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ClpX Shifts into High Gear to Unfold Stable Proteins

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Protein degradation by the ClpXP protease requires collaboration among the six AAA+ domains of ClpX. Using single-molecule optical tweezers, Sen et al. show that ClpX uses a coordinated succession of power strokes to translocate polypeptides in ATP-tunable bursts before reloading with nucleotide. This strategy allows ClpX to kinetically capture transiently unfolded intermediates.

ClpX, a member of the AAA+ superfamily, is a homomeric hexamer that harnesses nucleotide hydrolysis-dependent conformational changes to promote unfolding of engaged substrate proteins. ClpX forms a stacked-ring complex with ClpP and catalyzes degradation of intracellular proteins. The ATP-dependent reaction cycle begins with binding of the N or C terminus of the substrate within the axial channel of ClpX, after which ClpX repetitively pulls on the polypeptide chain, causing the protein to unfold and then processively translocates it through the channel into the degradation chamber of ClpP. In this issue of *Cell*, Sen et al. (2013) monitored the activity of single molecules and found that ClpX orchestrates its ATP use to drive unfolding of stable proteins.

Proteases like ClpXP face thermodynamic and kinetic challenges in assisting a substrate in navigating the energy landscape between native and unfolded states and then over the entropic barrier for translocation through the narrow pore in the hexamer. Single-molecule studies (Aubin-Tam et al., 2011; Maillard et al., 2011) have shown that the ClpX AAA+ machine performs mechanical work in overcoming these energy barriers, translocating a polypeptide against an opposing force and delivering a power stroke capable of unfolding stable domains.

The structure of ClpX provides a physical model for power stroke delivery in which nucleotide binding and hydrolysis lead to switching between subunit conformational states that is accompanied by a displacement of a conserved axial loop known to directly engage substrate proteins (Glynn et al., 2009). Movement of the central channel loop can deliver a power stroke estimated to be ~5 kT, corresponding to the force of ~20 pN applied during an ~1 nm displacement. By measuring translocation velocities using ATP, ADP, and phosphate concentrations, Sen et al. (2013) marshal a convincing argument that phosphate release, which is essentially irreversible under the experimental conditions, is the major force-generating step. The absence of a direct role for ATP binding in the force delivery step fits well with findings (described below) that translocation steps occur in bursts of 2 to 4, which are envisioned as resulting from rapid-fire ATP hydrolysis and phosphate release triggered after 2–4 ATPs are loaded on ClpX.

Because the central channel loop moves ~1 nm, 2–4 nm bursts represent the sum of multiple subunits acting in quick succession. Sen et al. (2013) found that burst size distributions depended on the concentration of ATP. The largest burst size was 4 nm, which correlates with findings that a maximum of four ATPs bind to ClpX hexamers (Hersch et al., 2005) and with single turnover studies showing that hydrolysis of four ATPs provide maximum activity of ClpXP (Martin et al., 2008). Using a competitive inhibitor of ATP binding, Sen et al. (2013) observed that three of the four ATP sites had to be blocked in order to stall translocation, meaning that just two functional sites per ring are sufficient to catalyze translocation and produce rapid 2 nm bursts. The prevalence of 2–4 nm bursts during single-molecule translocation suggests that ClpX must coordinate ATP hydrolysis and/or the accompanying

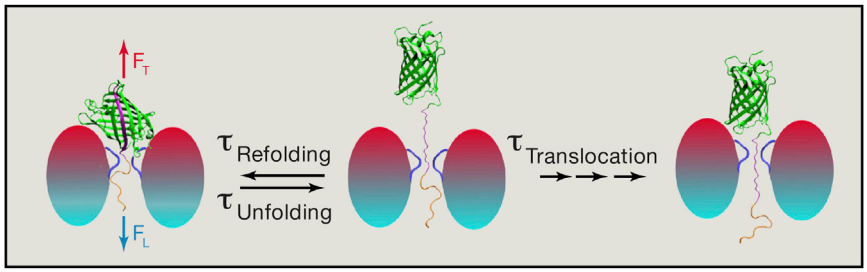

Figure 1. Kinetic and Thermodynamic Requirements of Protein Degradation by the ClpXP Machine in Optical Tweezer Experiments

Homohexameric ClpX (shown schematically as two subunits viewed from the side, where the cyan-colored regions correspond to the ClpP docking surfaces) attached to one bead engages the tagged GFP substrate (orange and green) linked to another bead (beads not shown). The central channel loop of each subunit (blue) interacts with the substrate during individual ATP hydrolysis events, and ClpX repetitively applies a force F_L pulling the substrate against the opposing force F_T generated by the optical tweezers. The initial unfolding event, extraction of strand β11 (purple) from the native GFP barrel, is accomplished by a single power stroke from ClpX but is spontaneously reversed on a rapid timescale $\tau_{refolding}$. To effect irreversible unfolding, ClpX must trap and translocate the unfolding intermediate using three additional ATP hydrolysis steps within $\tau_{translocation} < \tau_{refolding}$. The slower internal timing mechanism for resetting the machine dictates that translocation must be completed within a fraction of ClpX cycle time, requiring strong coordination between the catalytic steps of the nonconcerted cycle.

