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Clp chaperone–proteases: structure and function

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Abstract

Clp proteases are the most widespread energy-dependent proteases in bacteria. Their two-component architecture of protease core and ATPase rings results in an inventory of several Clp protease complexes that often coexist. Here, we present insights into Clp protease function, from their assembly to substrate recruitment and processing, and how this is coupled to the expense of energy.

Keywords: ATP-dependent protease; Endopeptidase ClpP; ClpAP; ClpXP; ClpCP; AAA ATPase; Degradation

1. Introduction

ATP-dependent proteases are responsible for selective degradation of various cellular proteins, influencing protein quality control and regulation of many cellular processes. In eukaryotes, the main energy-dependent processive protease is the 26S proteasome, whereas in bacteria this task is performed by a repertoire of proteases: the members of the Clp protease family (ClpAP, ClpCP, ClpEP, ClpXP, HslUV) as well as Lon protease and the membrane-bound FtsH protease [57]. All Clp degradation complexes have a ''stack-of-rings'' architecture and consist of two functional elements: a cylinder-like proteolytic core and ATPase-active chaperone rings (Fig. 1). The chaperone is responsible for substrate recognition, unfolding and threading of the extended polypeptide chain through a narrow pore into the protease compartment, where the proteolytic active sites are sequestered from the outside solution. All chaperones oligomerize into toroidal hexamers with a central pore and are related members of the large AAA (ATPases associated with various cellular activities) ATPase family. This family is defined by a P-loop domain with Walker A and B motifs preceding a smaller helical domain that communicates the different nucleotide states of the P-loop domain to other parts of the complex [52]. The proteolytic cylinder possesses multiple active sites of serine or threonine-type, which enable hydrolysis of proteins into 5–10 amino acid long peptide products [65].

The two-component architecture of Clp proteases and the existence of two different proteolytic cores and multiple different chaperone rings, results in an inventory of several possible Clp protease complexes. The ClpP protease core can interact with different chaperones, namely ClpA, ClpC, ClpE, and ClpX, forming the respective active chaperone–protease complexes. In contrast, ClpQ exclusively interacts with the ClpY chaperone forming the ClpYQ (also known as HslUV) protease (Fig. 1). Amongst the Clp-type ATPases, two distinct classes exist: class I contains two consecutive AAA modules per protomer (e.g. ClpA, ClpC and ClpE) and class II contains only one such AAA module (e.g. ClpX and ClpY).

Almost all bacteria contain the ClpXP chaperone-protease often alongside the also very widespread Lon and FtsH proteases. This makes ClpXP the most ubiquitous of the Clp proteases (Table 1). ClpA and ClpC are orthologs and usually bacteria harbor either one or the other. ClpA is found in the Gram-negative proteobacteria, while ClpC is found in Gram-positive bacteria and cyanobacteria. ClpYQ exists alongside ClpAP in most proteobacteria and is also found in certain

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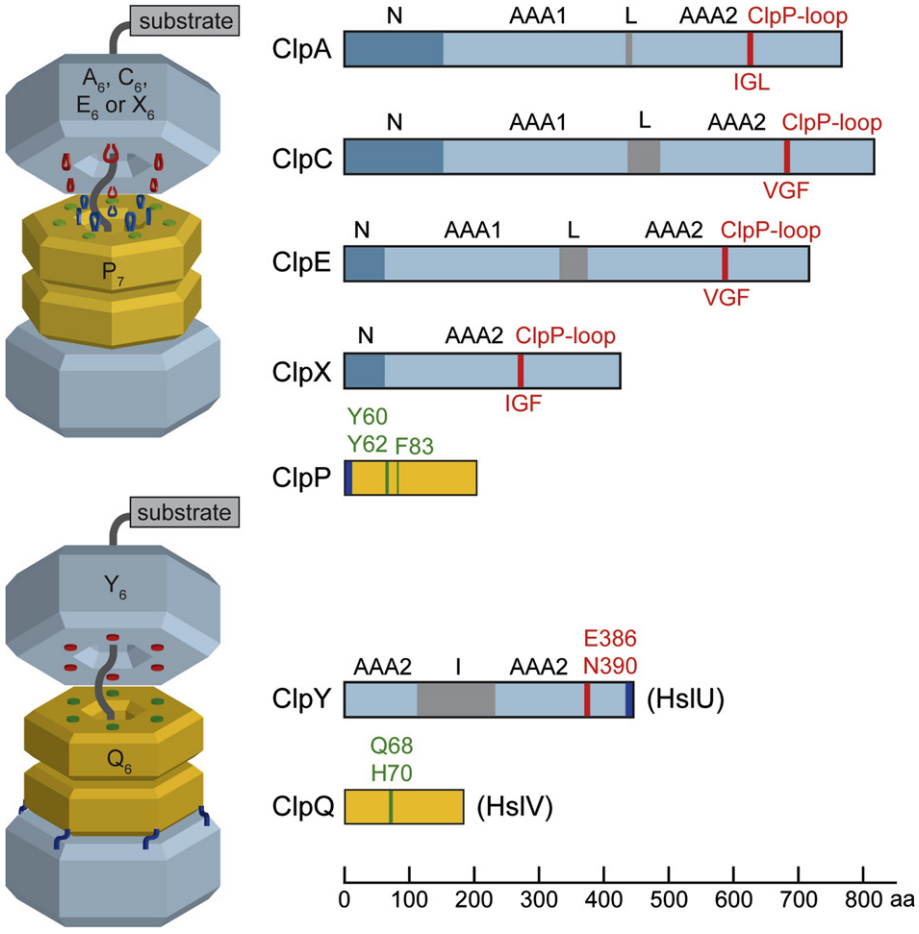

Fig. 1. Overall architecture of Clp chaperone–proteases. All Clp chaperones (blue) form homohexameric ring structures shaping a central pore through which substrate molecules (gray) are spooled into the associated Clp protease (orange). The protease ClpP is a barrel-shaped tetradecamer bearing the proteolytic sites inside its cavity, and interacts with several class I (ClpA, ClpC, ClpE) and class II (ClpX) AAA proteins. ClpY (class II) binds to both sides of the dodecameric protease ClpQ. Structural features important for chaperone–protease interaction are shown in red, green and dark blue.

Gram-positive bacteria. Energy-dependent protein degradation has been studied in great detail in the Gram-negative bacterium *Escherichia coli*, which contains four cytosolic protease complexes, the three Clp proteases ClpAP, ClpXP and ClpYQ and the Lon protease, as well as the membrane-associated FtsH protease. The ClpCP degradation system has been characterized particularly in the Gram-positive, spore-forming, bacterium *Bacillus subtilis*.

