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Mechanistic insights into bacterial AAA+ proteases and protein-remodelling machines

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Preface

To maintain protein homeostasis, AAA+ proteolytic machines degrade damaged and unneeded proteins in bacteria, archaea and eukaryotes. This process involves ATP-dependent unfolding of a target protein and subsequent translocation into a self-compartmentalized proteolytic chamber. Related AAA+ enzymes also disaggregate and remodel proteins. Recent structural and biochemical studies, in combination with direct visualization of unfolding and translocation in single-molecule experiments, have illuminated molecular mechanisms and suggest how remodelling of macromolecular complexes by AAA+ enzymes could occur without global denaturation. In this Review, we discuss the structural and mechanistic features of AAA+ proteases and remodeling machines, focusing on bacterial ClpXP and ClpX as paradigms. We also consider the potential of these enzymes as antibacterial targets and outline future challenges for the field.

Introduction. In all domains of life, cellular compartments are packed with proteins, many of which are in the process of folding, are intrinsically disordered, or contain both natively folded and unstructured regions[1]. Because the peptide bonds in an unstructured polypeptide are highly sensitive to proteolytic cleavage**,** the cytoplasm of bacteria and archaea, and most eukaryotic cellular compartments, do not contain indiscriminate proteases. Instead, specific proteins in these intracellular environments are degraded by proteolytic machines that sequester the active sites for peptide-bond cleavage within a protected chamber. These enzymes are known as AAA+ proteases, owing to the presence of a AAA+ unfoldase that recognizes specific substrates and uses the chemical energy of ATP hydrolysis to mechanically unfold the target protein and then translocate it into the degradation chamber[2–4].

AAA+ proteases present in bacteria, mitochondria, and chloroplasts include ClpXP, ClpAP, ClpCP, HslUV, Lon and FtsH[2]. Other proteases in the AAA+ family consist of the 20S peptidase, which is found in all three domains of life, in combination with different AAA+ unfoldase partners, such as Mpa (bacteria), PAN or Cdc48/p97 (archaea) or the $Rpt_{1\text{-}6}$ ring of the 26S proteasome (eukaryotic cytosol and nucleus)[3–5]. These AAA+ proteases enforce protein quality control by recognizing and destroying proteins that have been damaged by oxidation and heat stress[6,7] and protein fragments that have been generated by endoproteolytic cleavage or failures in translation[8-10]. Cellular processes can also be controlled by AAA+ proteases that degrade regulatory proteins, including the bacterial stationary-phase sigma factor[11,12], cell-division checkpoint inhibitors of the DNA-damage response[13], and proteins that regulate cell-cycle

progression[14]. For example, DNA damage in *Escherichia coli* results in synthesis of SulA, a cell-division inhibitor that must be degraded by the Lon protease before growth can resume[13], and ClpXP degradation of CtrA, a master regulator of transcription in *Caulobacter crescentus*, is required for cell-cycle progression and initiation of chromosomal replication[14]. Developmental transitions frequently involve degradation of specific proteins by AAA+ proteases, altering the composition of the intracellular proteome. ATP-dependent degradation of specific target proteins can also facilitate development of genetic competence, sporulation, virulence and biofilm formation[15,16].

In this Review, we discuss recent work that has shed light on the molecular mechanisms of bacterial AAA+ proteases. In particular, we highlight biochemical, structural, and single-molecule studies of protein degradation by *E. coli* ClpXP that illuminate the principles and dynamic interactions that enable the unfolding, translocation and degradation of a wide variety of structurally diverse protein substrates. Related principles explain how AAA+ enzymes can also function to remodel macromolecular complexes. We also examine the diversity of AAA+ proteases present in the bacterial domain and the potential of some of these enzymes as targets for antibacterial therapy. Finally, we outline future challenges for the field and the technological advances that will be needed to address them.

Bacterial AAA+ proteases. Most bacterial phyla utilize ClpXP, ClpAP or ClpCP, HslUV, Lon and FtsH to execute ATP-dependent protein degradation, whereas Actinobacteria also employ the Mpa•20S proteasome[2,3]. Mycoplasma, which have the smallest bacterial genomes, typically encode only the Lon and FtsH proteases[17,18].

ClpXP, a paradigm for AAA+ proteases. ClpXP, the best characterized AAA+ protease, consists of the ClpX unfoldase and ClpP peptidase[19]. Each ClpX subunit contains a large AAA+ domain and a small AAA+ domain, which together form the ATP-hydrolysis and motor module. In the ClpX hexamer, the AAA+ domains pack together to form a ring with an axial channel or pore that serves to initially engage a portion of the target protein, has an active role in unfolding, and is the conduit for translocation into the degradation chamber of ClpP[19]. ClpX also contains a family-specific N domain required for efficient recognition of adaptors and auxiliary signals in some substrates[20,21]. Like ClpX, subunits of the HslU, Mpa, PAN, Lon and FtsH unfolding enzymes contain a single ATP-hydrolysis and motor module, whereas subunits of the ClpA, ClpC, and Cdc48 enzymes have two ATP-hydrolysis and motor modules, which form discrete stacked rings in the hexamer[2].

ClpP consists of two heptameric rings that enclose a chamber containing the active sites for peptide-bond cleavage (Fig. 1a)[22–24]. A portal at the centre of each ring controls access to the degradation chamber and allows entry only to small peptides when ClpP is not bound to a AAA+ unfoldase partner[25]. The peptidases of other AAA+ proteases differ from ClpP in subunit structure, in the number of subunits in each ring, in the number of rings, and in the chemistry of the active-site residues that catalyse peptide-bond cleavage (Figs. 1b-f)[2]. Nevertheless, each of these peptidases also sequesters its active sites within a self-compartmentalized chamber, with access controlled by a AAA+ unfoldase partner, and thus uses the same strategy as ClpP to limit non-specific degradation.

ClpX rings can bind to one or both ClpP rings to form singly or doubly capped complexes in which the portals of ClpP are aligned with the axial channels of ClpX, allowing substrates translocated through the ClpX channel to enter ClpP[26]. Assembly of these proteolytic complexes, which have an inherent symmetry mismatch owing to different numbers of subunits in the ClpX and ClpP rings, is stabilised by flexible loops from one face of a hexameric ClpX ring that dock into clefts on a heptameric ClpP ring[20,27,28]. Mismatched docking of a hexameric AAA+ ring with a heptameric proteolytic ring also occurs in ClpAP, ClpCP, Mpa•20S, PAN•20S, Cdc48•20S and the 26S proteasome (Figs. 1a-b). In HslUV, Lon and FtsH, by contrast, the AAA+ ring and proteolytic ring are both hexamers (Figs. 1c-f).

E. coli ClpP consists of 14 identical subunits, as do most ClpP enzymes in other bacteria. However, Actinobacteria, Cyanobacteria and some species from other phyla contain multiple ClpP paralogues, which are often differentially expressed[23,24]. In *Mycobacterium tuberculosis*, discrete ClpP1 and ClpP2 rings form the proteolytic barrel[29,30], whereas in some Cyanobacteria and plants, each heptameric ring contains multiple paralogous subunits[31,32]. Potential biological advantages of using more than one type of ClpP subunit to form active tetradecamers include altering specificity for different AAA+ unfoldase partners, developmental regulation of activity, enhancing the diversity of peptide-bond cleavage specificity and increasing resistance to antibiotics that target ClpP[29,33]. In many species, ClpP assembles into active tetradecamers without assistance, but interactions with a AAA+ unfoldase partner and substrate delivery can also be required to stabilize active double-ring barrels[28,34,35]. Crystal structures of inactive and active ClpP tetradecamers reveal alterations in the geometry

of the active sites and substrate-binding pockets as a consequence of different packing arrangements at the ring–ring interface[23,24]. It has been suggested that peptide products exit the ClpP chamber through ring–ring interface windows that open transiently[36].

Both ClpP and its AAA+ unfoldase partners are targets for antibacterial drugs, including small molecules that prevent partner binding and open the portals into the ClpP chamber, suicide inhibitors of the peptidase active sites and cyclic peptides that act via the AAA+ enzymes[33,35,37–45] (Box 1).

Principles of ATP-fuelled proteolysis. ClpXP and other AAA+ proteases share a common basic mechanism (Fig. 2a)[2]. ClpX or another AAA+ unfoldase ring hexamer engages a recognition tag attached to a target protein that is too large in its native state to pass through the pore. Cycles of ATP binding and hydrolysis power conformational changes in the AAA+ unfoldase ring that pull on the native substrate, typically resulting in failed unfolding and/or substrate release but occasionally causing unfolding. Once unfolding is successful, the polypeptide is translocated through the pore and into the chamber of the associated self-compartmentalized peptidase, where it is cleaved into peptides, typically 5–15 amino acids in length[46,47].

The specificity of degradation by AAA+ proteases is controlled in several ways. In the simplest case, binding sites in the pore of the AAA+ unfoldase ring recognize an amino-acid sequence in the unstructured engagement tag of the substrate (Fig. 2a), although tag access can also be regulated by unfolding, dissociation or post-translational modification reactions[2]. Specificity can also depend on additional recognition sequences

in the protein substrate or by ubiquitination or pupylation[48,49]. For example, pupylation and degradation of pupylated substrates by Mpa•20S allows *Mycobacterium smegmatis* to survive nitrogen starvation[50]. Secondary recognition signals on substrates typically bind to auxiliary domains on the AAA+ unfolding ring either directly or via adaptor proteins (Fig. 2b). Such interactions can mediate recognition by increasing the effective concentration of the engagement tag with respect to the pore, improving the probability of engagement, or by strengthening affinity, which may help keep substrates bound after failed unfolding attempts[2]. These secondary recognition signals can also be used to direct the unfoldase to a specific oligomeric or conformational state of the substrate, and thus serve a regulatory function. Adaptor-mediated degradation can also be influenced by “anti-adaptor” proteins or post-translational modification to enhance or inhibit protease recognition[51–53].

