ClpP: A structurally dynamic protease regulated by AAA+ proteins

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Abstract

Proteolysis is an important process for many aspects of the bacterial physiology. Clp proteases carry out a large proportion of protein degradation in bacteria. These enzymes assemble in complexes that combine the protease ClpP and the unfoldase, ClpA or ClpX. ClpP oligomerizes as two stacked heptameric rings enclosing a central chamber containing the proteolytic sites. ClpX and ClpA assemble into hexameric rings that bind both axial surfaces of the ClpP tetradecamer forming a barrel-like complex. ClpP requires association with ClpA or ClpX to unfold and thread protein substrates through the axial pore into the inner chamber where degradation occurs. A gating mechanism regulated by the ATPase exists at the entry of the ClpP axial pore and involves the N-terminal regions of the ClpP protomers. These gating motifs are located at the axial regions of the tetradecamer but in most crystal structures they are not visible. We also lack structural information about the ClpAP or ClpXP complexes. Therefore, the structural details of how the axial gate in ClpP is regulated by the ATPases are unknown. Here, we review our current understanding of the conformational changes that ClpA or ClpX induce in ClpP to open the axial gate and increase substrate accessibility into the degradation chamber. Most of this knowledge comes from the recent crystal structures of ClpP in complex with acyldepsipeptidase (ADEP) antibiotics. These small molecules are providing new insights into the gating mechanism of this protease because they imitate the interaction of ClpA/ClpX with ClpP and activate its protease activity.

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1. Introduction

Macromolecular assemblies comprised of multiple protein components perform most enzymatic reactions in bacteria, including proteolysis (Baumeister, 2005). These complexes are dynamic macromolecular machines that continuously change conformation in order to perform their functions. Visualizing these conformational changes and the dynamics of these protein complexes is necessary to understand the molecular mechanisms that allow these enzymes to perform work or catalyze reactions. However, obtaining a three dimensional view of the motions that these complexes undergo during the course of a reaction represents a challenge for X-ray crystallography or cryo-electron microscopy (cryo-EM). These techniques immobilize the macromolecular assemblies for X-ray crystallography or cryo-electron microscopy (cryo-EM). These techniques immobilize the macromolecular assemblies in either crystal lattices (Chayen and Saridakis, 2008) or vitreous ice (Dubochet et al., 1988) as a necessary step to obtain their three-dimensional structures. Therefore, most frequently these techniques provide snapshots of macromolecular assemblies that represent ground low-energy conformers of the enzyme. These pictures are insufficient to describe the entire range of motions that macromolecular complexes experience during the course of a reaction. Nuclear magnetic resonance (NMR) spectroscopy is an ideal method to probe protein dynamics but traditionally this technique has been restricted to study small monomeric proteins or protein domains. Recently, impressive advances in instrumentation and experimental tools for the production of appropriately labeled samples (Kay, 2005) have made possible to use NMR to study dynamics in high-molecular weight complexes, which were previously considered to be outside the scope of this technique. Structural biologists have realized that a well-defined average structure obtained by X-ray crystallography or cryo-EM is no longer the endpoint goal, but rather the starting point of additional efforts to uncover the dynamics of macromolecular assemblies and how changes in structure relate to their function (Baldwin and Kay, 2009).

The bacterial Caseinolytic protease (ClpP) clearly exemplifies this scenario. The crystal structures of ClpP proteins from several organisms have been experimentally determined (Bewley et al., 2006; Gribun et al., 2005; Kang et al., 2004; Szyk and Maurizi, 2006; Wang et al., 1997). These structures constitute a starting point in our understanding of the function and regulation of the ClpP protease. They show that ClpP assembles into a tetradecameric barrel-shaped enzyme with an enclosed chamber containing
14 proteolytic active sites. Entry of the protein substrates into the ClpP degradation chamber occurs through the two axial pores (Ortega et al., 2000), which are gated by the N-terminal region of the protomers. These protein motifs must adopt multiple conformations to either prevent or permit the entry of protein substrates into the ClpP degradation chamber. However, the structure of the N-terminal region of ClpP in the open and closed state of the gate is unknown since in most of the crystal structures these motifs are partially or completely disordered. Likewise, how the degraded polypeptides are released from the proteolytic chamber also remains largely controversial. This process probably also involves mobile motifs of ClpP, either at the N-terminus or at the ‘handle’ region located at the equator of the tetradecamer (Sprangers et al., 2005). However, clear structural information on how the movements of these regions regulate the exit of proteolysis products remains to be determined. Consequently, the available structural information about ClpP only provides a partial understanding on how ClpP performs its function and by no means presents a full description on the motions of the protein during important steps of the degradation process including entry of substrates and exit of the products.