In this review, we present structural and functional insights into Clp proteases, from the assembly of these large, cylindrical complexes to the current mechanistic view on substrate processing.

Table 1
Clp chaperone–proteases in prokaryotes

Chaperone–protease	Occurrence	Cellular function (substrate example)
Class I		
ClpAP	*Proteobacteria*	Protein quality control (N-end rule, SsrA-tagged)
ClpCP	*Firmicutes*, *Actinobacteria*, *Cyanobacteria*	Competence development and sporulation (ComK), transcription (SpoIIAB) and regulation of stress response (CtsR)
ClpEP	*Firmicutes*	Thermotolerance (CtsR), cell division and virulence
Class II		
ClpXP	*Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Cyanobacteria*, *Deinococcus-Thermus*, *Fusobacteria*, *Spirochaetes*, *Aquificae*, *Thermatogae*	Protein quality control (SsrA-tagged), cell division (FtsZ, CtrA), transposition (MuA), virulence (Spx)
ClpYQ	*Proteobacteria*, *Firmicutes*, *Spirochaetes*, *Aquificae*, *Thermatogae*	Cell division (SulA), heat shock response (RpoH) and capsule transcription (RcsA)

2. Assembly and composition of Clp chaperone–proteases

Chaperone–proteases are composed of multiple subunits to form assemblies that are large enough to process their protein substrates. All Clp chaperones form homohexameric ring structures, shaping a central pore through which substrate molecules are translocated into the associated protease (Fig. 1). To date, only one crystal structure of a hexameric Clp-chaperone, ClpY (HslU), has been solved (Fig. 2e) [3,55,62]. Interestingly, in some HslU-structures, the subunits adopt different conformations and only some ATP binding sites are occupied by nucleotide [3,55,62]. Furthermore, titration studies on ClpY and ClpX (highly homologous to ClpY) show that only 3–4 ATP molecules bind per hexamer [19,64]. Hence, both, structural and biochemical data are indicative of a hexameric ring structure, in which the conformational status of individual subunits in the functionally active ring breaks down the sixfold symmetry.

Most class I Clp chaperones assemble into the oligomeric state only in the presence of nucleotide. One of the best studied members of this class, the *E. coli* ClpA chaperone, can dimerize in the absence of nucleotides, but does not form the biologically relevant hexameric state [44]. The first ATPase domain (D1) of ClpA promotes ATP-induced hexamerization, whereas the second ATPase domain (D2) contributes the main portion of ATP hydrolysis exhibited by the assembled ClpAP protease. A structural comparison of D1 and D2 explains why D1 mediates nucleotide-dependent assembly while D2 does not. In both D1 and D2, the large P-loop-domain and the small α-helical domain form a hinged structure with a concave ATP binding pocket on one side and a convex surface on the other [18]. In the oligomeric state, the convex surface of one monomer fits into the concave ATP binding site of the neighboring subunit (Fig. 2a). In D1, the ATP binding pocket is surrounded by negatively charged residues complementary to the positively charged convex surface of the adjacent subunit and several of the conserved positively charged side chains interact directly with the bound nucleotide (Fig. 2a, magnified view). In contrast, the proximate D2 subunits are neither complementary in shape nor in charge. Rapid kinetic analysis of the oligomerization reaction revealed that, upon binding of ATP, ClpA assembles from dimers via a tetrameric, transiently accumulating intermediate to the hexamer (Fig. 2a). The fully assembled hexamer then undergoes a conformational change to the ATPase-active state [33]. It is only in this conformational state that the ATPase can associate with ClpP to form the fully active complex.

Unlike ClpA, ClpX has only one ATPase domain, which is structurally similar to ClpY (HslU) and D2 of ClpA [28]. In the modeled ClpX hexamer the C-terminal subdomain of the ClpX ATPase domain interacts directly with the ATPase domain of the adjacent subunit (Fig. 2b). Therefore, ClpX exists partially as a hexamer in absence of nucleotide [51]. However, ATP binding to ClpX enhances oligomerization, because the nucleotide mediates an additional interaction between the subunits [28]. In the ClpY (HslU) hexamer, another class II member, the nucleotide is also bound at the interface between two subunits, thereby promoting hexamerization, which is a prerequisite for the interaction with the protease ClpQ (Fig. 2e) [3,55].

As shown by the examples above, all Clp ATPases have to be in their nucleotide-bound, hexameric forms to associate with the proteolytic core cylinders. This association likely occurs on a much faster time scale than the chaperone assembly itself, as has been shown for the association of ClpA with ClpP [33]. The only X-ray structural information of a fully assembled Clp chaperone–protease available to date is of the ClpYQ complex (Fig. 2e) [55]. One reason for this might be that ClpQ shares the sixfold symmetry with its chaperone partner, as it is assembled from hexameric rings. ClpP, the proteolytic partner of all other Clp chaperones (ClpA, ClpC, ClpE and ClpX), is composed of heptameric rings, resulting in a symmetry mismatch between chaperone and protease. Consequently, the mode of interaction in ClpYQ versus the ClpP-type complexes is different. The crystal structure of ClpY in complex with ClpQ revealed that the interaction here is mediated by a hydrogen-bond pattern between two helix-loop-helix motifs, one on ClpY, the other on the apical surface of ClpQ (Fig. 2e) [55]. Furthermore, the C-terminal 14 amino acids of ClpY form an ordered loop which binds between two ClpQ protomers (Fig. 2e, magnified view).