ClpX ring structure and function. Crystal structures of the AAA+ ring of single-chain variants of *E. coli* ClpX have been solved in different nucleotide states[54,55] (Fig. 3a). Differences in the orientations of the large and small AAA+ domains generate subunits with a “loadable” (L) conformation, which have a nucleotide-binding pocket in a cleft between the large and small domains, and subunits with an “unloadable” (U) conformation, which do not have a binding pocket (Fig. 3b). Subunits in most crystal structures are arranged in a pseudo-symmetric L-L-U-L-L-U pattern (Fig. 3a), which is unlikely to represent the major configuration of the working ring, as conformational assays support an asymmetric 5:1 ratio of L:U subunits (Fig. 3c)[55]. A 5:1 arrangement has also been observed by electron microscopy for the Rpt_{1-6} AAA+ ring of the 26S proteasome[56].

Dynamic switching of subunits between the L and U conformations is required for robust ClpX function. For example, locking one subunit in either conformation by crosslinking permits ATP hydrolysis in the ring but largely uncouples ATP hydrolysis from mechanical activity[55,57]. Different L subunits in the ring bind ATP in an asymmetric pattern with a wide range of affinities, presumably owing to structural variations. L⇔U conformational switching may prevent machine stalling when power strokes fail to unfold a stable protein by resetting the functional roles of subunits[55,57]. It is also possible that subunit switching is a way to avoid ring malfunction when ADP fails to dissociate or binds to an empty subunit instead of ATP.

In crystal structures of the ClpX hexamer, each small AAA+ domain packs against the large AAA+ domain of the neighbouring subunit in the same way, forming a ring with six identical rigid-body units connected by short hinges between the large and small AAA+ domains of each subunit (Fig. 3c)[54,55]. Engineering disulfide bonds across each rigid-body interface between subunits in the hexamer results in a topologically closed ring that is highly active in ATP-dependent protein unfolding and degradation[58]. Thus, ClpX function does not require an open ring or lock-washer conformation, and all functional ring conformations can be accessed by changing the hinge conformations.

How does ATP hydrolysis drive changes in the conformation of the ClpX ring? Bound ATP contacts both the AAA+ domains and the hinge, and thus sets the hinge conformation. When ATP is hydrolysed, and ADP and P_i are subsequently released, the

hinge conformation changes again. Thus, changes in the orientations of the large and small AAA+ domains in one subunit – caused by ATP binding, hydrolysis or product release – propagate conformational changes around the ring to drive substrate unfolding and/or translocation. In addition, altering the conformation of any single hinge will change the hinge conformations of flanking subunits to maintain a closed ring. The precise nature of these conformational changes is not yet known, but structures in different nucleotide states show movements of ~1 nm of the axial pore loops that are thought to drive unfolding and translocation[54].

Finally, one ATP hydrolysis event is sufficient to generate a power stroke and mechanical function, as a ClpX ring with a single hydrolytically active subunit supports protein degradation[59]. This result rules out mechanochemical models in which multiple ClpX subunits hydrolyse ATP synchronously or individual subunits hydrolyse ATP in a strictly coordinated sequence[60,61].

Watching unfolding and translocation. Single-molecule force spectroscopy has recently been used to visualize ClpX mechanical activity directly. Attachment of ClpXP to one laser-trapped bead and attachment of a multi-domain protein substrate to another bead enables the substrate to be captured by the protease, creating a tether. The positions and power of the lasers in the optical trap are set so that a small and constant force would pull the beads apart in the absence of this tether. As the degradation reaction progresses, ATP-fuelled unfolding and subsequent translocation of each domain changes the bead-to-bead distance, providing a direct readout of ClpXP

mechanical activity (Fig. 4)[62,63]. For example, unfolding of the structure of a domain causes a sudden increase in distance, which is followed by a slower decrease as the unfolded polypeptide is spooled through ClpX and into ClpP for degradation[62,63]. As substrate domains are translocated against resisting tension in these experiments, ClpXP must itself generate mechanical force. After completing translocation of one unfolded domain, ClpXP attempts to unfold the next domain, and no substantial change in distance is observed until unfolding occurs[62,63].

ClpXP unfolding of individual protein domains typically occurs in a cooperative all-or-none fashion. However, intermediates in unfolding are observed when ClpXP extraction of one structural element of a domain does not cause global denaturation[62–65]. As ClpXP attempts to unfold a domain in the optical trap, the length of the pre-unfolding dwell time provides information important for understanding how unfolding occurs. As expected for a reaction with a single rate-limiting step, these dwell times are exponentially distributed for a specific protein domain with a time constant that reflects its mechanical stability[65]. For rapidly unfolded domains, the unfolding time constant matches the time constant for ATP hydrolysis, indicating that one hydrolysis event and associated power stroke can drive unfolding[62]. For domains that unfold more slowly, many ATPs are hydrolysed during the pre-unfolding dwell time[62–65], a result consistent with biochemical studies showing that ClpXP hydrolyses hundreds of ATPs during degradation of a single protein substrate[66]. In these cases, most power strokes do not force unfolding and only an occasional power stroke succeeds. Nevertheless, the exponential distribution of unfolding dwell times implies that every power stroke has the same probability of causing unfolding, as a lag in the kinetics would be expected if

repeated power strokes were needed to weaken the structure to the point of failure or multiple ATP-hydrolysis events were required to build mechanical tension in the enzyme. Importantly, the average number of power strokes required for unfolding increases as the local stability of structural elements adjacent to the site of enzyme-mediated pulling increases[65–67]. Transient fraying of this local structure, caused by random thermal motions, would result in a subpopulation vulnerable to unfolding by a single power stroke. By taking advantage of the stochastic local dynamics of a substrate domain, enzymatic pulling by ClpXP and other AAA+ machines could enhance an unfolding pathway that relatively few protein molecules would normally follow in solution[67].

Following unfolding, ClpX translocates the denatured polypeptide into ClpP, resulting in decreased bead-to-bead distance in the optical trap. The average length of the smallest translocation steps corresponds to movement of ~5 amino acids of the substrate through the ClpX pore during a power stroke. However, steps ~2, ~3 and ~4 times longer than the smallest step are also observed, which appear to result from kinetic bursts of power strokes[64,65]. Interestingly, steps of different lengths do not occur in any specific pattern, indicating that some aspect of ClpX ring function is inherently probabilistic. One model to explain random stepping behaviour posits that step size depends on the number of ATP molecules bound to the ring and subsequently hydrolysed[64]. Another model proposes that an initial ATP hydrolysis event can occur in any one of the L subunits in an ATP-saturated ring, followed by a burst of ATP hydrolysis and translocation steps in neighbouring subunits until a U subunit is reached[65].

Substrate gripping and release. In ClpX and related AAA+ machines, conformational changes in the ring, which are powered by cycles of ATP hydrolysis, are analogous to the movements within the engine of a motor vehicle. However, additional mechanisms are still needed to grip the protein substrate, apply force and perform mechanical work. Loops with a highly conserved aromatic–hydrophobic–glycine motif in the axial pore of ClpX have crucial roles in substrate gripping during protein unfolding. These loops can be crosslinked to substrates, severe loop mutations eliminate mechanical activity, and reducing the number of wild-type loops in the ClpX hexamer slows unfolding, resulting in an increased ATP cost for degradation (Fig. 5a)[68–71]. Intriguingly, the relationship between unfolding activity and the number of wild-type pore loops suggests that five or six pore loops combine to grip the substrate and apply an unfolding force as a consequence of ATP hydrolysis in a single ClpX subunit[70,71]. Pore loops with very similar aromatic–hydrophobic–glycine motifs are found in all hexameric AAA+ machines that unfold or remodel proteins, implying that the role of these loops in the function of AAA+ unfoldases is highly conserved[2].

Experiments with peptide substrates show that ClpX does not recognize specific side-chain features or the regular spacing of peptide bonds in translocating polypeptides[72]. Translocation can proceed in an N→C or C→N direction, depending on the location of the engagement tag[67,73,74], and multiple polypeptides can be concurrently translocated through the axial pore[75,76]. How might gripping occur if ClpX does not recognize specific chemical features of a translocating polypeptide? One possibility is that the axial pore is elastic and can close tightly around one or multiple polypeptides, with the gripping mechanism dependent on nonspecific van der Waals interactions[54].

During the degradation of multi-domain substrates, ClpXP can release partially degraded protein fragments, which may regulate protein function in some cases[67,73,77–79]. Release of partially degraded protein fragments occurs when one or more domains have been unfolded and degraded, and another stable domain is encountered. Experiments with multi-domain substrates show that ClpXP has some chance of degrading each stable domain and some chance of releasing a partially degraded protein with an unstructured tail of ~40 residues, which would span the distance from the top of the ClpX pore to the active sites of ClpP. These released fragments escape further degradation if their tails do not contain engagement tags for ClpXP. Recent studies show that this mechanism allows ClpXP to partially degrade the *C. crescentus* DnaX clamp loader but then release a truncated fragment with a new activity when it encounters a glycine-rich sequence before a stable domain[79]. Earlier work demonstrated generation of specific truncation products of a few eukaryotic substrates by the 26S proteasome[80–82].

