Published in final edited form as:

J Struct Biol. 2009 February ; 165(2): 118–125. doi:10.1016/j.jsb.2008.10.005.

Turned on for degradation: ATPase-independent degradation by ClpP

Maria C. Bewley^a, Vito Graziano^b, Kathleen Griffin^a, and John M. Flanagan^{a,*}

^aDepartment of Biochemistry and Molecular Biology, Pennsylvania State University College of Medicine, Hershey, PA 17033, USA

^bBiology Department, Brookhaven National Laboratory, Upton, NY 11973, USA

Abstract

Clp is a barrel-shaped hetero-oligomeric ATP-dependent protease comprising a hexameric ATPase (ClpX or ClpA) that unfolds protein substrates and translocates them into the central chamber of the tetradecameric proteolytic component (ClpP) where they are degraded processively to short peptides. Chamber access is controlled by the N-terminal 20 residues (for *Escherichia coli*) in ClpP that prevent entry of large polypeptides in the absence of the ATPase subunits and ATP hydrolysis. Remarkably, removal of 10–17 residues from the mature N-terminus allows processive degradation of a large model unfolded substrate to short peptides without the ATPase subunit or ATP hydrolysis; removal of 14 residues is maximal for activation. Furthermore, since the product size distribution of $\Delta 14$ -ClpP is identical to ClpAP and ClpXP, the ATPases do not play an essential role in determining this distribution. Comparison of the structures of $\Delta 14$ -ClpP and $\Delta 17$ -ClpP with other published structures shows R15 and S16 are labile and that residue 17 can adopt a range of rotomers to ensure protection of a hydrophobic pocket formed by I19, R24 and F49 and maintain a hydrophilic character of the pore.

Keywords

ATP-independent degradation; Clp; ClpP; Substrate entry

1. Introduction

ClpP is the proteolytic component of the ATP-dependent protease machine and an architectural and functional homolog of the 26S proteasome. The ClpP oligomer is comprised of 14 identical subunits arranged into two, stacked, heptameric-rings (Flanagan et al., 1995; Kessel et al., 1996; Maurizi et al., 1990a,b; Wang et al., 1997; 1998). Alone, the assembled ClpP exhibits weak peptidase activity against short peptide substrates (<20 residues) but no intrinsic proteolytic activity. Degradation of folded proteins and larger peptide substrates requires binding of the hexameric ATPases, ClpA or ClpX, to activate proteolysis (Thompson and Maurizi, 1994; Thompson et al., 1994; Wickner et al., 1994). In the functional ClpAP and ClpXP complexes, the ATPase binds the protease at each end to give a barrel-shaped proteolytic complex (Kessel et al., 1995). ClpA and ClpX unfold and translocate protein substrates during degradation and confer different substrate specificities on ClpP (Neuwald et al., 1999; Patel and Latterich, 1998; Reid et al., 2001; Weber-Ban et

* Corresponding author. Fax: +1 717 531 7072. jmf27@psu.edu (J.M. Flanagan).

Appendix A. Supplementary data: Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsb.2008.10.005.

al., 1999). Protein substrates initially bind to the ATPase subunits in an ATP-hydrolysis-independent manner near the distal ends of the proteolytic complex. Once stably bound, they are unfolded and translocated through a narrow, central, substrate channel that runs the length of the hexameric ATPase before they enter ClpP. These two steps are energy-dependent and require multiple rounds of ATP hydrolysis (Hoskins et al., 2000b; 2000a,b; Ishikawa et al., 2001; Kim et al., 2000; Ortega et al., 2000; Reid et al., 2001; Singh et al., 2000; Weber-Ban et al., 1999). Substrates delivered to ClpP are hydrolyzed in an energy-independent manner, in a large (~50 Å in diameter), spherical, proteolytic chamber that houses the 14 active sites (Wang et al., 1997; 1998).