Association with ClpP is more complex due to the symmetry mismatch between chaperone and protease. ClpP exhibits two distinct structural features to recruit the chaperone rings. One is a hydrophobic patch on the apical surface comprising Tyr60 and Tyr62 from one subunit and Phe82 from the neighboring subunit, yielding a total number of seven binding sites on each cylinder face (Fig. 2d, green) [61]. The chaperone interacts with the ClpP-ring surface, presumably via the aforementioned hydrophobic patch, by means of the so-called ClpP binding loops protruding from the proximal chaperone ring face. The interaction is mediated through a conserved binding motif at the tip of this loop consisting of a glycine flanked by two hydrophobic residues (ΦGΦ) (Fig. 2d, red) [30]. The ClpP binding loop in ClpX was visualized in the crystal structure, revealing its flexible and rather extended nature that facilitates the asymmetric association with ClpP [28]. The other structural feature of ClpP contributing to interaction with the ATPase is a β-hairpin consisting of the N-terminal 20 residues (not counting the propeptide) [16,26]. This β-turn-β motif can adopt two opposite conformations: a ‘down’ conformation, where the N-terminal residues are entirely concealed within the substrate access pore, or an ‘up’ conformation, where the hairpin extends out of the pore (Fig. 2d). A recently published crystal structure revealed that only one N-terminus adopts the ‘down’ conformation and the remaining six N-termini adopt the ‘up’ conformation, shaping a dome-like structure (Fig. 2d) [1]. Interestingly, this architecture is roughly complementary to the concave faces of the modeled ClpA and ClpX rings (Fig. 2d). Several single amino acid substitutions in this region were reported to render ClpP incapable of forming a stable complex with ClpA or ClpX, underscoring the importance of the ClpP N-terminus for chaperone binding [1,16,26]. It was suggested

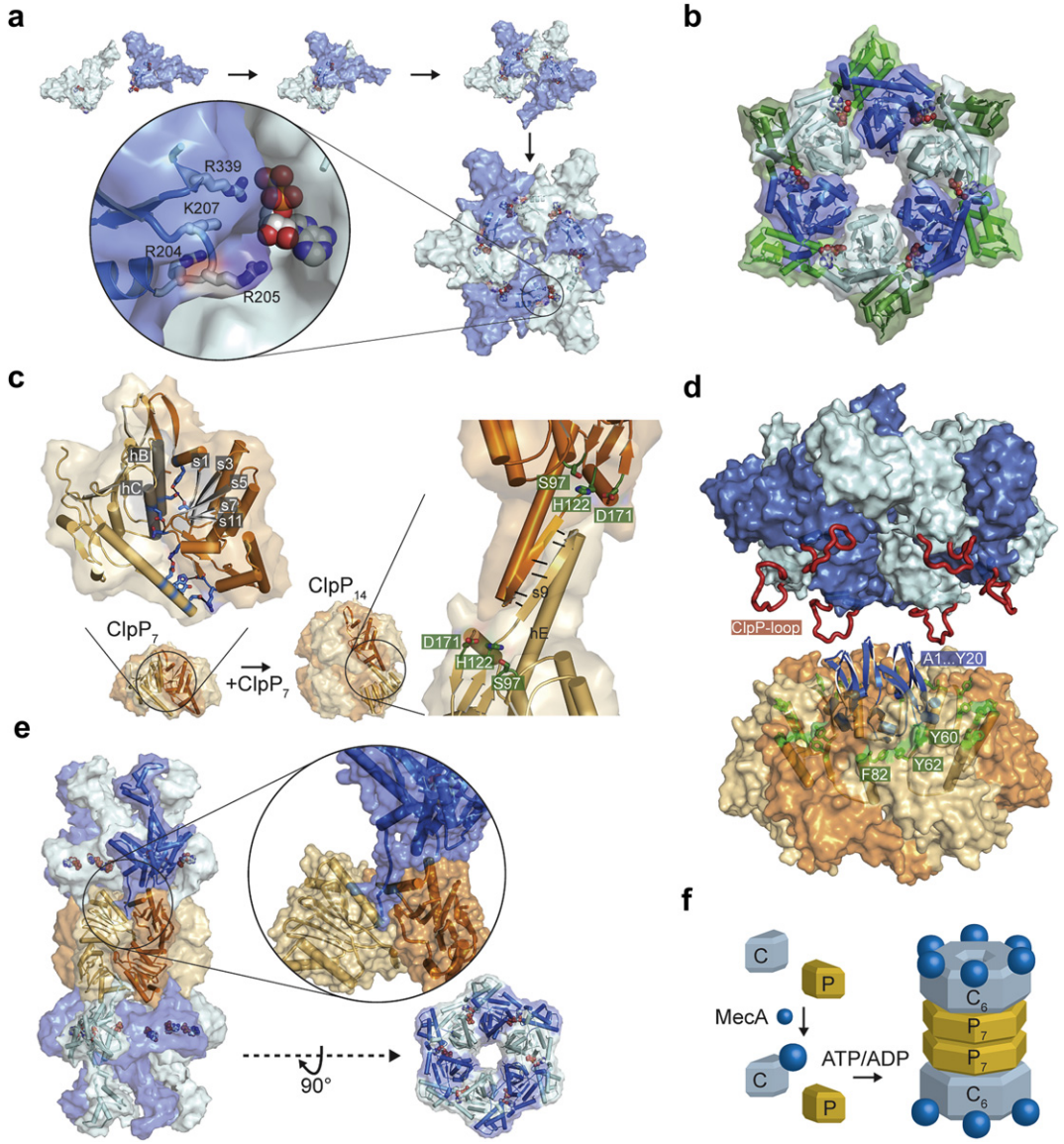

Fig. 2. Assembly of Clp chaperone–proteases. Chaperone and protease subunits are colored in shades of blue and orange, respectively. Nucleotide is shown in sphere representation. (a) ClpA hexamer assembly pathway. ClpA hexamer (pdb 1KSF; *E. coli*) was modeled as described [18]. In the presence of ATP, the equilibrium between the monomer (M) and dimer (D) is altered in favor of the dimer. Two dimers form a tetrameric (T) intermediate that binds another dimer to yield the hexamer (H). The magnified view depicts ATP bound to the first AAA module (D1) of a ClpA protomer (light blue). Several residues from the adjacent subunit (dark blue) make direct contact with the nucleotide. (b) The ClpX hexamer (pdb 1UM8; *H. pylori*) was modeled using hexameric ClpY (HslU; pdb 1HQY; *E. coli*) as template. The nucleotide binds in the subunit–subunit interface and the C-terminal sub domain (green) interacts with the ATPase domain of the adjacent subunit (light blue). (c) Proposed model of ClpP assembly (pdb 1YG6; *E. coli*). Seven ClpP molecules form a heptameric ring. The magnification shows how two helices (gray) of one protomer pack against a five membered β-sheet (gray) of the neighboring subunit. Amino acids involved in polar contacts are shown in stick representation (blue). The handle domains (s9-turn-hE) of two opposing heptameric rings intercalate to form the ClpP cavity. The magnification of the ring–ring interface shows a protomer of one ring forming an anti-parallel β-sheet with a protomer of the opposing ring. (d) Asymmetric interaction between the hexameric chaperone ClpX (pdb 1UM8; *H. pylori*) (blue) and the seven-fold symmetric protease ClpP (pdb 1YG6; *E. coli*) (orange). The long and flexible ClpP loop (red) likely facilitates binding of the conserved hydrophobic residues (LGF) to the hydrophobic patch on the ClpP surface (Y60, Y62, and F82 in green). The N-terminal 20 amino acids of ClpP form a β-hairpin (dark blue). Six out of seven N-termini adopt the 'up' conformation (N-terminus in 'down' conformation is in front); shaping a dome-like structure complementary to the concave binding surface of ClpX. (e) Side view of the ClpYQ (HslUV) complex (pdb 1G3I; *H. influenzae*) with the I-domain modeled using the *E. coli* ClpY structure (pdb 1HQY). One ClpY protomer (blue) and two ClpQ protomers (orange) per hexameric ring are shown in ribbon representation. Lower right: Top view of ClpY with nucleotides bound to the subunit interfaces. The magnified view depicts the ClpY-ClpQ interface where ClpY (blue) binds to a ClpQ subunit (dark orange) and the C-terminal extension of ClpY binds into a cleft between two ClpQ subunits (dark and light orange). (f) Adaptor-protein mediated assembly of *B. subtilis* ClpC. ClpC (light blue) and MecA (dark blue) form a heterodimer which activates ClpC and allows oligomerization in the presence of the nucleotide. Hexameric ClpC binds to both sides of ClpP (orange).

