer of force and increasing the probability of unfolding certain proteins.

Lessons for Solution Degradation

For multiple substrates, ClpXP degradation is substantially slower than predicted based on single-molecule rates of unfolding and translocation (Figure 6A), indicating that commitment is the slowest step in solution degradation. Indeed, experiments suggest that native titin substrates are bound and released many times before being unfolded by ClpXP (Kenniston et al., 2005). Two factors can affect commitment times for ClpXP. First, the SspB adaptor, which binds both to the ssrA tag and to ClpX, increases V_{max} for protein degradation (Levchenko et al., 2000; Wah et al., 2002). If commitment is the slow step in degradation at substrate saturation, then SspB must make this step faster. Consistently, SspB reduces the time required for binding and unfolding in single-turnover experiments. For example, in single-turnover experiments at 30°C, the time required for ClpXP binding and unfolding of GFP-ssrA is ~ 34 s in our experiment but ~ 17 s with SspB present (Martin et al., 2008a). Thus, SspB is likely to increase the probability of unfolding by increasing the average number of ClpXP-unfolding attempts that occur before substrate dissociation and the need for rebinding. Second, the length of polypeptide bound in the axial pore of ClpXP influences commitment. For example, this length is ~ 15 residues for ssrA-tagged titin, Halo, and GFP substrates but 35–40 residues for the nontagged domains of multidomain substrates, including those in single-molecule experiments (Lee et al., 2001; Kenniston et al., 2005; Martin et al., 2008a). In single-turnover experiments performed in the presence of SspB at 30°C, ClpXP degraded GFP followed by an unstructured C-terminal

titin-ssrA domain almost twice as fast as GFP-ssrA and at rates similar to those observed for single-molecule unfolding (Martin et al., 2008a; Maillard et al., 2011; Sen et al., 2013). From a mechanistic perspective, a longer region of polypeptide in the axial pore of ClpXP should allow a tighter grip by the enzyme and thus reduce the probability of dissociation following a failed unfolding attempt. If longer unstructured degrons can speed degradation and result in a lower net cost in terms of ATP hydrolysis, then why are relatively short degrons used so often in biological systems? One possibility is that protein degradation typically occurs in energy-rich cellular environments and that longer degrons would open the possibility for truncation of the degron by nonspecific proteases, preventing targeted degradation of the proper substrates by ClpXP and other AAA+ proteases.

EXPERIMENTAL PROCEDURES

Proteins were expressed and purified as described in the [Extended Experimental Procedures](#). For single-molecule experiments, biotinylated ClpXP was attached to a laser-trapped bead, substrate containing a Halo domain covalently linked to biotinylated DNA was attached to another bead, and enzyme-substrate tethers were formed. Measurements were performed under constant force at 18°C–22°C using 2 mM ATP with ATP-regeneration and oxygen-scavenging systems as described (Aubin-Tam et al., 2011). Data were collected at 3 kHz sampling frequency and decimated for further analysis. Custom MATLAB scripts were used to calculate interbead distances, measure unfolding distances, measure preunfolding dwells from the end of one translocation event to the next unfolding event, determine average translocation velocity, and detect pauses. Individual translocation steps were identified using the chi-square method (Kerssemakers et al., 2006), which requires input of the expected number of steps, estimated by taking the pairwise distribution of decimated data subjected to a step-smoothing algorithm based on L1 regularization with independent noise (Little et al., 2011). We set a minimum detectable step-size threshold of 0.75 nm and combined smaller steps, including backward steps or slips, with previous and following steps by adding the dwell-weighted average (S_{avg}) of a small step (S) to the previous step (S–1) and subtracting S_{avg} from the following step (S+1). The dwell-weighted average is defined as

$$S_{\text{avg}} = S \cdot (d_{S+1}) / (d_S + d_{S+1})$$

where d_S is the dwell preceding a small step and d_{S+1} is the dwell following a small step. Kinetic simulations of the models shown in [Figure 7](#) are described in the [Extended Experimental Procedures](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and eight figures and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2014.05.043>.

AUTHOR CONTRIBUTIONS

J.C.C., A.O.O., and Y.S. performed and/or analyzed optical-trapping experiments. B.M.S. performed the [Figure 6](#) experiments. S.C., K.R.S., and A.O.O. constructed and purified substrates and enzymes for single-molecule experiments. A.O.O., M.-E.A.T., and M.J.L. built the optical traps. R.T.S. developed the [Figure 7](#) models. All authors contributed to writing the manuscript.

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