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Protease Power Strokes Force Proteins to Unfold

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ATP-dependent proteases degrade proteins in the cytosol of cells. Two recent articles, by Aubin-Tam et al. (2011) and Maillard et al. (2011 [this issue]), use single-molecule optical tweezers to show directly that these molecular machines use the energy derived from ATP hydrolysis to mechanically unfold and translocate its substrates into the proteolytic chamber.

Most proteins have transient lives inside the cell, which end when they are degraded by ATP-dependent proteases. However, prior to degradation, the protein must first be unfolded by the protease and then threaded through the narrow mouth of the protease's channel. A long-time mystery has been, how does the same molecular machine efficiently unfold a wide variety of proteins with different topologies and stabilities? Although most proteins are readily denatured by high concentrations of chemical denaturants, such as urea, these methods of protein denaturation are not feasible in the mild environment of the cytosol. Thus, mechanical force has been proposed as the most plausible mechanism that unfolds proteins targeted for degradation in the cytosol (Hochstrasser and Wang, 2001).

Now two independent studies, one in this issue of *Cell* (Maillard et al., 2011) and one in the previous issue (Aubin-Tam et al., 2011), demonstrate that a prokaryotic ATP-dependent protease, ClpXP, is a power stroke molecular machine that can generate forces of up to 20 pN to unfold and translocate polypeptides into its proteolytic active site. The results presented in both reports represent a breakthrough in our understanding of protein unfolding in vivo and pave the way for future studies that will reveal a protein's final destination in unprecedented detail. Furthermore, these results now conclusively place the mechanical unfolding of proteins as a keystone in biology.

Since the advent of single-protein force spectroscopy (Figure 1A), a mechanical stretching force has been shown to accelerate exponentially the unfolding rate of a protein (Schlierf et al., 2004), albeit the mechanical stability varies greatly from protein to protein. Thus, a mechanical force is an ideal mechanism for triggering the unfolding of proteins by ATP-dependent proteases. Nevertheless, direct evidence that these macromolecular complexes use mechanical forces to unfold their substrates has been missing.

To study the mechanism of protein unfolding and translocation by the ClpXP motor, both Aubin-Tam et al. and Maillard et al. use a single-molecule optical tweezer setup (Figure 1B). This allows them to apply a calibrated force to the distal end of ClpXP's substrate, which counteracts the "pull" by ClpXP as the substrate threads through the protease's mouth. Both groups then measure the distance from the ClpXP to the distal end of the substrate. These measurements reveal abrupt elongations of the substrate at the nanometer scale, which have become the signature for proteins unfolding under force (Fernandez and Li, 2004). The elongation events are immediately followed by translocation of the unfolded polypeptide into the protease, against the pulling force. In addition, both groups find that the translocation speed decreases with increasing pulling forces, with the stall force of the AAA^+ motor estimated at 20–30 pN.

In the study of Aubin-Tam and colleagues, the protease substrate is a protein with eight human filamin A domains connected by flexible linkers. The β sandwich fold of this domain is mechanically stable, and its unfolding has been studied by single-protein force spectroscopy (Furuike et al., 2001). Using this polyprotein as a substrate provides a clear mechanical fingerprint for degradation: peaks of unfolding are followed by a rapid translocation of the protein and then a pause that unambiguously represents the time needed to unfold the next protein in the chain. There are many satisfying details in these recordings. For example, after the protease fully consumes each filamin domain, the overall end-to-end length of the polyprotein shortens by ~4 nm. This is approximately the length of a folded filamin domain, confirming its full disappearance from the polyprotein. Another striking finding is that the dwell times marking the duration of the translocation pauses between unfolding events are independent of the pulling force. This result contradicts more than 10 years of force spectroscopy studies on single proteins, which predict that unfolding dwell times should drop to less than a third when the pulling force is increased from ~4 pN up to 20 pN. Furthermore, at high probe forces (>15 pN), distal filamin modules that are not bound to ClpXP's mouth are occasionally seen to unfold in the experiments by Aubin-Tam and colleagues. At these same high forces, the unfolding dwell times of the proximal domains abutting ClpXP remained unchanged. What could explain these puzzling results?