that the dynamic N-termini are not only a structural feature of the binding interface, but that they might play a functional role as well by forming hydrogen bonds with incoming unfolded proteins as part of the central substrate channel [1,26].

Since oligomerization is a prerequisite for all biological activities mediated by Clp chaperones, assembly can be used as a regulatory feature of chaperone activity. An interesting case for such a regulatory mechanism was reported for the ClpCP complex of *B. subtilis*. ClpC does not form a hexameric ring even in the presence of nucleotide. Only when the small protein MecA binds to the chaperone ClpC is it able to oligomerize into the hexameric ring [31]. Thus, the biological activity of this chaperone directly depends on the presence of another protein that mediates the assembly and thereby activates ClpC (Fig. 2f). Two additional such regulatory proteins (YpbH and McsB) were suggested to play similar roles in activating ClpC in *B. subtilis* [31].

The assembly pathway and the composition of the ClpP core cylinder itself vary among prokaryotes. In *E. coli*, ClpP is produced with a 14 amino acid propeptide which is released in an intermolecular autocatalytic cleavage reaction. Although cleavage of the propeptide is not required for ClpP oligomerization, it is crucial for its proteolytic activity [43]. The crystal structure of the ClpP 14-mer [61] suggests that single, heptameric ClpP-rings are formed first. This reaction is mainly driven by burying a significant portion of the solvent-accessible surface upon packing of two helices (hB and hC) of a protomer against the β-sheet (s1, s3, s5, s7, s11) of an adjacent subunit. The resulting subunit–subunit interface is predominantly hydrophobic, with several hydrogen bonds contributing as well (Fig. 2c, left). Two heptameric ClpP-rings then associate into a double-ring, shaping a roughly spherical chamber of about 51 Å in diameter with two axial pores of ~10 Å diameter. Consequently, the 14 catalytic sites (triad: Ser97, His122, Asp171) are placed inside the cavity close to the equatorial plane of the ClpP barrel (Fig. 2c, right). Formation of the ClpP 14-mer is due to the intercalation of the handle domains (s9, hE) from opposing ClpP-rings, thereby forming an anti-parallel β-sheet (Fig. 2c, right). In the fully assembled state each protomer buries ~42.5% of its surface in forming a tetradecamer. However, only ~8.5% of the ClpP surface is buried in the ring–ring interface rendering ClpP prone to ring–ring dissociation under certain conditions *in vivo* [37,44].

3. Substrate recognition and selectivity

As their name implies, *in vivo*, most Clps recognize and process the model substrate casein due to its low content of tertiary structure. This rather non-specific recognition of poorly structured proteins is one important feature of many Clp chaperone–proteases. Under stress (temperature, pH, salt, etc.), cellular proteins are prone to partial or complete unfolding which leads to the exposure of extended regions that are normally buried inside the native protein. Clp chaperone–proteases, along with Lon protease, thus help to rid the cell of non-functional unfolded proteins before they can form larger aggregates.

However, various mechanisms have evolved in order to target proteins for degradation. These mechanisms usually rely on features contained in or introduced at the N- or C-terminal ends of proteins destined for degradation. For example, the SsrA-tag is attached co-translationally to the carboxy-terminus of nascent peptide chains stalled on ribosomes, often due to truncated mRNA. The SsrA-tag consists of 11 amino acids encoded on a small rescue RNA molecule that acts as both tRNA and mRNA. The stalled chain is thus already destined for destruction upon release from the ribosome [46].

Both ClpAP and ClpXP exhibit loops in the central channel of the chaperone that interact directly with the SsrA-peptide [13,20,40]. *In vivo*, however, the bulk of ClpA is in a complex with ClpS, which inhibits recognition of SsrA-tagged substrates by ClpA [9,14]. Indeed, SsrA-tagged proteins are generally directed to ClpXP with the aid of SspB, a small delivery protein that forms a dimer [35,36,54]. SspB binds SsrA-tags at its N-terminal domain and docks to ClpX through a C-terminal binding motif. The substrate binding domain and the ClpX interaction motif are separated by a highly disordered stretch of ~40 amino acids [10,60]. A single SspB dimer binds per ClpX hexamer at a time (Fig. 3a). Each of the two ClpX binding domains of SspB binds to one ClpX N-domain dimer. This bidentate tethering is required for efficient substrate delivery [5]. The SsrA peptide exhibits different, but partially overlapping determinants for its interactions with ClpX, ClpA, and SspB (Fig. 3a, right). The SsrA-tag can interact simultaneously with SspB and ClpX, so that processing can commence while the substrate is still attached to SspB. However, ClpA recognizes residues 1–2, also recognized by SspB, in addition to residues 8–11 and, therefore, SspB binding inhibits SsrA substrate recognition by ClpA [15].

Another class of substrates contains an amino-terminal degradation signal, the so-called N-degron. The N-end rule describes the dependence of the *in vivo* half-life of a protein upon the identity of its amino-terminal residue(s) [45,59]. In bacteria, ClpAP in combination with the adaptor protein ClpS is responsible for removal of this class of substrates [11]. ClpS binds proteins bearing at their very N-terminus one of the four primary destabilizing residues (Leu, Phe, Tyr, and Trp) and directs them to the ClpAP complex through interaction with the ClpA N-domain (Fig. 3b). Secondary destabilizing amino acids exist (Arg, Lys) that are recognized by an L/F-transferase that catalyzes the attachment of primary destabilizing residues (Leu or Phe) to these residues, generating the N-degron sequences FR, LR, FK and LK [53,58,63].