ClpXP degrades some protein substrates at a rate proportional to the ATP-hydrolysis rate but ceases to degrade other substrates when hydrolysis falls below a critical threshold value as a consequence of low ATP concentrations or ClpX mutations[12,77,83]. This ATP dependence provides a mechanism for specific regulation of cellular processes by ClpXP. For example, *E. coli* σ^S, which regulates entry into stationary phase, is constitutively degraded by ClpXP during exponential growth when ATP levels are high but is not degraded when ATP levels fall, signalling a need to change the transcriptional program to deal with a low-energy environment[12]. Why does ClpXP degradation of some but not all substrates stall at low ATP concentrations? A likely

possibility is that grip on the substrate is reduced when the ClpX ring is only partially saturated with ATP, which differentially affects more mechanically stable proteins that require a stronger grip to initiate unfolding[70].

Degradation energetics. Because cleavage of a peptide bond is energetically favourable, the cost of ClpXP degradation depends on how much ATP must be hydrolysed to ensure unfolding and translocation of a given protein substrate. This value can vary substantially for closely related substrates. For example, ClpXP degradation of a titin^{I27} domain, a model substrate, consumes ~600 ATPs, whereas degradation of the less stable V15P and V13P titin^{I27} variants consumes ~230 and ~120 ATPs, respectively (Fig. 5b, 5c)[66]. To place these costs into context, synthesis of a titin domain of ~100 amino acids requires an energetic investment comparable to hydrolysis of ~400 ATPs. As translocation of a titin domain by ClpXP requires ~20 ATPs[65], the vast majority of ATP hydrolysis during ClpXP degradation of titin^{I27} occurs as the ClpXP engages and attempts to unfold the substrate. Comparison of single-molecule and solution-biochemical experiments shows that ~25% of the ATP required for eventual unfolding is hydrolysed while ClpXP remains bound to the substrate[65]. Another ~75% is consumed in reactions in which the substrate is bound but eventually released after unsuccessful unfolding[66,73].

ClpA, a double-ring AAA+ unfoldase. In the ClpAP protease, the ClpP peptidase partners with the AAA+ ClpA unfoldase to degrade protein substrates[2,3]. Because ClpAP and ClpXP use different unfoldases, they have different functional properties, as discussed beow. Each ClpA subunit contains two AAA+ modules, D1 and D2, which

form distinct stacked rings in the hexamer[26]. The D2 ring, which is proximal to ClpP, seems to play the major role in unfolding and translocation, as inactivation of ATP hydrolysis in this ring abrogates robust degradation of stable native substrates[84], and axial pore loops in this ring contact the engagement tags of substrates[85]. By contrast, ClpA retains substantial degradation activity when the D1 ring cannot hydrolyse ATP, suggesting that this ring has a secondary role in force generation[84,86].

In optical-trap assays of mechanochemical activity, ClpAP translocates unfolded polypeptides more slowly than ClpXP, largely because the average steps are smaller, corresponding to movement of ~5 or ~10 amino acids per step[87]. Despite slower translocation than ClpXP, ClpAP unfolds most protein domains substantially faster[78,87,88]. This enhanced unfolding does not occur because ClpAP applies more force than ClpXP[87]. However, because the double-ring architecture of ClpA creates a longer axial pore, loops from both the D1 and D2 rings could cooperate in gripping substrates more tightly, resulting in more efficient transfer of force to the substrate during a power stroke. Alternatively, as ClpAP and ClpXP pull on a native protein, differences in the enzyme surfaces that the substrate contacts may allow better unfolding of certain classes of proteins by one enzyme compared with the other. For example, ClpAP might be more efficient than ClpXP at initial extraction of beta strands from proteins. Whether evolution has matched degradation of a given substrate to a specific proteolytic machine that can unfold the protein more rapidly or at lower energetic costs remains to be determined, as information about natural ClpXP and ClpAP substrates is currently limited. Interestingly, ClpX unfolds proteins with roughly the same efficiency as ClpXP, whereas ClpA alone is a substantially poorer unfoldase

than ClpAP[86]. This difference occurs, in part, because ClpP binding doubles the rate of ATP hydrolysis by ClpA but modestly suppresses the ATPase activity of ClpX[84,86]. It is unclear if these opposing effects of ClpP on ATP hydrolysis by its AAA+ unfoldase partners are biologically significant.

AAA+ protease diversity. Depending on genome size, bacteria typically have two to five AAA+ proteases. Similarly, eukaryotic organelles of bacterial origin often have multiple AAA+ proteases. By contrast, ATP-dependent degradation in the cytosol and nucleus of eukaryotic cells depends exclusively on the 26S proteasome, perhaps because the ubiquitin system with its highly diverse E3 ligases is largely responsible for substrate identification in these compartments[4,56]. The roles of specific AAA+ proteases can change between organisms. In most bacteria, for example, ClpXP degrades ssrA-tagged proteins produced by tmRNA-mediated rescue of stalled ribosomes, whereas Lon serves this function in *Mesoplasma florum*, *Mycoplasma pneumoniae* and other *Mycoplasma* spp.[9,10,17,18].

The use of multiple AAA+ proteases by bacterial cells or eukaryotic organelles may, in part, be related to the diverse subcellular locations of substrates. For example, bacterial FtsH and its mitochondrial homologues are membrane bound, and some of their substrates are peripheral or integral membrane proteins[89,90]. In archaea, LonB-family proteases are also membrane bound, whereas most Lon orthologues in bacteria are cytoplasmic and those in mitochondria function in the matrix[7]. FtsH and Lon are unique among AAA+ proteases because they consist of a single polypeptide in which the AAA+ subunit is fused directly to a peptidase subunit. It is unclear whether this fused

architecture has advantages in comparison with complexes that have distinct ClpP, HslV or 20S peptidases that operate with different AAA+ unfoldase partners. Interestingly, the architecture of Lon hexamers allows them to assemble into a dodecamer in which access to the engagement pores may be restricted by portals at the hexamer–hexamer interface (Fig. 1e)[91]. If dodecamer assembly alters the substrate repertoire, increased cellular Lon concentrations would increase the proportion of dodecamers and thus influence which proteins are degraded. Lon substrates also have a more active role in controlling AAA+ unfoldase ring activity than substrates of other AAA+ proteases[92]. As natural antibiotics can target both the peptidase and unfoldase components of AAA+ proteases[37,45,93], using multiple proteases with different peptidase active site architectures and distinct AAA+ unfoldase enzymes may minimize susceptibility to any single inhibitor. In a given cell, different AAA+ proteases are also likely to function optimally under different conditions. For instance, *E. coli* HslUV is overexpressed under heat-shock conditions and has a temperature optimum for substrate degradation of ~50 °C *in vitro*, whereas ClpXP is not under heat-shock control and is optimally active at ~30 °C[94]. Furthermore, specific protein substrates are often degraded by more than one AAA+ protease in bacteria. For example, the SulA inhibitor of cell division in *E. coli* is a substrate of both Lon and HslUV, which may ensure proper degradation following DNA damage over a range of temperatures[13,95].

As discussed above, certain AAA+ proteases are likely to be better at unfolding and degrading specific cellular substrates. Moreover, almost all AAA+ proteases have family-specific auxiliary domains that function directly in substrate recognition or bind adaptors that alter activity and/or substrate preference, enabling diversity in the

evolution and control of degradation[2,96]. For example, both ClpXP and ClpAP degrade ssrA-tagged proteins efficiently *in vitro*. In *E. coli*, by contrast, the SspB adaptor enhances ClpXP degradation of ssrA-tagged substrates, whereas the ClpS adaptor represses ClpAP degradation of ssrA-tagged substrates but facilitates degradation of N-end-rule substrates, which typically begin with Leu, Phe, Tyr, or Trp[2].

AAA+ remodelling machines. Some AAA+ enzymes that function in proteolysis also remodel protein structures and complexes. For instance, *E. coli* ClpX disassembles a tetramer of the bacteriophage MuA transposase bound to recombined DNA by unfolding at least one protein subunit in the complex[97]. In this case, target recognition involves multiple MuA sequence elements, some of which bind in the ClpX pore and others to the N-terminal domain[98]. Bacteria and fungi also use double-ring AAA+ ClpB, ClpV and Hsp104 hexamers, which do not have peptidase partners but function as dedicated remodelling enzymes, sometimes to resolubilize aggregates or fragment amyloid fibers[99,100]. ClpV, for example, disassembles and recycles components of a contractile injection apparatus important for delivery of effector proteins in the type VI secretion system of *Vibrio cholerae* and other Gram-negative bacteria[99]. In eukaryotes, the single-ring AAA+ katanin and spastin enzymes sever microtubules by extracting tubulin subunits[101]. During cell division in bacteria, ClpX performs a similar function by disassembling the Z-ring, which is composed of tubulin-like subunits[102]. In each of these cases, it is likely that the AAA+ unfoldase engages and unfolds one or more subunits in the ring or polymer (Fig. 6a), leading to disassembly.