In what follows, we will review the available structural information and describe the conformational changes that occur in the structure of ClpP to allow the access of protein substrates to the digestion chamber. Aspects of our research in this area are highlighted. We will also comment on some models proposed by different research groups to mechanistically describe how ClpP regulates substrate entry. The current literature shows that we only have a fragmented understanding of the molecular mechanisms of the ClpP protease.

2. ClpP is structured as a self-compartmentalized protease

Proteases are involved in virtually every aspect of the bacterial physiology, including timing of the cell division cycle and responses to heat shock and other stresses (Gottesman et al., 1997; Sauer and Baker, 2011). Proteases are also key contributors to the maintenance of protein homeostasis, a process that involves removing damaged, denatured and aberrantly folded proteins that are harmful to the cell (Wickner et al., 1999). They are critically important for the overall fitness of bacteria and contribute to virulence (Butler et al., 2006). ClpP carries out a large proportion of protein degradation in the bacterial cell. This enzyme is a highly conserved serine protease present throughout bacteria and is also found in the mitochondria and chloroplasts of eukaryotic cells (Corydon et al., 1998; Katayama-Fujimura et al., 1987; Maurizi et al., 1990).

ClpP orthologs (Bewley et al., 2006; Gribun et al., 2005; Kang et al., 2004; Szyk and Maurizi, 2006; Wang et al., 1997) are structurally very similar (Fig. 1A). In all these species, the 21-kDa ClpP monomer is folded in three subdomains: the “handle”, the globular “head” and the N-terminal region (Fig. 1A). ClpP is considered a self-compartmentalized protease because in Escherichia coli and many other bacterial species it oligomerizes as two stacked heptameric rings enclosing a central chamber containing 14 proteolytic active sites, each comprised of a canonical Ser-His-Asp catalytic triad (Wang et al., 1997). The handle region of the protomers forms the area of interaction between the two heptameric rings and the heads comprise the main body of the rings (Fig. 1B and C). The N-terminus is located at the axial regions of the tetradecamer (Fig. 1B), but in most crystal structures the electron density of this region has been uninterpretable; hence, they are not modeled (Fig. 1A). Based on these observations, this region of ClpP is believed to be highly flexible.

ClpP on its own can degrade small peptides (<5 amino acids) (Thompson et al., 1994; Woo et al., 1989) and also with low...
efficiency full-length unfolded proteins (α-casein) (Jennings et al., 2008b). ClpP requires association to the ClpA or ClpX ATPases to efficiently process larger peptides and folded proteins (Grimaud et al., 1998; Kessel et al., 1995). ClpX and ClpA belong to the AAA+ protein family (ATPases associated with various cellular activities) and they assemble into hexameric rings that bind coaxially to the ClpP tetradecamer forming a barrel-like holoenzyme complex (Fig. 1D, left panel) (Beuron et al., 1998). Binding of these hexameric ATPases to the ClpP tetradecamer mediates unfolding of substrates in an ATP-dependent manner (Hoskins et al., 1998; Sauer et al., 2004; Singh et al., 1999). The unfolded polypeptide is threaded into the ClpP inner chamber through the axial channel and degraded (Fig. 1E) (Ishikawa et al., 2001; Ortega et al., 2002, 2000). This implies the existence of a gating mechanism controlled by the ATPase.

3. The enigmatic N-terminal region of ClpP gates substrate entry

The N-terminal regions of the ClpP proteases occupy a near axial location in the tetradecamer (Fig. 1B), which initially suggested that these regions would form the gate that control substrate access. Subsequently, it was shown that deletion of the first 10–17 N-terminal amino acids of ClpP allows access and rapid degradation of large unfolded proteins in the degradation chamber without assistance of ClpA or ClpX (Bewley et al., 2005; Jennings et al., 2008a). These results revealed that indeed this region of ClpP is involved in the gating mechanism.