Crystal structures of a binary complex between ClpS and the isolated N-domain of ClpA show that ClpS is a cone-shaped molecule made up of a layer of three helices packing over a small anti-parallel β-sheet [17,66]. On its N-terminus, it carries a coiled extension of roughly 26 amino acids. The cone-shaped body carries the N-degron binding site on its base and a binding site for the ClpA N-domain at its tip (Fig. 3b).

N-end rule substrate recruitment occurs due to the hydrophobic nature of the amino-terminal residue and is not

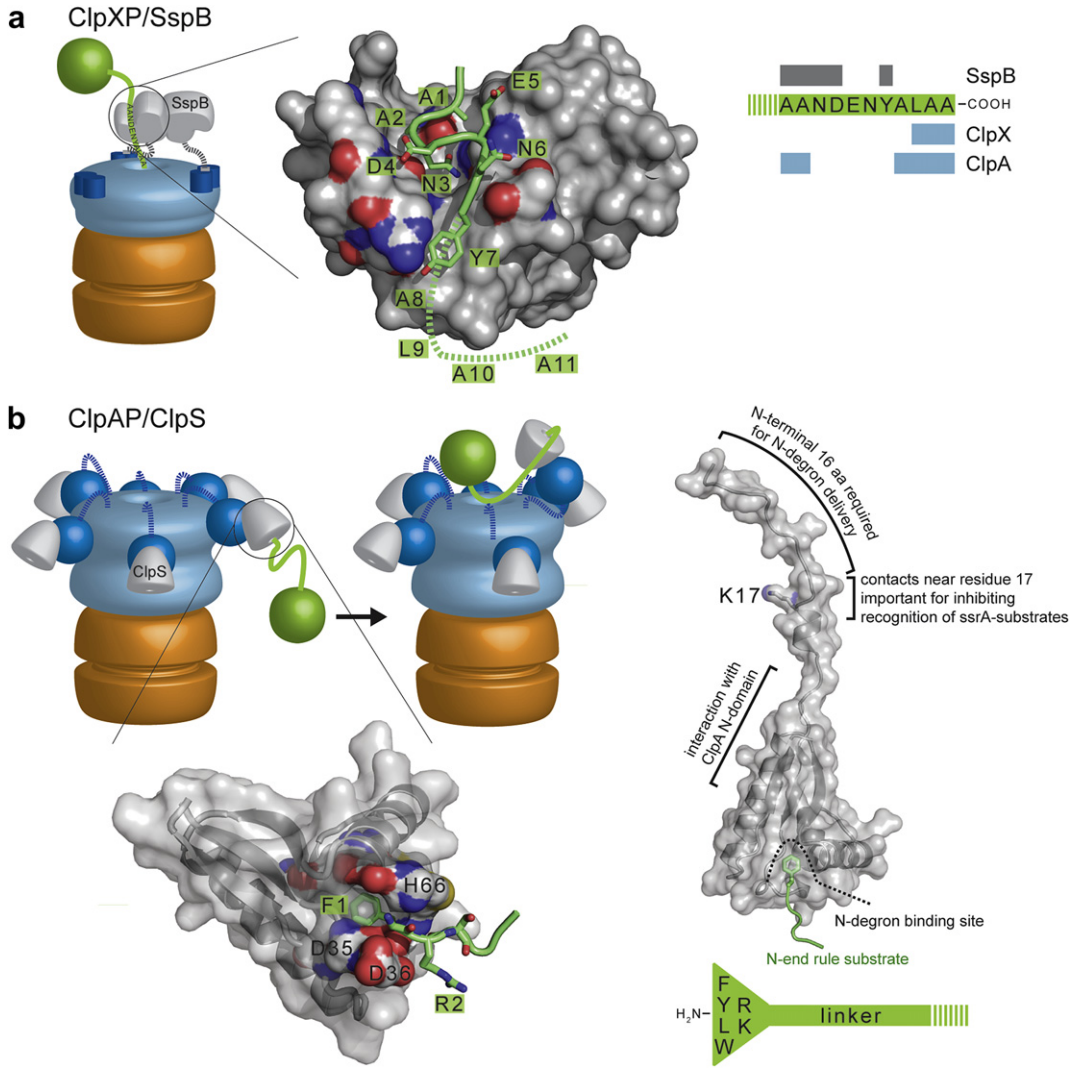

Fig. 3. Adaptor-mediated substrate recruitment to ClpXP and ClpAP. The chaperones and ClpP are shown in light blue and orange, respectively. (a) The SspB dimer binds to the first four (AAND) and the 7th residue (Y) of the SsrA peptide and interacts with the ClpX N-domains (dark blue) through the highly mobile C-terminal ClpX binding region (XBR). The last three amino acids of the SsrA tag (LAA) are not masked by SspB and are therefore available for capturing by ClpX. The surface representation of SspB (pdb 1OU8; *H. influenzae*) depicts how the SsrA-peptide (green) is bound to the binding groove of SspB (carbon = gray, oxygen = red, nitrogen = blue). The determinants for ClpA binding to SsrA are located at the amino- and carboxy-termini of the SsrA-tag and overlap with the determinants for SspB binding. (b) Left panel: ClpS binds to the ClpA N-domain (dark blue), thereby furnishing ClpA with a specific N-degron binding site. The magnified view depicts the hydrophobic binding pocket of ClpS (pdb 2WA8; *E. coli*) (carbon = gray, oxygen = red, nitrogen = blue) with a bound N-degron (FR, green). Our current understanding of N-end rule substrate delivery suggests that the highly mobile ClpA N-domain allows the bound substrate to approach the ClpA pore where the unstructured N-terminus is captured. Right panel: ClpS exhibits an amino-terminal coil region crucial for the allosteric regulation of ClpAP activity.

sequence-specific. However, in addition, conserved polar residues at the rim of the ClpS binding pocket (Asn34, Asp35, Asp36, His66) form hydrogen bonds with the free α-amino group of the substrate and several residues (Asp35 and Asp36) were shown to be crucial for efficient substrate binding [11,49,50]. Although it appears that the hydrophobic binding pocket of ClpS is optimized for Phe, Tyr and, to a lesser extent Leu, it is big enough to accommodate an N-terminal tryptophan [49,50]. Residue specificity might also be imposed by a methionine side chain of ClpS at position 40 (position 53 in *Caulobacter crescentus*), as it has been proposed that it excludes binding of β-branched amino acids (Iso and Val) [50].