Some AAA+ machines appear to remodel substrates by a mechanism that does not

require complete translocation of the target subunit through the axial pore. For example, ClpX orthologs in mitochondria, some of which lack ClpP partners, catalyse incorporation of a cofactor into a haem biosynthesis enzyme[103], possibly by pulling on it to populate a more open insertion-competent structure (Fig. 6b). Similar functions have yet to be documented in bacteria but seem likely to emerge given the evolutionary relationship between mitochondria and α-proteobacteria. In other cases, disassembly of a protein or protein-DNA complex might occur simply by coupling multipoint substrate binding to a large change in conformation in the AAA+ unfoldase ring (Fig. 6c). In eukaryotes, disassembly of soluble NSF attachment protein receptor (SNARE) complexes during membrane fusion by the double-ring N-ethylmaleimide sensitive factor (NSF) enzyme seems to occur by this mechanism. Structural characterization of NSF-SNARE complexes by cryo-electron microscopy (cryo-EM) [104] and single-molecule experiments suggest a spring-loaded mechanism of disassembly[105]. ClpB may remodel some bacterial substrates by a similar mechanism, as a recent study suggests that it dissociates from an unfolded substrate after only one or two translocation steps[106].

Future challenges. A combination of structural, biochemical and single-molecule studies have outlined the basic mechanisms of several bacterial AAA+ enzymes that unfold and/or remodel proteins. In most cases, however, it is not understood at a detailed structural level how target proteins are initially bound or how conformational changes coupled to ATP binding, hydrolysis and product release drive unfolding, translocation or remodelling. High-resolution cryo-EM or crystal structures may answer some of these questions. The development and application of single-molecule assays that simultaneously monitor ATP binding, conformational changes in the AAA+

unfoldase ring and mechanical activity may be needed to address other questions. What dictates whether AAA+ proteases processively degrade or release partially processed substrates is incompletely understood. Moreover, it is not known if tension-relief mechanisms, such as that proposed for NSF, are fundamentally different than power-stroke mechanisms, and whether a single AAA+ unfoldase enzyme is limited to using one mechanism or the other. Finally, our knowledge of the repertoire of natural substrates and degrons recognized by the different bacterial AAA+ proteases and remodelling machines is limited, as is our understanding of machine and target regulation by adaptors or modification reactions. Thus, there is ample room for continued discovery and exciting progress in our understanding of these fascinating molecular machines.

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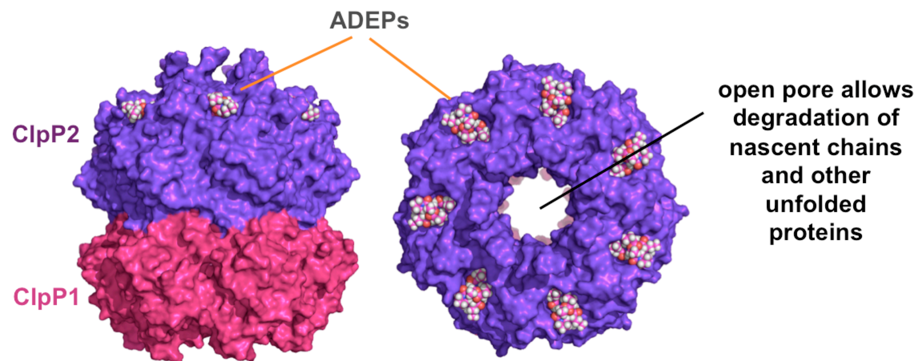
Competing interests statement

The authors declare no competing interests.



Box 1 | Clp proteases as drug targets

Clp-family proteases have emerged as promising antibacterial targets over the past decade. Brötz-Oesterhelt and colleagues first discovered that Gram-positive bacteria could be killed by small-molecule acyldepsipeptides (ADEPs)[37], which activate ClpP degradation of proteins with little stable native structure in the absence of a AAA+ partner. These results suggested that ADEP killing of bacterial cells results from uncontrolled proteolysis of unfolded proteins and newly synthesized nascent chains. As shown in the figure, ADEPs bind in the ClpP clefts that normally serve as partner-docking sites, preventing binding of AAA+ unfoldases, and also widen the axial entry portal into the degradation chamber, allowing unfolded proteins to enter and be degraded[35,38–40]. As expected, *clpP* null mutations confer ADEP resistance in bacteria, like *Escherichia coli*, in which ClpP is not essential[37]. Although ClpP is also not essential in *Staphylococcus aureus*, combining an ADEP variant with rifampicin, an inhibitor of RNA polymerase, eradicated *S. aureus* biofilms in a chronic infection mouse model[42]. ADEP derivatives with enhanced antibacterial potency have been synthesized[44], but their ultimate use as therapies remains to be determined.

In *Mycobacterium tuberculosis* and related actinobacteria, ClpP, ClpX and ClpC (a homologue of ClpA) are all essential enzymes[107,108]. Thus, ADEP-induced lethality might, in principle, result from inhibition of ClpXP or ClpCP proteolysis as well as from uncontrolled ClpP degradation[35]. Suicide inhibitors that react specifically with the active-site serine of ClpP also kill *M. tuberculosis* and other bacteria in which ClpP function is essential[33,41]. Finally, natural cyclic peptides can kill strains of *M. tuberculosis* that are otherwise drug resistant by binding to the N-terminal domain of ClpC, hyper-activating ATP hydrolysis and uncoupling this hydrolysis from ClpCP degradation[43,45,93].

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Figure legends

Figure 1. **Self-compartmentalized peptidases are the degradation components of AAA+ proteases**. In each panel, a single representative structure is shown. (***a***) The ClpP peptidase from *E. coli* (pdb 1TYF) consists of two heptameric rings, uses a Ser–His–Asp catalytic triad for peptide-bond cleavage, and functions with one of three homohexameric AAA+ partners (ClpX, ClpA, or ClpC). In different species, ClpP can consist of 14 identical subunits, distinct homomeric rings or a mixture of subunits in each ring. (***b***) The 20S proteasome from *Thermoplasma acidophilum* (pdb 1PMA) has an $\alpha_7\beta_7\beta_7\alpha_7$ structure, uses a Thr nucleophile for peptide-bond cleavage, and partners with homohexameric Mpa in bacteria, homohexameric PAN or Cdc48/p97 in archaea, or the heterohexameric $Rpt_{1\text{-}6}$ ring in the eukaryotic 26S proteasome. The α and β rings have seven identical subunits in bacteria and archaea and seven distinct α or β subunits in eukaryotes. (***c***) The HslV peptidase from *Haemophilus influenzae* (pdb 1G3I) consists of two homohexameric rings (each subunit is homologous to a β subunit of the 20S proteasome), uses a Thr nucleophile for peptide-bond cleavage, and partners with an HslU homohexamer. (***d***) The homohexameric Lon protease from *Thermococcus onnurineus* (pdb 3K1J) is assembled from subunits in which the AAA+ module is fused to the peptidase domain and uses a Ser–Lys dyad for peptide-bond cleavage. (***e***) Two *E. coli* Lon hexamers can combine to form a dodecamer, which is stabilized by N-domain interactions that form portals of ~45 Å into the enzyme lumen. The panel shows the *E. coli* 3LJC and *B. subtilis* 3M6A structures modeled into a low-resolution electron-density map[91]. (***f***) The homohexameric *Thermotoga maritima* FtsH protease (pdb 3KDS)

also assembles from subunits in which the AAA+ module is fused to the peptidase domain. FtsH uses an Asp–Zn^{++} active site for peptide-bond hydrolysis.

Figure 2. **Substrate recognition and degradation**. (***a***) Minimal model for recognition, unfolding, translocation and degradation of a single-domain protein by a AAA+ protease. Reaction steps in the forward direction are ATP dependent. In the initial recognition step, a disordered engagement tag in the native protein substrate is bound in the axial pore of the AAA+ ring hexamer. ATP-fuelled conformational changes in the ring then pull on the substrate, which can result either in failed unfolding, substrate release or substrate denaturation. The probability of each of these outcomes depends on substrate stability, as a very stable protein might be bound and released many times resulting in unproductive hydrolysis of a substantial amount of ATP. Following forced unfolding, the denatured polypeptide is processively translocated through the pore and into the peptidase chamber for degradation. (***b***) Efficient recognition of some protein substrates requires secondary recognition signals, which are substrate sequences that bind to the AAA+ enzyme either directly or via adaptor proteins. In principle, these secondary signals might affect any of the pre-unfolding steps shown in panel ***a***.

Figure 3. **ClpX ring structure**. (***a***) Views of the AAA+ ring of *E. coli* ClpX (pdb 3HWS). In each subunit, the large AAA+ domain is coloured dark or light grey and the small AAA+ domain is coloured purple. Hinges between the large and small domains of each subunit are coloured red. (***b***) Subunits can adopt either a loadable (L) or an unloadable (U) conformation. In L subunits, ATP binds in a cleft between the large and small AAA+ domains. In U subunits, rotation of the small domain destroys the binding pocket. (***c***)

Cartoon of a 5L:1U ClpX ring showing how six rigid-body units connected by six hinges are created by packing between the small AAA+ domain of a subunit (coloured blue) and the large AAA+ domain of a neighbouring subunit (dark grey for L subunits; light grey for U subunits). Because the ring is topologically closed, changes in the conformation of any single hinge — caused by ATP binding, ATP hydrolysis or product release — propagates around the ring.

Figure 4. **Single-molecule force spectroscopy of ClpXP**. (***a***) Cartoon of an optical-trapping experiment. Micron-sized beads, trapped by infrared lasers, are tethered to either ClpXP or a multi-domain substrate via a DNA linker. When ClpXP engages the substrate, ATP-fuelled mechanical activity can be monitored by measuring bead movements relative to the centre of laser focus (dotted lines). (***b***) ClpXP unfolding of an individual substrate domain (panels ***a***2 and ***a***5) results in an increase in bead-to-bead distance. Translocation of the substrate (panels ***a***3 and ***a***6) results in a decrease in bead-to-bead distance that corresponds to the length of the translocated domain. Periods of no movement are dwells (panels ***a***1 and ***a***4) in which ClpXP tries to unfold the next native domain in the substrate.