By itself, ClpP shows a very limited degradation activity, even for peptides that are a few amino acids long. Therefore, in the absence of the ATPases the N-terminal region of ClpP must mainly adopt a closed conformation that excludes efficient entry of substrates into the degradation chamber. Subsequent binding of ClpA or ClpX serves to relieve the interactions that stabilize the closed conformation, inducing an open configuration of the N-terminus of ClpP that facilitates translocation of large unfolded substrates.

Biochemically, it has been described that interactions mediated by charged residues in the N-terminal region of ClpP (Glu8, Arg12, Glu14 and Arg15) are important to stabilize the closed conformation of the gate. Adjacent protomers in the ClpP heptamer ring also stabilize the closed conformation by forming a hydrophobic cluster between Pro4, Val6 and Ile19 in one protomer and Leu24’ and Phe49’ in the adjacent ClpP molecule (Lee et al., 2010b). All these interactions seal the gate and prevent the translocation of substrates. However, the structural conformation of the N-terminal region of ClpP in the open and closed conformation is enigmatic because of the high degree of variability observed in the conformation of the N-terminal region among available X-ray structures.

ClpP structures have been solved from thirteen different organisms including E. coli (Bewley et al., 2006; Szyk and Maurizi, 2006; Wang et al., 1997), Bacillus subtilis (Lee et al., 2011, 2010a), Helicobacter pylori (Kim and Kim, 2008) and Mycobacterium tuberculosis (Ingvarsson et al., 2007). Multiple crystal structures exist for some of these proteins. In many of the structures the N-terminal regions are disordered (Fig. 1A). In structures for which electron density is observable, the N-termini are in either an ‘up’ or ‘down’ conformation. In the ‘up’ conformation (Fig. 2A), the N-terminus forms a β-hairpin loop containing a two-stranded antiparallel β-sheet in which the first seven residues line the axial pore, while residues 8–16 form a flexible loop extending out of the apical surface of ClpP (Bewley et al., 2006; Kang et al., 2004; Szyk and Maurizi, 2006). The ‘down’ conformation (Fig. 2B) is less well defined but in this case no residues protrude from the apical surface and the N-terminal regions are contained within the pore, forming poorly defined hairpins oriented perpendicular to the sevenfold axis (Bewley et al., 2006). Unfortunately, it is unclear how the ‘up’ or ‘down’ configuration of the N-terminal regions of ClpP relate to the open and closed states of the tetradecameric enzyme.

Fig. 2. Asymmetric crystal structure of E. coli ClpP. (A) Close up view (left) of the N-terminal regions of first heptameric ring in the ClpP tetradecamer. This ring contains six of these regions in the ‘up’ conformation and one (drawn in yellow) in the ‘down’ conformation. Top view (right) of the axial channel in the first hexameric ring shows how the N-terminal regions collapse into the axial channel and cause its closure. (B) Close up view (left) of the N-terminal regions of second heptameric ring in the ‘down’ conformation. A surface representation of this ring shows an open axial pore of ~12 Å diameter, as the first 12 N-terminal residues of each protomer are not represented in the X-ray structure. Figure produced from the structure of E. coli PDB ID: 1YG6.

4. Interactions of ClpA and ClpX ATPases with ClpP induce the open conformation of the axial gate

Contributions from several groups (Gribun et al., 2005; Hoskins et al., 1998; Martin et al., 2007; Ortega et al., 2004; Singh et al., 1999) have established that the interaction between ClpA and ClpX with ClpP induces the open state of the ClpP tetradecamer, which allows translocation of large protein substrates into its digestion chamber.