The role of the ClpS N-terminal extension is still poorly understood. The presence of the first 16 amino acids, but not the sequence, is crucial for N-degron delivery and for suppressing ClpAP ATPase activity, suggesting that these two effects might be related (Fig. 3b, right) [21].

Although recruitment of N-end rule substrates to the ClpAP complex through ClpS is crucial, it is not sufficient for substrate degradation. Interestingly, an unstructured N-terminal stretch of around 20 amino acids following the N-degron is required on the substrate for it to be processed [11]. This unstructured region could be needed to overcome steric restrictions during substrate transfer form ClpS to ClpA or, more likely, the N-end rule substrates bind simultaneously to ClpS and to the ClpA pore.

The N-domain of ClpA is attached to the first AAA module through a 25-residues-long linker. It was suggested that this

linker and the attached N-domain are highly flexible, which might facilitate the approach of the bound substrate (via ClpS) to the chaperone pore where substrate processing is initiated (Fig. 3b) [23]. Deletion of this linker impairs ClpS-dependent degradation of N-end rule substrates, underscoring its importance for efficient substrate delivery [8]. The ClpAP complex also recruits SsrA-tagged substrates directly to the pore when ClpS is absent [20]. Notably, deletion of the linker together with the N-domain increases catalytic efficiency for SsrA-substrate degradation, indicating that the N-domains partially mask the pore [8]. Hence, a highly mobile N-domain permits active delivery of ClpS-dependent substrates to the pore, allowing other substrates to directly reach the chaperone by moving away from the pore.

In *E. coli*, SsrA-tagged substrates are mainly directed to the ClpXP/SspB complex, whereas their recruitment to ClpAP is inhibited by ClpS [9]. However, under conditions where SsrA-tagged proteins become abundant (stress), binding of SsrA-substrates to ClpAP stabilizes ClpA against autodegradation, leading to an increased ratio of ClpAP over ClpAPS. Consequently, ClpAP can degrade a significant amount of SsrA substrates when the ClpXP/SspB system is overwhelmed [14,38].

4. Molecular details of chaperone activity

After the substrate has been recruited by the chaperone, it needs to be unfolded in order to pass through the narrow protease pore. There are three key structural elements present in all Clp ATPases that enable this process: a narrow gate that restricts passage of a structured protein sequence into the central channel, a long inert central channel showing no clear sequence preference and ATP-hydrolysis-powered loops positioned along this channel that are in contact with the engaged substrate chain. The mechanism of unfolding/translocation has mostly been investigated in ClpAP and ClpXP complexes, but other chaperone–protease complexes are expected to function in a similar manner, since they all share the key elements required for this process (Fig. 4a–c).

The narrow entrance at the substrate-interacting face of the chaperone ring is lined with loops implicated in substrate binding [20,40]. Whether and how these loops contribute to substrate unfolding is still unclear (Fig. 4a–c). In ClpX and ClpY (HslU), there is no evidence to support their active involvement in substrate unfolding, most likely because they are not coupled to the ATP hydrolysis site. ClpA in contrast, has two AAA modules, and even though ATP hydrolysis in D1 is considerably slower than in D2, it is possible that the D1 loops change conformation depending on the D1 ATP hydrolysis state. Indeed, recent data show that ATP hydrolysis in D1 is sufficient to process small, single domain proteins of low stability [67].

Despite the possible involvement of the D1 loops, a loop in D2 of ClpA, and equivalent loops in ClpX and ClpY (HslU) characterized by a conserved GYVG motif, are considered to be predominantly responsible for substrate unfolding and translocation into the protease cylinder [20,41,47]. In general, loops with a conserved aromatic-hydrophobic (Ar-φ) dipeptide are common in AAA+ chaperones and are implicated in transmitting mechanical force to their substrates. The tyrosine residue in the GYVG-loop of ClpA, ClpX, and ClpY (HslU) was identified as essential for unfolding and translocation of native substrate proteins [20,41,47]. Single-molecule fluorescence microscopy studies demonstrated that the conserved tyrosine is also important for the formation of the prehydrolytic, high substrate affinity conformation in ClpA [12], supporting a mechanism where tyrosine residues grip substrates to drive unfolding and translocation [41]. Several lines of evidence suggest that ATP hydrolysis enables these loops to cycle between ‘‘up’’ and ‘‘down’’ conformations, thereby exerting a mechanical force that mediates pulling of substrates from their attachment point. The most direct evidence for these two conformations came from the ClpY (HslU) crystal structure solved in the absence and in the presence of the two nucleotides, ATP and ADP. The GYVG-loop faces up in the nucleotide-free state, is oriented perpendicular to the central channel in the ATP state and points downwards in the presence of ADP [62]. Furthermore, synchrotron hydroxy-radical footprinting experiments comparing the nucleotide-free, unassembled state of ClpA with the ATPγS-bound hexamer, also indicate that the ClpA GYVG-loop faces upwards and possibly even contacts D1-loops in its prehydrolytic state [4].

The most detailed investigation of nucleotide-dependent changes in the pore loops was described for ClpX in several recent publications [40–42]. In these studies, covalently linked ClpX subunits were used with mutations in only a subset of the linked subunits in order to mimic asymmetric states during the ATPase cycle (ATP-bound, ATP-hydrolyzing or nucleotide-free). In addition, the key tyrosine residue in the GYVG-loop was replaced by alanine in some of the subunits [41]. By combining these two types of mutations, it was possible to probe the relationship between the nucleotide state of a subunit and the function of its pore loop. The data demonstrated that the conserved tyrosine residues link ATP hydrolysis to mechanical work by gripping the substrate to transmit a pulling force to the polypeptide chain [41]. Removal of the tyrosine in only a few ClpX subunits resulted in a large increase in the energetic cost of substrate unfolding, likely due to failed attempts to exert a grip on the substrate, resulting in ‘‘slippage’’.