Figure 5. **Factors influencing the energetic cost of degradation**. The average number of ATPs hydrolysed by the ClpXP protease during degradation of a single protein substrate depends upon the protein's stability and how well it is gripped by ClpX. (***a***) The axial pore of ClpX contains a loop from each subunit that grips the substrate during protein unfolding. Mutating one or two loops decreases the maximal rate of degradation of a GFP substrate and increases the ATP cost[70]. Thus, maximal unfolding

and degradation efficiency requires the combined gripping action of five or six pore loops. (***b***) Structure of the I27 domain of the human muscle protein titin (pdb 1TIT) and the locations of two mutations (V13P and V15P) that destabilize this domain by removing or disrupting hydrogen bonds. Also shown is an engagement tag at the C-terminus that allows ClpX to recognize and pull on $titin^{I27}$ variants. The bar graph shows the average ATP cost of ClpXP degradation for the wild-type (WT), V15P and V13P $titin^{I27}$ domains[65,66]. ATP hydrolysed during translocation, terminal unfolding attempts and unfolding attempts that result in substrate release are indicated.

Figure 6. **AAA+ remodelling of proteins and protein complexes**. (***a***) A AAA+ remodeling machine breaks a polymer into two pieces by unfolding an interior subunit. Remodelling of the polymer but not the monomer is possible if signals for AAA+ recognition are only properly arranged in the polymer. Related mechanisms may explain severing of microtubules, cell-division rings and amyloid fibers by AAA+ enzymes. (***b***) Model in which a AAA+ machine enhances the rate of cofactor insertion into a metabolic enzyme by inducing a conformational change in the cofactor binding site. This mechanism appears to be used by mitochondrial ClpX to catalyse incorporation of a cofactor into a haem biosynthesis enzyme[103]. (***c***) Multi-point binding of a complex to a AAA+ ring, followed by a major conformational change in the ring, might pull the complex apart. This mechanism may account for ClpB remodeling of some bacterial substrates and for disassembly of complexes required for membrane fusion in eukaryotic cells.

Glossary

AAA+ enzyme

AAA+ (pronounced “triple A plus”) enzymes use the chemical energy of ATP binding, hydrolysis and product release to perform mechanical work in cells.

Adaptor

A protein that binds a substrate and delivers it to a AAA+ protease for degradation.

Acyldepsipeptides (ADEPs)

Antibacterial compounds that kill bacteria by activating non-specific ClpP degradation of unfolded proteins and nascent polypeptide chains.

Cryo-electron microscopy (cryo-EM)

A technique to visualize single macromolecules in a thin layer of vitreous ice. Recent advances in direct electron detectors and data-processing algorithms now allow some structures to be determined at atomic resolution.

Degradation chamber

The interior of a barrel-like compartment that contains the active sites for peptide-bond cleavage in a AAA+ protease.

Degron

Any sequence or structural element, including the engagement tag, that is required for recognition and degradation of a substrate by a AAA+ protease.

E3 ligases

Eukaryotic enzymes responsible for the addition of ubiquitin chains, which in some cases target proteins for degradation by the proteasome.

Engagement tag

A specific but intrinsically disordered sequence that binds in the pore of the AAA+ unfoldase ring and allows the enzyme to pull on an attached native protein to unfold it.

Loadable subunit

A subunit of ClpX or any other AAA+ unfoldase ring that can bind ATP and other nucleotides.

Lock-washer conformation

AAA+ unfoldase ring with the subunits arranged in a helical conformation, creating an open interface between the first and last subunits.

N-ethylmaleimide sensitive factor (NSF)

AAA+ remodelling machine that disassembles soluble NSF attachment protein receptor (SNARE) complexes, which are required for vesicle fusion.

Persisters

Microbial cells in a non-dividing dormant state that escape killing by conventional antibiotics but are not inherently antibiotic resistant.

Power stroke

A conformational movement of the AAA+ ring – generated by ATP binding, hydrolysis or product release – that pulls on or propels a peptide segment of a substrate through the axial channel.

Proteasome

AAA+ protease that uses the 20S peptidase for degradation.

Pupylation

A post-translational modification that attaches a Pup (prokaryotic ubiquitin-like protein) to a lysine in a protein to direct its degradation by the Mpa•20S proteasome.

Rigid-body unit

A structure that shows little structural variation formed by packing of the small AAA+ domain of one ClpX subunit against the large AAA+ domain of a neighbouring subunit.

Self-compartmentalized peptidase

An enzyme in which multiple subunits assemble to form a barrel-shaped structure in which the active sites for peptide-bind cleavage are located in an internal chamber.

Single-molecule force spectroscopy

A biophysical method that uses optical tweezers, magnetic tweezers or atomic-force microscopy to study the behavior of a single macromolecule under force.

Suicide inhibitors

Substrate analogues that cause irreversible inhibition of an enzyme by forming an irreversible covalent bond with the active site of an enzyme.

Time constant

The reciprocal of the kinetic rate constant for any reaction. For example, if the rate constant for ATP hydrolysis by a single ClpX enzyme is 4 s^{-1}, then the time constant is 0.25 s.

Titin^{I27} domain

One of the many related β-sheet immunoglobulin-like domains, each comprising ~100 amino acids, in the titin protein, which is responsible for muscle elasticity. The titin^{I27} domain has been used for many biochemical and biophysical studies of protein folding.

tmRNA

A bacterial RNA molecule that acts both like a tRNA and an mRNA molecule to rescue ribosomes stalled during translation and to add the ssrA degradation tag to the partially synthesized nascent polypeptide.

van der Waals interactions

Electrostatic interactions that occur between atoms of all types and have an attractive component, resulting from transient induced dipoles (typically maximal at inter-atomic distances of 2-3.5 Å), and a repulsive component at closer distances where the electron shells of the two atoms overlap.

Online only

Author biographies

Adrian O. Olivares is a research scientist at MIT, Cambridge, USA. His graduate work at Yale, New Haven, USA, focused on the molecular mechanism of myosin motors. At MIT, he has spearheaded a multi-laboratory effort to apply single-molecule optical trapping to understanding how the ClpXP and ClpAP proteases unfold and translocate specific protein substrates.

Tania A. Baker is Professor of Biology and an investigator of the Howard Hughes Medical Institute, at MIT, Cambridge, USA. Her laboratory studies mechanisms of direct and adaptor-mediated substrate recognition of protein substrates by AAA+ proteases and protein-remodelling chaperones.

Robert T. Sauer is Professor of Biology at MIT, Cambridge, USA. His lab studies the structures and molecular mechanisms of protein unfolding, translocation and degradation by AAA+ proteases and also studies PDZ proteases involved in signalling and protein quality control.

Key points

• In ATP-dependent proteases, a ring-shaped AAA+ machine harnesses the chemical energy of ATP binding and hydrolysis to mechanically unfold target proteins by translocating them through an axial pore and into the degradation chamber of a compartmental peptidase.

• Recognition of specific target proteins involves direct binding of amino-acid sequences to the axial pore of the AAA+ ring, binding of sequences to auxiliary domains, and/or binding mediated by adaptor proteins. Degron sequences can be revealed or added to substrates by protein-modification reactions.

• Novel antibiotics kill some bacteria by binding to the ClpP compartmental peptidase and transforming it into a rogue enzyme that indiscriminately degrades nascent polypeptides and unstructured cellular proteins.

• Single-molecule optical trapping has directly visualized the unfolding and translocation activities of the ClpXP and ClpAP AAA+ proteases. These experiments and solution studies support a probabilistic model of AAA+ ring function and show that each power stroke has a constant and typically low probability of unfolding a stable protein domain.

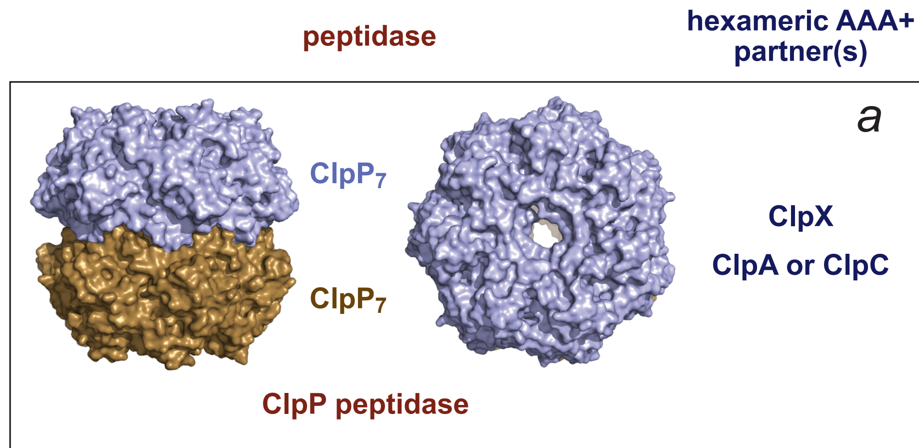
• Although protein degradation by AAA+ proteases is typically highly processive, multi-domain substrates are sometimes partially proteolyzed, with the released products having new biological functions.