Biochemically it has been shown that ClpP communicates with the ATPases through two distinct sets of interactions. The six IGF/L loops of a ClpA and ClpX hexamer (Kim et al., 2001) dock in the hydrophobic pockets located near the outer edge of the apical surface of each ClpP heptamer ring (Fig. 1D, right panel). These interactions are static high-affinity contacts that maintain a stable ClpAP or ClpXP complex and coaxially align the ATPase component with ClpP. Importantly, all six loops in the ATPase hexamer are required for strong ClpP binding (Martin et al., 2007), although how it accommodates the symmetry mismatch is not clear. In addition, the N-terminal region of ClpP interacts with the axial pore-2 loops of the ATPase. These are relatively weak and dynamic interactions that vary with the nucleotide state of the protomers in the heptameric ring of the ATPase (Martin et al., 2007). The 6:7 symmetry mismatch between ClpAP/ClpX and ClpP (Beuron et al., 1998) has hindered crystallographic efforts and single particle analysis of cryo-EM images to obtain high-resolution structures of ClpAP and ClpXP. Therefore, we presently do not have a three dimensional view of how these static and dynamic interactions with ClpA or ClpX induce the opening of the axial gate in ClpP and allow substrates access to the degradation chamber.
As outlined previously, our structural understanding of the open and closed conformations of ClpP is limited. Current models are mainly based upon one of the crystal structures of ClpP reported by John Flanagan’s group (Bewley et al., 2006) and a three-dimensional structure of ClpP bound to ClpA obtained by cryo-electron microscopy (cryo-EM) reported by Alasdair Steven’s group (Effantin et al., 2010).

Interestingly, in this crystal structure of ClpP (Bewley et al., 2006), the N-terminal regions of six of the subunits in the first heptameric ring of the tetradecamer adopt the ‘up’ conformation (Fig. 2A). The remaining protomer in this ring and all the subunits in the second ring, the N-terminals are present in the ‘down’ conformation (Fig. 2B). The particular conformation of the N-terminal regions in the first ring (Bewley et al., 2006) suggested that this structure might have similarities to the structure of ClpP in complex with ClpA or ClpX. It also provided a model to explain how the heptameric ring of the protease interacts with the hexameric ring of the ATPases in spite of the symmetry mismatch. According to this model, it is possible that the six N-terminals in the ‘up’ conformation adopt a pseudo-6-fold symmetric arrangement that establishes contacts with the axial loops of the ClpA or ClpX hexamers. The seventh N-terminal region in the ‘down’ conformation is perhaps engaged in translocating substrate.

The conformational difference of the N-terminal regions in the protomers of ClpP of this structure is caused by differences in the crystal-packing environment of each ring (Bewley et al., 2006). In particular, residues 8–11 in the ‘up’ conformation establish crystal contacts with residues in the side surfaces of the adjacent tetradecamer. Conversely, the N-terminals in the ‘down’ conformation are not involved in crystal contacts. In the six protomers in the ‘up’ conformation, the conformation of the backbone and side chain atoms of residues in the two N-terminal β-strands is very similar and clearly defined in the electron density map. Weaker density is present for residues in the loop area of the N-terminals and frequently extends out to the lumen causing the closing of the axial pore (Fig. 2A, right panel). It is possible that these surface exposed loops of the N-terminal regions (Fig. 2A) act as a flap that opens when ClpP interacts with the axial loops of the hexameric ClpA or ClpX. This model would predict that a perfect 6-fold symmetry arrangement of these loops in an open conformation is only observed upon interaction with the ATPases. For the ClpP protomers in the second ring adopting the ‘down’ conformation, density for the first 12 N-terminal residues is diffuse and delocalized and only residues 12–20 are generally interpretable. Consequently, a surface representation of this ring shows an open axial pore of ∼12 Å diameter (Fig. 2B, right panel) (Bewley et al., 2006).

An important caveat of this model is that the N-terminal regions in the ‘up’ conformation are stabilized by crystal contacts. However, these interactions do not occur in solution and may be different from the ones established by the protease and ATPase in the context of the ClpAP and ClpXP complexes. Therefore, it is unclear from this work whether the open and closed conformations of the axial channel of ClpP in the ClpAP and ClpXP complexes are truly portrayed by the ‘up’ or ‘down’ conformations described by this asymmetric crystal structure of ClpP (Bewley et al., 2006).