Another loop, described for ClpX, located at the bottom of the central channel (residues 220–233 in *Helicobacter pylori* ClpX equivalent to residues 190–203 in *E. coli* ClpX) and referred to as pore-2 loop, is also dependent on the nucleotide states of individual subunits and assists in unfolding of native protein substrates (Fig. 4b) [39]. As different nucleotide states influence pore-2 loop movements, inversely, pore-2 residues might play a role in controlling the ATPase rate of ClpX. Interaction of ClpP with ClpX, facilitated through N-terminal ClpP residues and the ClpX pore-2 loops, causes a 50% decrease in the ClpX ATPase rate, whereas a ClpX variant lacking the pore-2 loop is not inhibited by ClpP [39]. Thus, it was suggested that the ClpP N-terminus interacts with the

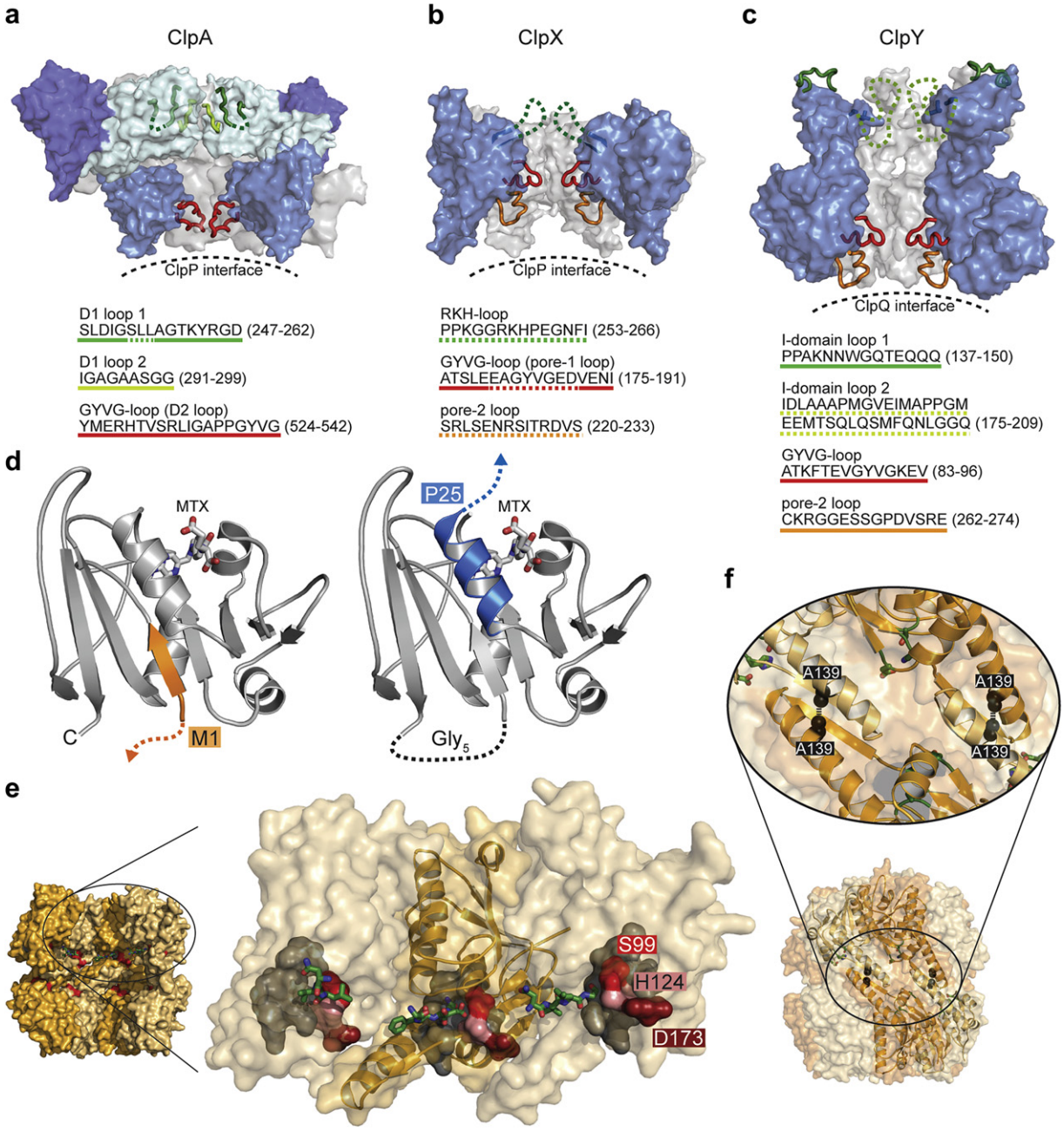

Fig. 4. Chaperone–protease mediated substrate unfolding and degradation. (a–c) Loops in the central channel of ClpA, ClpX and ClpY. Two subunits of the hexameric chaperone ring are omitted in order to visualize the central unfolding channel. Two opposing chaperone subunits are shown in blue. The N-domain of ClpA (a) is shown in dark blue and the two AAA modules are shown in light blue (D1) and blue (D2). Loops in the central channel of the chaperone ClpA (pdb 1KSF; *E. coli*) (a), ClpX (pdb 1UM8; *H. pylori*) (b), and ClpY (pdb 1HQY; *E. coli*) (c) involved in substrate binding and translocation are shown in light and dark green (distal to the protease interface) and in red and orange (proximal to the protease interface). The GYVG loop (red) and the pore-2 loop (orange) of ClpX (b) were modeled using ClpY (c) as template. Unresolved parts of the loops are drawn as dashed lines. (d) DHFR (gray) in complex with methotrexate (MTX) (pdb 1RG7; *E. coli*). Left panel: N-terminally-tagged wild-type DHFR/MTX is not processed by ClpAP. Right panel: A circular permutated variant of DHFR exhibiting a new N-terminus at position P25 is efficiently degraded by ClpAP even when stabilized by MTX. (e) ClpP in complex with peptides (pdb 2ZL2; *H. pylori*). Protomers are alternately colored in light and dark orange. Peptides are shown in green. Three subunits of each heptameric ClpP-ring are omitted to visualize the ClpP cavity with the catalytic triad shown in red. The magnified view depicts three neighboring ClpP subunits with tetrapeptides bound to the active sites. The S1 binding pocket (gray) is mainly hydrophobic and sufficient in size to accommodate all amino acids. (f) ClpP side pores were suggested to be crucial for peptide release. When Ala139 (black) of ClpP (not counting the propeptide) is replaced by cysteine, a disulfide bond is formed under oxidizing conditions that links the intercalated handle regions of the two opposing ClpP-ring leading to retardation of peptide release.

ClpX pore-2 loop, thereby inhibiting loop movements causing a decreased rate in ATP hydrolysis.