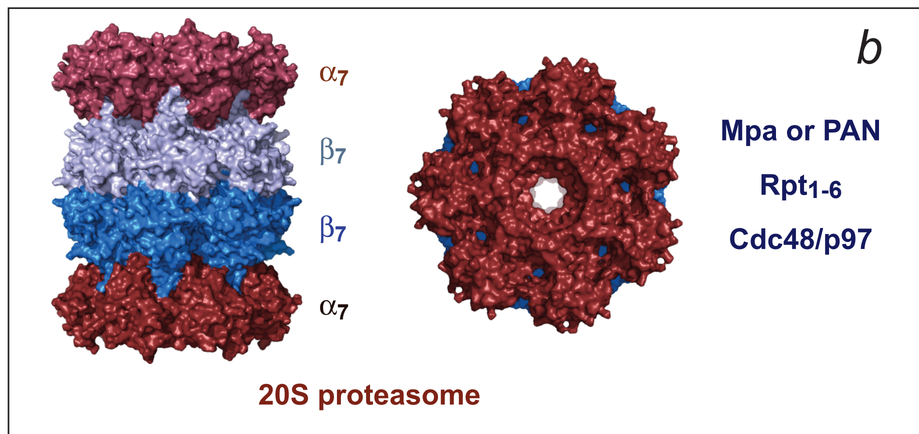
• AAA+ enzymes can function independently to solubilize aggregated proteins, disassemble macromolecular complexes, and catalyze incorporation of cofactors into enzymes.

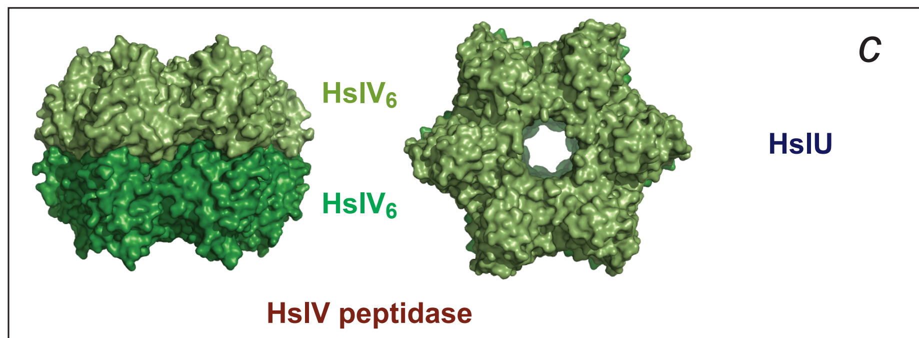
TOC blurb

AAA+ proteolytic machines unfold and degrade damaged and unneeded proteins in all domains of life. In this Review, Olivares and colleagues discuss the molecular mechanisms and structures of bacterial AAA+ machines, focusing on recent studies of ClpXP as a paradigm.

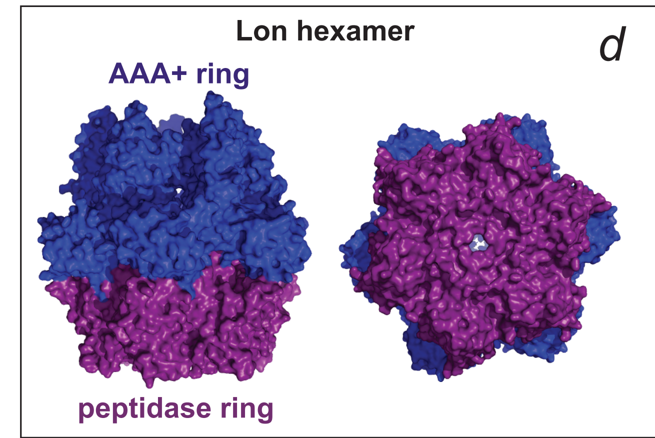
Distinct peptidase and AAA+ partners

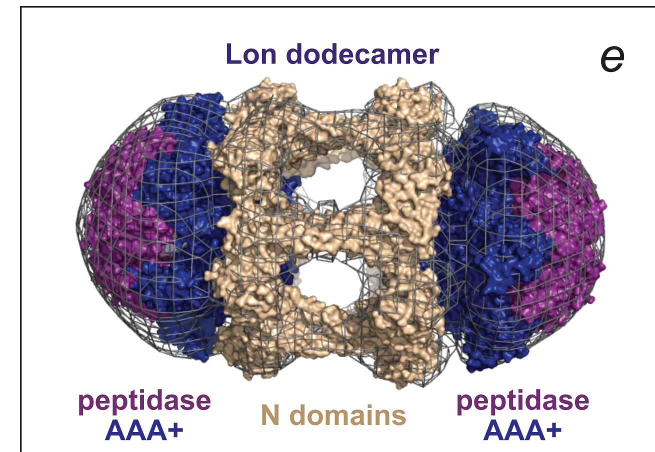






Fused peptidase and AAA+ subunits





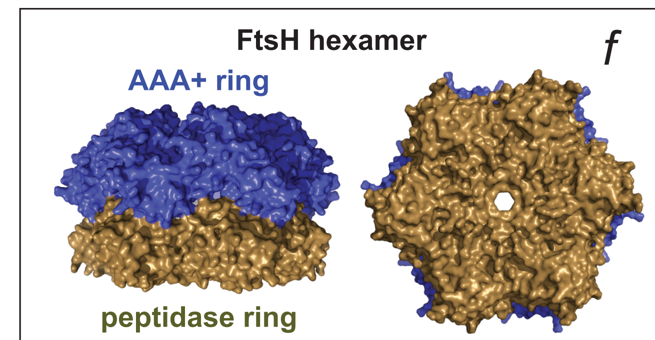
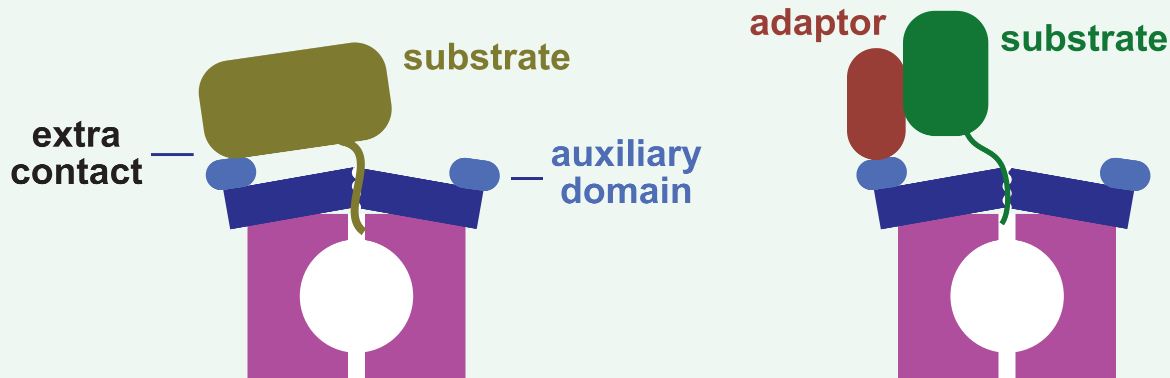


Figure 1



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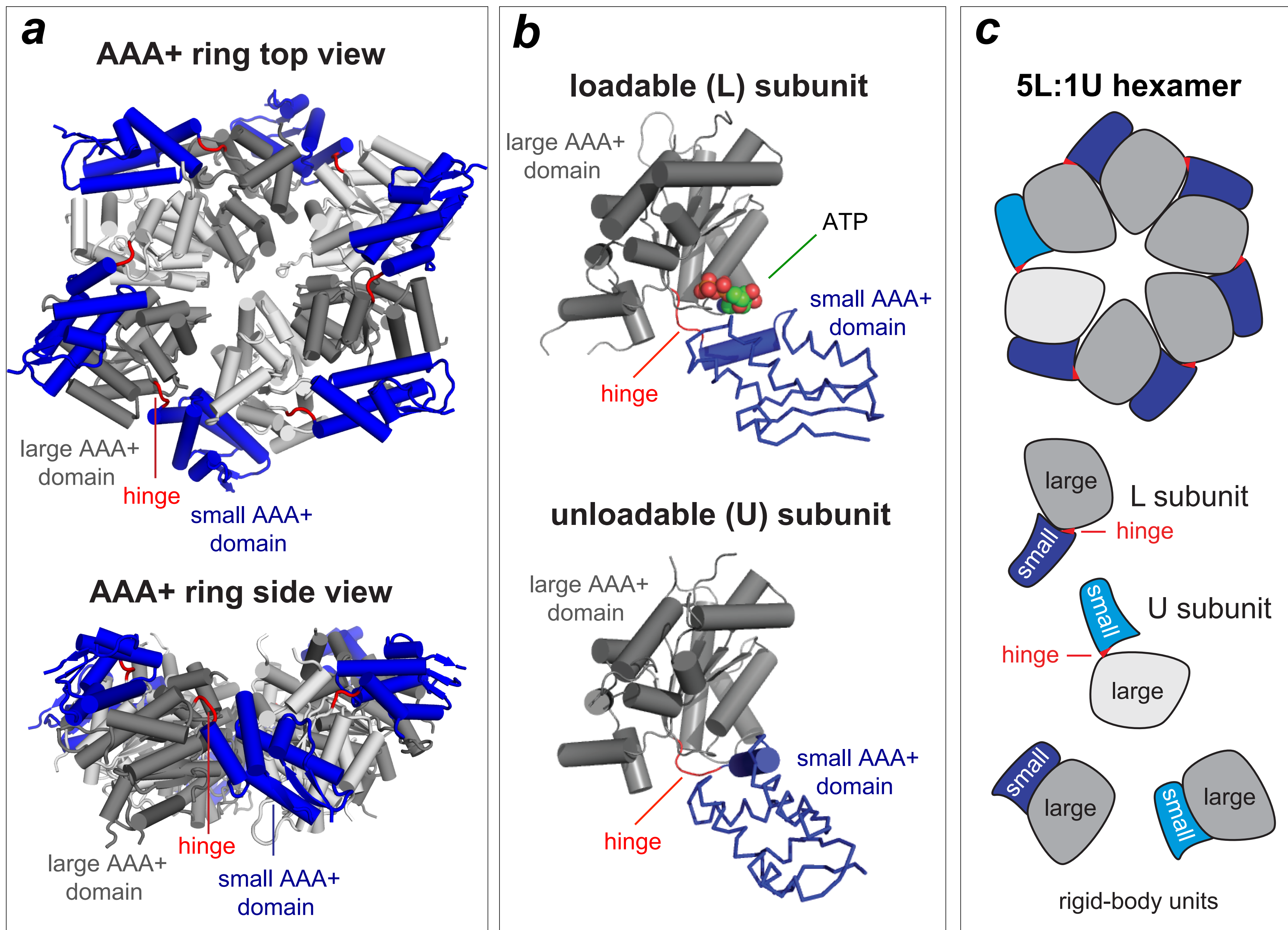