In single-protein force spectroscopy, the force is applied between the protein's

two termini (Figure 1A), but the pulling geometry of ClpXP is dramatically different (Figure 1B). When pinned against the mouth of the protease channel, the doomed protein is subject to two opposing forces: the constant force exerted by the molecular motor (F_M), pointing in the direction of translocation into the protease, and an opposing normal force arising from the steric interaction between ClpXP and the substrate (F_N) (which points perpendicular to the surface of ClpXP). These are the two forces that eventually unfold the target protein in vivo. Application of an external force (F_P) to the pinned substrate protein will simply reduce the value of the normal force, while keeping the sum of the forces on the protein constant ($F_P + F_N = F_M$). If we assume that the effects of F_N and F_P are equivalent, this simplified model predicts that a folded protein pinned at the mouth of the protease will show a lifetime that is independent of the external force. This is valid until that external force is strong enough to pull the substrate out of the channel. The observation that the external force does not alter the rate of ClpXP-mediated protein unfolding also implies that the lifetimes measured by the two groups should closely correspond to the in vivo values, in the absence of external forces.

After a protein unfolds, the struggle is far from over. Such freshly unfolded proteins may not always become submissive substrates that easily surrender against the pulling traction of the translocase. On the contrary, the protein can fight back by attempting to refold against the pulling force of the translocase and regain its native structure (Figure 1C). It is well documented that mechanically unfolded polypeptides collapse rapidly (Fernandez and Li, 2004) and form molten globule structures with weak mechanical stability, which nonetheless generate a significant opposing force that could easily counter that of the translocase (Garcia-Manyes et al., 2009). Such struggles are not observed in current recordings, but they may become readily apparent if more avid folders are chosen as substrates (Kubelka et al., 2004).

The path-breaking experiments reported in these two *Cell* papers invite biophysicists to expand the reach of these techniques, in hopes of answering long-standing fundamental questions about proteolysis inside the cytosol, such as: How do ATP-dependent proteases deal with more complex protein substrates that contain disulfide bonds? How does the eukaryotic proteasome handle indigestible substrates, such as amyloidogenic proteins, which can lead to serious human diseases? Tracts of polyglutamines, which have been linked to the pathogenesis of Huntington's disease, are known to inhibit the proteasome machinery (Bence et al., 2001). Notably, these polypeptides have also been shown to possess a remarkably high mechanical stability (Dougan et al., 2009). The key to answering these questions and the other mysteries of protein-mediated degradation may very well lie in the rapidly expanding world of single-protein force spectroscopy.

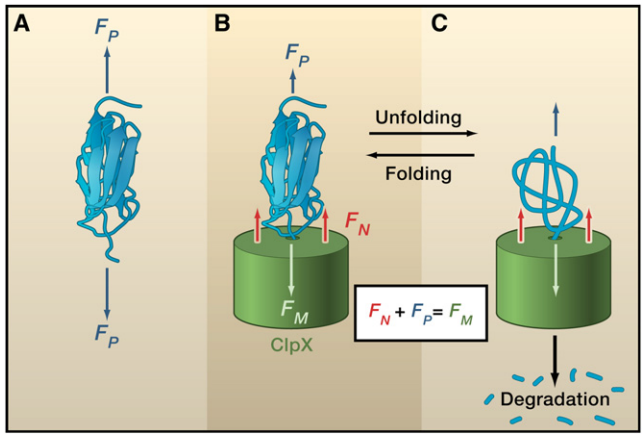

Figure 1. Force-Induced Protein Unfolding by ATP-Dependent Proteases

(A) In the standard geometry for single-protein force spectroscopy experiments, a stretching force is applied to the two termini of a protein.

(B) The ATP-dependent protease ClpXP generates a force F_M, which pulls degradation-targeted substrates into its central pore. A normal force, F_N, arises as the folded substrate is pinned against the narrow opening of ClpXP. These opposing forces trigger unfolding of the substrate in vivo. In the optical tweezer setup used by Maillard et al. (2011) and Aubin-Tam et al. (2011), a probe anchored to one end of the substrate introduces a third force in the system, F_P, which counteracts the pull of ClpXP. Changing F_P shifts the balance between F_N and F_P, according to the equation shown.

(C) Mechanical unfolding of the substrate reduces its resilience and enables degradation. However, in vivo, the substrate probably remains collapsed. Refolding from this collapsed state allows the protein to survive in an ongoing struggle against degradation.

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