The role of ATP hydrolysis in driving unfolding and translocation was addressed by using covalently linked ATPase-active and -inactive ClpX subunits to build the hexamer. Effects of diverse geometric arrangements of individual ClpX subunits on unfolding and translocation of protein substrates were tested, showing that the ClpX power stroke is generated by ATP hydrolysis in a single subunit. A probabilistic rather than a concerted or strictly sequential sequence of nucleotide hydrolysis events around the hexameric ring driving substrate threading into ClpP was suggested [42].

ATP consumption is largely independent of the global stability of the substrate, as was shown by a study using Arc repressor variants of varying stability as substrates [6]. ClpXP degraded each variant at a similar rate and consumed similar amounts of ATP per degraded substrate molecule. However, it is not the global thermodynamic stability of the substrate proteins, but rather the local stability of the protein structure adjacent to the targeting signal, that determines the rate of substrate processing. This was demonstrated using circular permutations of dihydrofolate reductase (DHFR) in complex with methotrexate as substrates for the ClpAP protease (Fig. 4d) [34]. These DHFR variants only differed in the location of the N- and C-termini within an otherwise identical structure of the same global stability. When ClpAP first encountered an α-helical region, loosely packed against the protein core, the degradation rate was the same regardless of whether methotrexate was present or not. If, however, it first encountered a stable β-sheet conformation, degradation was dramatically reduced (Fig. 4d). The same study demonstrated that, in multidomain proteins, independently stable domains are unfolded sequentially. Identical behavior was demonstrated for ClpXP protease, since destabilizing the substrate structure near the degradation tag accelerated degradation and reduced ATP consumption [27]. Comparison of the degradation rates for native and unfolded protein substrates indicates that, for stable substrates, unfolding is rate-limiting, whereas translocation becomes rate-limiting for processing unfolded substrates and substrates displaying low local protein stability near the degradation tag [27].

Recently, a FRET-based approach characterizing the main steps in SsrA-tagged substrate processing by the bacterial ClpAP and ClpXP complexes was reported [32]. This study shows that a substrate with moderate stability is rapidly unfolded and that substrate unfolding in this case does not limit degradation. Unfolding coincides with directional threading of the substrate through the narrow channel of the chaperone ring towards the protease, indicative of a mechanical unfolding mechanism. Interestingly, this FRET study revealed transient substrate recompaction, after fast initial unfolding, that can be explained by a kinetic model where initiation of translocation into the protease is the rate-limiting step in the reaction cycle. Once substrate transfer into ClpP has commenced, translocation and degradation occur rapidly and in a processive manner.

5. Mechanism of substrate transfer and degradation

After the substrate has been unfolded and threaded through the chaperone, it has to be translocated inside the protease core for degradation. The ClpP protease pore is narrow, and in the absence of a chaperone partner it allows efficient passage only of very small peptides up to 5 amino acids in size. The entry of larger substrates is blocked by the N-terminal ClpP loops that change conformation only upon interaction with the chaperone, allowing the threading of larger substrates. This is supported by the observation that in the absence of a chaperone partner, the deletion of the N-terminal ClpP loops increases the degradation rate of substrates [2,16,24]. Recently, it was demonstrated that unfolded proteins can also enter wild-type ClpP and become degraded; however, this occurs on a time scale several orders of magnitude slower than in the presence of the chaperone [25].

Substrates that enter the proteolytic cavity bind within hydrophobic binding grooves positioned at the equatorial plane of both ClpP and ClpQ (HslV) protease [55,61]. The grooves of the ClpP subunits in one heptameric ring are linked, resulting in a continuous, circular, substrate binding surface. Along these grooves, the catalytic triads are spaced ~25 Å apart so that an extended hepta or octapeptide substrate can easily span the distance between adjacent active sites [61]. Structures in complex with peptides were solved for wild-type and mutant ClpP from *H. pylori* and showed that substrates are sandwiched between the two β-strands of ClpP, resulting in a three-stranded, anti-parallel β-sheet [29] (Fig. 4e). The S1 binding pocket in ClpP is formed by residues Phe103, Ser106, Ile123, His124, Pro126, Lys148, Met151, Asn152 and Leu155, making it highly hydrophobic and big enough to accommodate all amino acids. The crystal structure of apo-ClpP revealed that, in this state, the three residues of the catalytic triad are not in an active conformation, as they are too far apart [29]. However, in the ClpP in complex with peptides, the distances are within hydrogen bonding range, suggesting that the catalytic triad of ClpP is rearranged into an active conformation upon peptide binding. In contrast, the ClpQ (HslV) catalytic triad of the threonine-type is inactive unless bound to ClpY (HslU). It was shown that the C-terminal extension of ClpY, which binds in between two neighboring ClpQ subunits, is crucial for ClpQ-activation [48].

ClpP degrades proteins in a processive manner, producing peptides with a discrete length distribution. However, the exact mechanism leading to such a peptide size distribution is poorly understood. Since the peptide product length is not altered due to inactivation of up to 70% of ClpP active sites, a mechanism was proposed in which translocation alternates with proteolysis, suggesting allosteric activation and inactivation between the ATPase and protease [7]. However, comparison of the size distribution of peptides generated by ClpAP and ClpP suggests that ClpP alone controls the length of the peptide products [25]. Furthermore, a ClpP variant lacking the N-terminus, hence unable to bind a chaperone partner but still able to degrade polypeptides, showed a similar peptide length distribution as the wild-type complex in the presence of ClpA or

ClpX, indicating that the ATPases do not play a significant role in generating peptide products of a certain length [2].

The release of the formed peptide products is the last step in the substrate degradation cycle. The mechanism of this process has been studied by various techniques and the accumulated evidence suggests that dynamic side pores which form at the interface of the two heptameric ClpP-rings function as the peptide exit sites. These pores were observed in the *Mycobacterium tuberculosis* ClpP crystal structure, potentially due to a higher level of flexibility of this region than in ClpPs from other species [22]. In solution, NMR spectroscopy of ClpP from *E. coli* demonstrated two structurally distinct conformations of the ring-ring interface [56]. In agreement with a side-pore release model, restricting the motional freedom of the ClpP handle region through the introduction of a disulfide bond (Fig. 4f), a significant retardation of substrate release is observed relative to the rate observed under reduced conditions [56].

Over the past decades, bacterial Clp proteases have become the best studied examples of barrel-shaped compartmentalizing proteases. The uncovered functional principles demonstrate that these sophisticated assemblies have evolved to ensure the processive degradation of a large range of cellular proteins into well-defined peptide products of a narrow size distribution, without compromising other soluble proteins that are needed to support cellular function.

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