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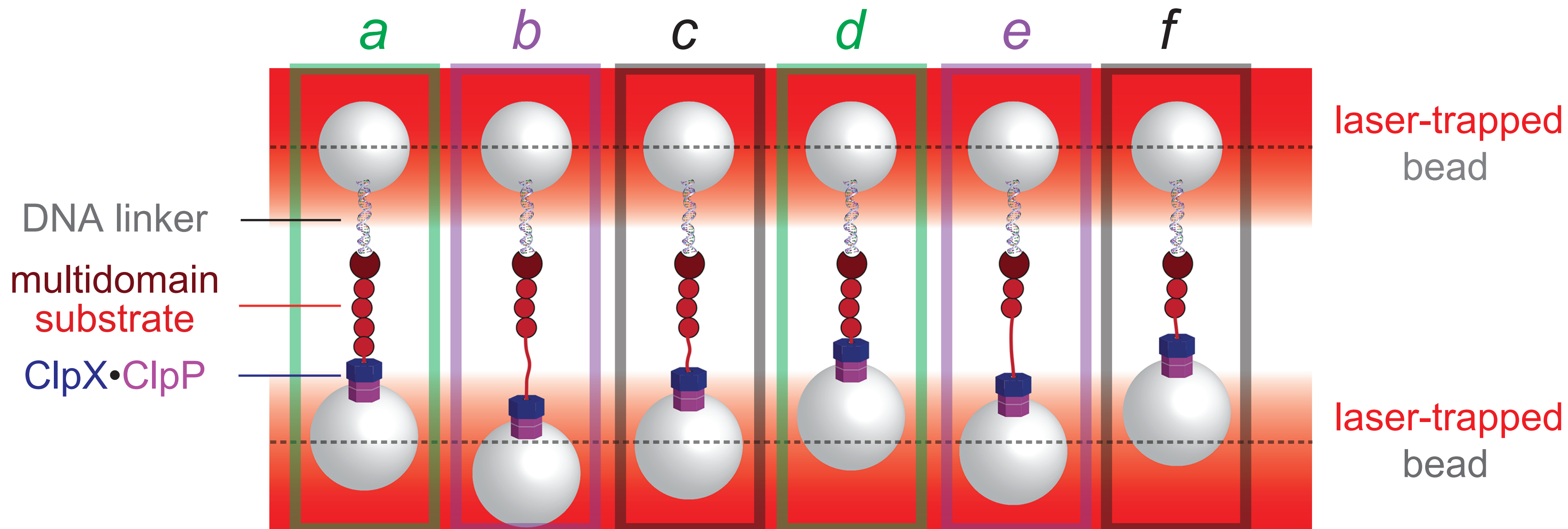


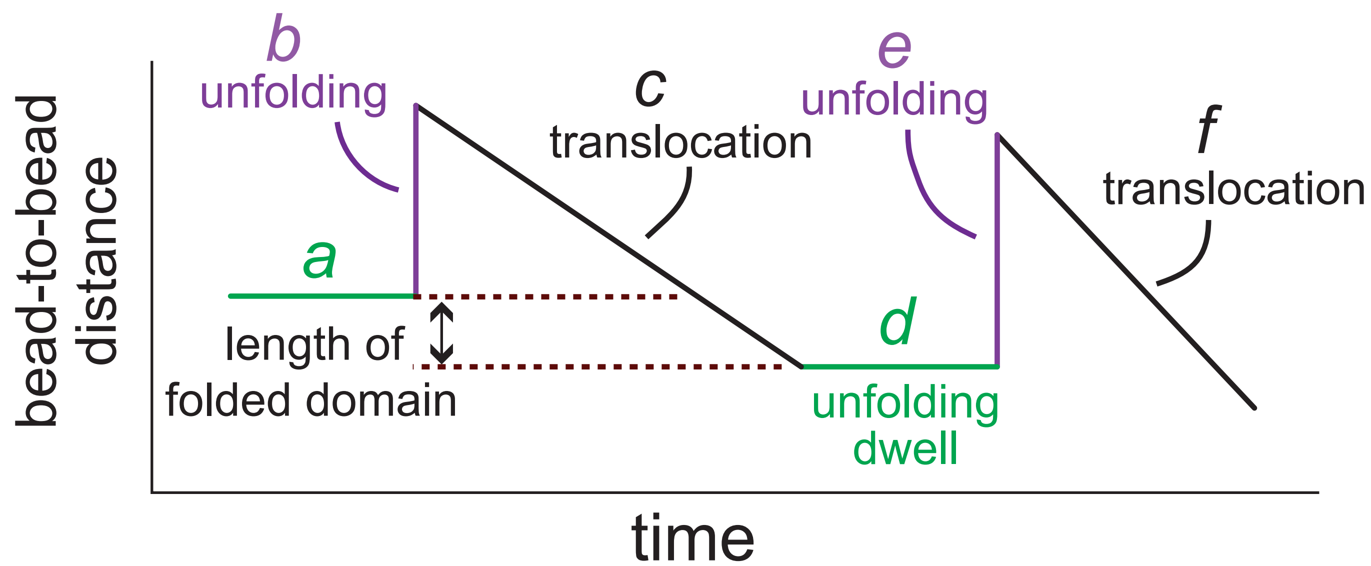
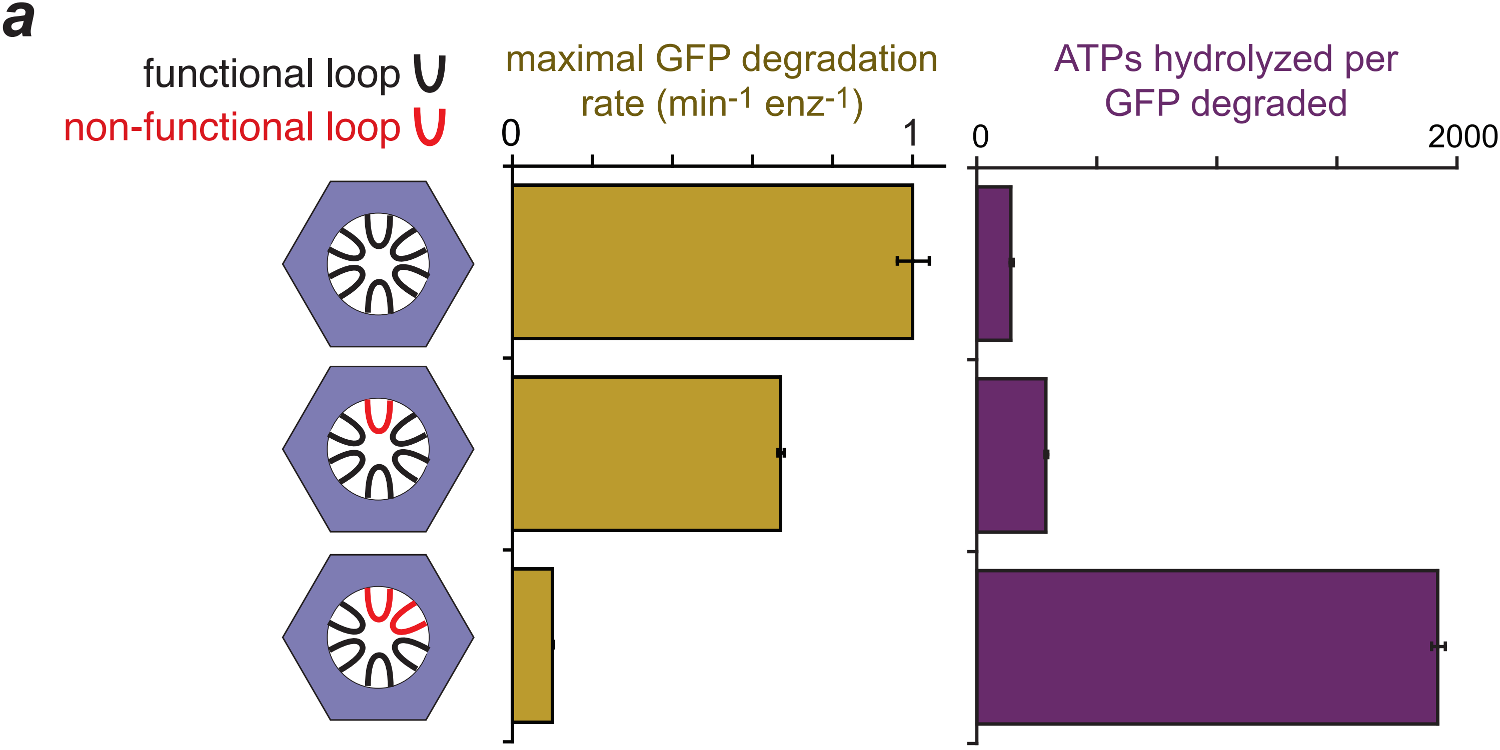
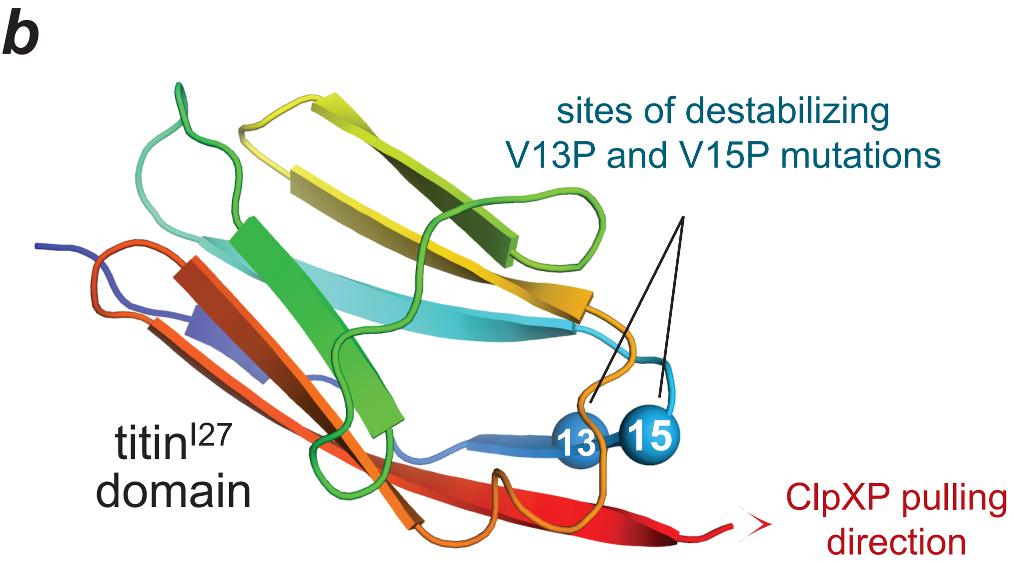
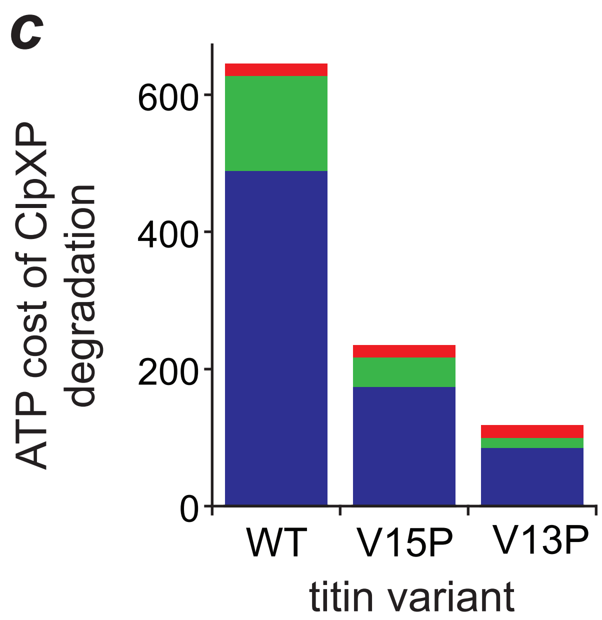