Additional structural insights into the open and closed conformations of the ClpP axial channel are provided by the three-dimensional reconstructions of ClpP bound to ClpA (Fig. 3) (Effantin et al., 2010) obtained by cryo-EM. In this work, the ClpP component of 2:1 (Fig. 3A) and 1:1 (Fig. 3B) ClpAP complexes (with ClpA bound to both or only one end of ClpP, respectively) was extracted from the particle images and used separately to obtain 7-fold symmetrized three-dimensional reconstructions of ClpP in these two type of complexes. Both ends of ClpP in the 2:1 complex and the ClpA-bound end of ClpP in the 1:1 complex showed the existence of an open axial channel (Fig. 3). However, this region was closed in the ClpA-free end of ClpP in the 1:1 complex (Fig. 3B and 3C, right panel), confirming that the interaction with the ATPase induces a conformational change in the N-termini regions of ClpP and opens the axial gate to facilitate the access of substrates to the digestion chamber.

5. How are the ‘up’ and ‘down’ conformations of the N-terminal region of ClpP related to the open and closed states of the axial gate?

A comparison of the asymmetric crystal structure of ClpP (Bewley et al., 2006) with the cryo-EM density maps of the 1:1 and 2:1 ClpAP complexes (Effantin et al., 2010) has provided insights on how the ‘up’ an ‘down’ conformation observed in the crystal structure of ClpP may relate to the open and closed conformations of the axial gate (Effantin et al., 2010).

To this end, central sections of the cryo-EM density maps of the 1:1 and 2:1 complexes were calculated (Fig. 4A) and compared to equivalent sections obtained from the crystal structure of ClpP (Bewley et al., 2006) after the resolution of the atomic coordinates was limited to 12 Å (Fig. 4B). The central sections of the cryo-EM...
change the appearance of the axial channel (Fig. 4B, right panel). Therefore, from this exercise it was not possible to determine by comparison whether any of the N-terminal regions of ClpP in the open axial channel are present in the ‘down’ conformation in the cryo-EM structure (Effantin et al., 2010).

In contrast, the appearance of the opposite end of ClpP in the calculated section (Fig. 4B, left panel, asterisk) was not consistent with the ClpA-free end of the 1:1 ClpAP complex (Fig. 4A, right panel, asterisk). The blocked axial channel in the cryo-EM map of the 1:1 ClpAP complex contrasted with the open channel observed in the calculated section. This is explained because the first 8–12 N-terminal residues of the ClpP protomers are not present in the crystal structure, preventing the authors (Effantin et al., 2010) from concluding whether the conformation of the N-terminal regions of ClpP in the closed axial channel have any resemblance to the observed ‘down’ conformation in the crystal structure.

The described cryo-EM reconstructions (Effantin et al., 2010) also provided some insights regarding the interactions between ClpP and ClpA in the 1:1 and 2:1 complexes. The ClpA-bound ends of ClpP showed seven fingers of density protruding from its surface, which were peripherally located at a radius of ~30 Å from the symmetry axis (Fig. 3C, left panel). It was proposed that they represent the high-affinity contacts that the IGL motifs of the ATPase establish with the hydrophobic pockets in the apical surfaces of ClpP. This feature of the cryo-EM reconstruction provided a structural confirmation to previous biochemical work (Kim et al., 2001; Martin et al., 2007) regarding the mechanisms of interaction between ClpP and its cognate ATPases. However, it is important to note that this feature of the cryo-EM map is to some extent artifactual. The 6:7 symmetry mismatch between ClpA and ClpP and the 7-fold symmetry imposed during the reconstruction process caused the structural details at the interface between the two proteins to be erased or distorted. This explains the presence of seven fingers of density in spite of only six IGL motifs existing in the ClpA hexameric ring. In addition, it would also explain why the dynamic interactions between the N-terminal region of ClpP and the pore-2 loops of the ATPase described biochemically (Martin et al., 2007) are not observed in these cryo-EM reconstructions, since they are erased during the 7-fold symmetrization of the 3D reconstructions (Figs. 3 and 4A).