power strokes in multiple subunits. How coordination is accomplished and what determines burst size are challenging questions, but they can now be rigorously addressed by single-molecule studies. The ability to design ClpX hexamers with active subunits and mutated subunits with various functional defects interspersed in multiple configurations (Martin et al., 2005) will provide a powerful means of obtaining deeper insight into the modes and mechanisms of subunit communication.

One of the most intriguing discoveries reported by Sen et al. (2013) is that the ability of ClpX to initiate protein unfolding was correlated with the frequency of 4 nm bursts. The data support a kinetic trapping model for unfolding by ClpX in which the unfolded parts of the substrate are rapidly separated to prevent refolding. GFP unfolding by ClpX is initiated by the extraction of the β11 strand (Figure 1), but this step is spontaneously reversible on a timescale of 240 ms. Irreversible unfolding can only be achieved if translocation events of ~4 nm occur within a subcycle timescale because the bursts are known to be separated by dwell times of ~350 ms during which no substrate movement occurs (Aubin-Tam et al., 2011; Maillard et al., 2011). By coordinating bursts to deliver a 4 nm power stroke within <10 ms, ClpX effectively translocates the unfolded structural element away before it can refold into the native structure. Such kinetic conditions for substrate remodeling have also been described in assisted protein folding by GroEL, which must undergo multiple allosteric cycles with timescales that are shorter than the folding times of its stringent substrates (Thirumalai and Lorimer 2001; Stan et al., 2007). The novel aspect of the findings of Sen et al. (2013) is the ability of the ClpX AAA+ machine to meet kinetic requirements by tuning its translocation capacity in nonconcerted ATP hydrolysis cycles.

What is involved in tuning ClpX to perform bursts of a given size? Sen et al. (2013) found that, even though rounds of hydrolysis could involve different numbers of subunits, the dwell time between rounds remained constant as ATP concentrations changed, preserving the overall cycle time. Simultaneously, the overall translocation rates nearly doubled, leading to the proposal that the ClpX machine acts at a constant rpm but in different gears depending on the number of ATP loaded or hydrolyzed. Translocation rates therefore depend on the degree of coordination among a variable number of ATP hydrolyzing subunits. Rapid bursts also require that the substrate be productively engaged in each catalytically active step of any cycle. Especially during the 4 nm bursts needed for unfolding, subunits must collaborate to ensure that the substrate is in a position to receive and react to each of the four power strokes.

The invariance of the cycle time with respect to ATP concentration can be explained by a rate-limiting reaction that occurs in each cycle and is independent of ATP binding. In fact, the authors calculated that there should be two such events. Such events must occur whether one, two, three, or four subunits have just delivered a power stroke, suggesting that they begin after the last subunit has hydrolyzed ATP. The division of the reaction cycle between a rapid burst phase and a longer dwell phase is consistent with the ring-resetting subunit switching cycle recently proposed by Stinson et al. (2013). The dwell periods observed by Sen et al. (2013) would represent the time during which the posthydrolysis ring loads ATP and isomerizes to a state in which a new burst of ATP hydrolysis can be initiated. A novel finding of this study is that allostery within the ClpX hexamer is not limited to adjacent subunits and must, at least at times, be communicated among all four ATP loaded subunits to account for coordinated 4 nm bursts. The variance in dwell times might reflect differential rates of ADP release or subunit switching in response to allosteric influence from the portion of the substrate occupying the central channel at any given stage, a proposal that can easily be tested by translocating multiple copies of an identical substrate domain and examining the distribution of bursts sizes along the trajectories.

Overall, the single-molecule results support the unified model of translocation and unfolding for proteases originally hypothesized by Matouschek (Lee et al., 2001) and present evidence that ClpX coordinates power stroke bursts involving multiple subunits. Many details of the burst phases and dwell times remain to be clarified, including what initiates the bursts and what are the rate-limiting reactions occurring during the dwell. Another important question not specifically answered by these studies is whether ClpX hexamers act by an ordered or a stochastic mechanism. That question can now be answered unambiguously using the methods employed here because the introduction of one nonhydrolyzing subunit into a hexamer should give a different distribution of burst sizes

in each of the models. Last, more needs to be known about how asymmetric interactions with ClpP affect ClpX burst sizes and help complete polypeptide translocation through the access channel of ClpP. Future work probing the intricacies of the mechanism of ClpXP will surely yield insights into the mechanics and kinetics of other ATP-dependent proteases and the entire family of AAA+ proteins.

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