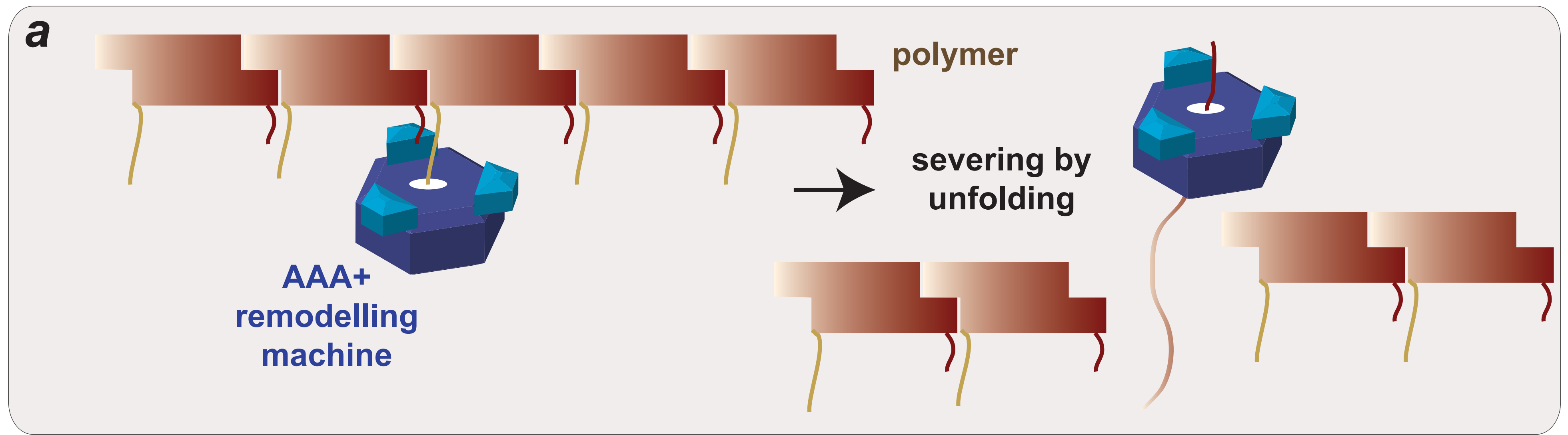
Figure 4

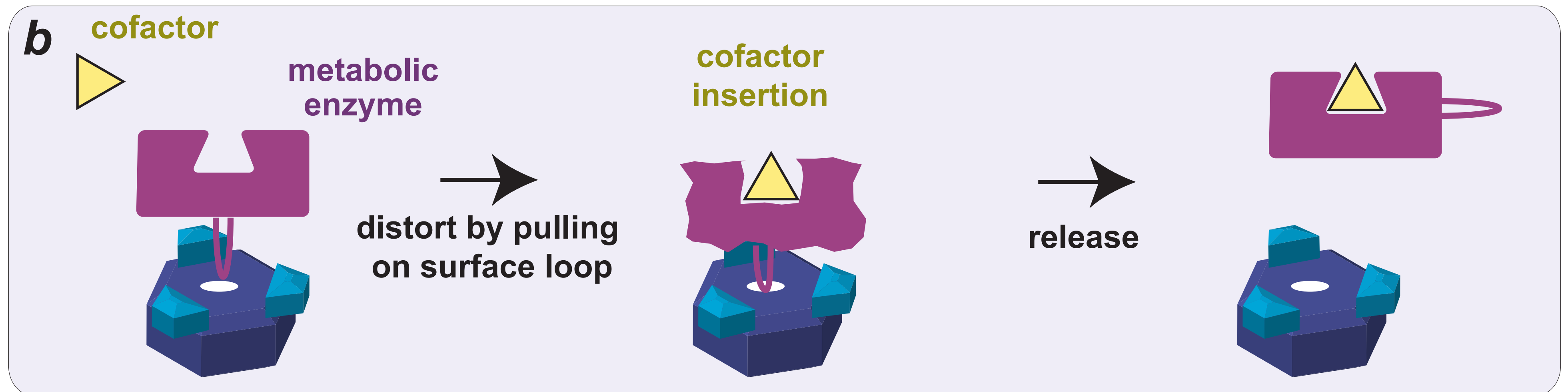
Figure 5











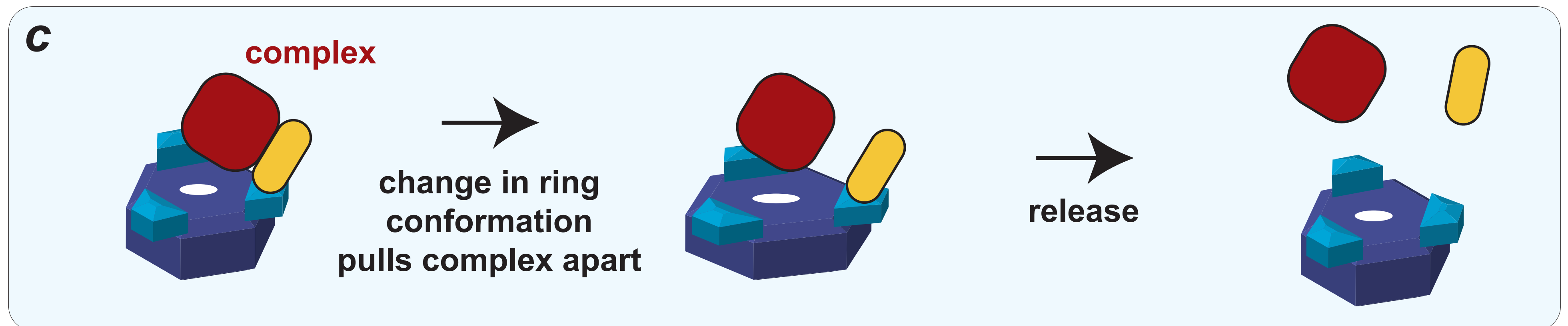


Figure 6