This cryo-EM work (Effantin et al., 2010) elegantly showed that binding of ClpA or ClpX causes the opening of the axial channel. However, it was not possible to establish from these structures whether this conformation was triggered by the peripheral interactions between the two proteins (IGL loop-mediated) or if it was caused by axial interactions (mediated by the N-terminal regions of ClpP and the axis loops in ClpA). Similarly, it was not possible to determine whether the open conformation of the axial channel is caused by either all or a specific number of the N-terminal regions of ClpP adopting an ‘up’ conformation. It is probable that the open state of the axial pore has some resemblance to the ‘up’ conformation observed in the asymmetric crystal structures of ClpP (Bewley et al., 2006). However, it is most likely not identical. Finally, because of the limited resolution of the 3D reconstructions (Effantin et al., 2010), these cryo-EM maps do not provide information on the important amino acids in the N-terminal region of ClpP that are involved in stabilizing the open or closed conformation.

6. Structures of ClpP in complex with small molecules provides atomic resolution details on the open conformation

Recent structural work (Lee et al., 2010a; Li et al., 2010) with acyldepsipeptide (ADEP) antibiotics that bind ClpP and mimic interactions of the IGL/L loops of the ATPases with the protease offer additional insights into the gating mechanism of the enzyme.
Crystal structures of ClpP in complex with these small molecules provide atomic resolution details of the open conformation and clues about the essential amino acids that may stabilize the N-terminal region of the protein in this configuration.

ADEF antibiotics are produced by *Streptomyces hawaiiensis* and target ClpP in bacteria (Brotz-Oesterhelt et al., 2005). The binding of ADEF to ClpP causes the enzyme to degrade unfolded proteins independently from its cognate ATPase, ClpA or ClpX (Kirstein et al., 2009). *In vivo*, ClpP redirects its activity away from its physiological substrates to target nascent polypeptide chains (Kirstein et al., 2009) and other proteins, including the essential cell division protein FtsZ (Sass et al., 2011). This causes the cell division process to stall and bacterial cells to die.

Crystal structures of ClpP in complex with ADEF compounds (Lee et al., 2010a; Li et al., 2010) determined that ADEF compounds bind to the same hydrophobic pockets at the outer edge of the ClpP rings where the IGF/L loops of ClpA and ClpX bind to form the ClpAP or ClpXP complex (Figs. 1D and 5A). Interestingly, one of these crystal structures (Li et al., 2010) showed that binding of ADEF1 (the parent ADEF compound purified from *S. hawaiiensis*) to *E. coli* ClpP stabilizes the N-terminal region, locking this region in a β-hairpin conformation (Fig. 5B, left panel). This conformational change creates a structured 20 Å diameter axial pore allowing entry of large substrates (Fig. 5A, left panel). Stabilization of the N-terminal loop seems to be mediated by interactions between four charged residues. First, Glu8 latches the β-hairpin at the N-terminal to the globular domain of ClpP through polar interactions with the side-chain of Lys25 in helix A (Fig. 6A) retracting the loop from the axial lumen. In addition, intermolecular interactions mainly between Glu14 and Arg15 staple the N-terminal loops from
adjacent protomers and create a rigid rim for the axial pore (Fig. 6B).

In contrast, a different group reported the structures of B. subtilis ClpP in complex with ADEP1 and ADEP2 (an optimized ADEP congener) and proposed a very different mechanism for the ADEP-mediated activation of ClpP (Lee et al., 2010a). The activation model proposed from this structure involves mainly the three hydrophobic side chains from Pro4, Val6 and Ile19 in each protomer, which interact with the side chains of Leu24' and Phe49' in the adjacent subunit. In the absence of ADEP1 or ADEP2, these five residues form a hydrophobic cluster (Fig. 6C) that stabilize each N-terminal region in the axial channel into a closed conformation that restrict passage of all but the smallest peptides into the digestion chamber of ClpP (Lee et al., 2010b). However, binding of ADEP1 or ADEP2 to B. subtilis ClpP initiates a structural change right on the binding site that is transmitted to the hydrophobic cluster region weakening these interactions (Fig. 6D). Ultimately, loosening these stabilizing interactions increases the flexibility of the N-terminal regions, which become invisible in the crystal structure (Fig. 5B, right panel). This event opens the axial channel facilitating the passage of substrate into the ClpP chamber (Fig. 5A, right panel).

Both models agree in that opening of this axial channel is what facilitates the passage of the substrate into the ClpP degradation chamber for degradation. However, whether the opened axial pore is "structured" (Fig. 5A, left panel) or "unstructured" (Fig. 5A, right panel) constitutes a major point of discrepancy between the two models. In spite of this controversy, the finding that ADEP1 and ADEP2 mimic the interaction of ClpP with its cognate ATPases, makes it likely that these structures provide snapshots at atomic resolution of the open conformation of ClpP bound to a Clp ATPase.

A recent high-throughput screening of compounds (Leung et al., 2011) found five additional compounds that also activate ClpP to degrade unfolded protein substrates in the absence of Clp ATPase. These compounds were generically named Activators of Self-Compartmentalizing Proteases (ACP). Although structurally different from the ADEP compounds, the existing biochemical data suggest that these compounds bind to the same hydrophobic pockets occupied by the ADEP molecules, as well as to an additional pocket, the C pocket. This second binding pocket is more charged than the hydrophobic pocket and it is located between ClpP protomers. Residues Y90, M94, Q95, D100, V101 and H170 of one ClpP protomer and residues H205 and N207 of the neighboring protomer constitute this pocket. X-ray structures of ClpP with bound ACP compounds are still not available. However, the dissimilar chemical structure of the ACP compounds compared to ADEP compounds and their possible binding in a different location of the ClpP tetradecamer (C pocket), makes these small molecules highly likely to provide additional structural insights into the gating mechanisms of ClpP.

7. Does the N-terminal region of ClpP in E. coli and B. subtilis adopt a different conformation in the open state of the axial channel?

An important concern when considering the ADEP-bound ClpP X-ray structures as possible models describing the open conformation of the N-terminal regions of ClpP in the context of the ClpAP or ClpXP complexes is to determine whether the observed conformation of the N-terminal loops is induced by crystal packing or binding of ADEP, as only the latter would mimic the interaction with the ATPases.

The crystal packing of the E. coli ClpP in complex with ADEP1 (Li et al., 2010) shows that the N-terminal regions of ClpP adopt a structured β-hairpin conformation upon interaction with the antibiotic (Fig. 5B, left panel). However, analysis of all of the N-terminal loops in the two tetradecamers of the asymmetric unit reveals that this conformation is adopted only when the N-terminal region is not engaged in crystal contacts (Fig. 7A), whereas they are disordered in protomers that have close packing contacts in this region (Fig. 7B). In contrast, in the crystal structures of B. subtilis ClpP in complex with ADEP1 or ADEP2 (Lee et al., 2010a) there is virtually no space left between the apical surfaces of adjacent tetradecamers in the B. subtilis ClpP structure (Fig. 7C). These surfaces establish multiple crystal contacts. Therefore, it is plausible that in this structure the tight packing in the crystals of B. subtilis ClpP may be preventing the N-terminal loops from forming a structured β-hairpin upon interaction with ADEP1 or ADEP2. Failing to adopt this conformation, the N-terminal region of B. subtilis ClpP in complex with ADEP1 or ADEP2 is delocalized. Therefore, it is possible that the increased flexibility of the N-terminal region of

![Figure 7](image-url)
B. subtilis ClpP observed in the presence of ADEP compounds, presumed to mediate the activation of ClpP, might be a consequence of crystal packing rather than ADEP binding. Nevertheless, it has not been formally ruled out that the open conformation of the axial channel in E. coli and B. subtilis may be different. However, this is unlikely because the high homology between the two proteins (82%) and in particular the 68% identity existing for their first 30 N-terminal residues.

In addition, analysis in the structure of E. coli ClpP in complex with ADEP1 (Li et al., 2010) of the side chains of Pro4, Val6, Ile19, Leu24 and Phe49 in adjacent protomers of ClpP showed that these residues are still forming the hydrophobic cluster that stabilizes the N-terminal regions in the axial channel into a closed conformation in free ClpP (Fig. 6E) (Lee et al., 2010b). Measuring the distances between the Cβ atoms of amino acids forming the hydrophobic cluster showed that the placement of their side chains is almost identical between structures of free ClpP and the structures of B. subtilis and E. coli ClpP in the presence of ADEP compounds (Fig. 6F and Table 1). Therefore, it is clear that at least in E. coli ClpP, the open conformation of the axial channel that allows substrate translocation coexists with properly formed hydrophobic clusters. Biochemical data has shown that these clusters are necessary to restrict the passage of substrates in free ClpP and to stabilize the closed state of the axial channel. However, in light of the existing evidence, it seems that the most important event in opening the axial pore is retraction of the N-terminal regions of ClpP from the lumen by formation of a defined β-hairpin conformation (Li et al., 2010) rather than the disruption of the hydrophobic clusters.

In any case, further studies must determine whether the structured β-hairpin conformation adopted by the N-terminal regions of E. coli ClpP in the presence of ADEP or the unstructured configuration observed in B. subtilis ClpP bound to ADEP have any resemblance to the open conformation of ClpP in the ClpAP and ClpXP complexes.

8. Comparison of the structures of ClpP bound to ADEP and to ClpA suggests that the open axial pore is ‘structured’

To determine the level of agreement between the X-ray structures of the E. coli and B. subtilis ClpP in complex with ADEP compounds (Lee et al., 2010a; Li et al., 2010) and the cryo-EM structure of ClpP bound to ClpA (Effantin et al., 2010), we viewed gray scale sections of the cryo-EM map of ClpP with ClpA bound at both apical surfaces and compared those to similar sections of the crystal structures limited to the resolution of the cryo-EM 3D reconstruction. Overall, the agreement of the two crystal structures with the cryo-EM structure is good (Fig. 4A, left panel and Fig. 4C). However, the 12 Å diameter axial pores surrounded by a sleeve of density observed in the section of the cryo-EM map (Fig. 4A, left panel) agrees better with the section produced from the E. coli ClpP in complex with ADEP (Fig. 4C, left panel). In the case of E. coli ClpP bound to ADEP1, an open axial pore surrounded by a sleeve of density was also observed but a wider channel, ~21 Å across was seen. The section produced from B. subtilis ClpP in complex with ADEP2 also showed an open axial channel of a diameter of ~27 Å; however the density sleeve surrounding the pore was absent (Fig. 4C, right panel). It follows that the N-terminal regions of ClpP in complex with ClpA (Effantin et al., 2010) probably adopt a structured conformation that more resembles the configuration observed in the structure of the E. coli ClpP in complex with ADEP1 (Li et al., 2010) than the one observed in the equivalent complex from B. subtilis. The cryo-EM structure of ClpP bound to ClpA was produced with E. coli protein. Therefore, whether the discrepancy between this structure and the structure from B. subtilis is indicative of differing conformations between species must still be determined.

Finally, an interesting observation we made when comparing the sections of the cryo-EM structure of ClpP bound to ClpA to the X-ray structures of the E. coli ClpP in complex with ADEP1 (Li et al., 2010) was that the diameter of the axial channel in the ClpP + ADEP structure was wider (~21 Å) than in the ClpA bound ClpP (~12 Å) (Fig. 4A, left panel and Fig. 4C, left panel). This observation may suggest that the diameter of the axial channel is somewhat variable and that in the ClpAP complex the ATPase limit the diameter of the induced open pore. Alternatively, ClpP may be able to change the diameter of its axial pore to adapt the translocation of substrates of different dimensions.

9. Conclusion

ClpP is the paradigm of self-compartmentalized proteases and the principal degradation machine inside the cell. Many aspects of the bacterial physiology require proper regulation and function of ClpP. The available crystal and cryo-EM structures of ClpP alone and in complex with small molecules, such as the ADEP antibiotics, provide a partial description of how ClpP regulates the entry of substrates into the degradation chamber. Recent high-throughput screens have found additional compounds that activate the protease activity of ClpP (Leung et al., 2011). These compounds are structurally distinct from ADEP antibiotics and provide exciting new tools to investigate the ClpP gating mechanisms.

In conclusion, the current knowledge offer an initial framework for further structural and biochemical studies to unveil the gating mechanism that regulates the access of substrates to the inner cavity of ClpP. Understanding how this process is regulated is essential to control ClpP activity and ultimately to use this enzyme as an antimicrobial target.

Acknowledgments

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Table 1

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