In the current structure-based model, protein substrates entering the proteolytic chamber must pass through one of two small (~12 Å) axial access/egress pores in ClpP followed by movement down a ~15 Å channel/pore lining formed by a subset of the first 20 residues of the ClpP subunits (Wang et al., 1997, 1998). The small diameter of these pores dictate that protein substrates must be in an unfolded, largely extended conformation to access the proteolytic chamber. In the ClpAP and ClpXP complexes, these pores are aligned with the central substrate channels in the ATPases providing a direct and continuous path through the entire proteolytic complex (Kessel et al., 1995). The detailed mechanism by which these larger substrates are efficiently shuttled through the set of pores in Clp is currently unknown. However, for the ATPase component, substrate translocation is thought to involve ATP-hydrolysis-driven conformational changes in ClpA/ClpX. By contrast, since ClpP does not possess an intrinsic ATPase activity, substrates continuing through its pores must either passively diffuse down the channel to the proteolytic chamber, or more likely, be actively translocated by conformational changes within ClpP. We and others have shown that a subset of the first 20 residues (N-terminal region) forms an antiparallel β -sheet that creates the axial pore and channel/pore lining and that some of these residues are essential in stabilizing the ClpAP and ClpXP complexes (Bewley et al., 2006; Gribun et al., 2005; Kang et al., 2004). Thus, they are ideally positioned to assist in substrate entry into ClpP. We observed two general conformations (up and down) in the structure of wt-ClpP that differ in the location of the turn between the β -strands and proposed a model for ATPase gated substrate translocation based on the observation that the conformation of this region in the free ClpP oligomer is relatively plastic and can adopt a range of discrete conformations that alter the size of the axial pore and channel/pore lining (Bewley et al., 2006).

We wondered which secondary structure elements were more important in substrate entry. Accordingly, in this study, we constructed a series of N-terminal deletion mutants based on the observed positions of the secondary structure elements to explore their roles in substrate entry and characterized them biochemically and crystallographically. We show that the ATPase components are not an absolute requirement for substrate entry into ClpP. Our results suggest that conformational changes in this region facilitate the gated access and processive degradation of large unfolded substrates that have been delivered by the ATPase. This idea is consistent with the model proposed for the role of the N-terminal 12 amino acids in the α -subunits of the 20S proteasome in its 26S complex (Benaroudj et al., 2003).

2. Methods and materials

Unless noted, nucleotides were purchased Boehringer Mannheim and all other chemicals from Sigma. *Escherichia coli* ClpP, ClpA and ClpX proteins were purified by modifying standard procedures (Banecki et al., 2001; Bewley et al., 2006; Grimaud et al., 1998; Singh et al., 2001). Unlabeled α -casein and the BODIPY- α -casein-FL conjugate were obtained from Pierce and Molecular Probes (E-6658), respectively.

2.1. N-terminal deletion variants

$\Delta 4$ -ClpP and $\Delta 6$ -ClpP are the result of autoproteolytic degradation of P4A-ClpP and V6A-ClpP single site variants (Bewley et al., 2006). The $\Delta 10$, $\Delta 14$, $\Delta 17$ and $\Delta 24$ constructs were generated by amplification (PCR) using primers that introduce an *Nde I* site inframe into the first codon of each construct and a *BamHI* site after the termination codon. The amplified DNA was cloned into the expression plasmid, pET-9a, under the control of the T7 promoter. Each variant had the initiator methionine as determined by MAL-DI-TOF spectrometry and N-terminal analysis. The active site substituted $\Delta 14$ -ClpP+S97A and $\Delta 14$ -ClpP+H122A variants were constructed by site directed mutagenesis using the Quickchange kit (Stratagene) and expressed and purified as previously (Bewley et al., 2006).

2.2. Tetradecamer assembly and ClpAP complex formation

Analytical size exclusion chromatography (Superdex-200) was used to assess the oligomeric state of each construct (Bewley et al., 2006). The ability of each ClpP variant (wild type, $\Delta 4$ -, $\Delta 6$ -, $\Delta 10$ -, $\Delta 14$ -, and $\Delta 17$ -ClpP) to form stable complexes with wild type ClpA₆ was examined by light scattering as described (Singh et al., 1999).

2.3. ATPase assay

ATPase activity of ClpA was measured by determining the free phosphate liberated during the reaction with a continuous spectrophotometric-based assay (Webb, 1992) using Molecular Probes, E6646. Reactions were conducted in 96-well microtiter plates using our standard ATPase assay buffer (25 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM MgCl₂, 2 mM ATP and the appropriate reagents from the EnzCheck phosphate assay kit) and, when indicated, ClpP or the appropriated deletion construct as discussed previously.

2.4. Peptidase and protease assays

The ClpA and ATP-independent peptidase activity of each ClpP construct was measured in an absorbance-based assay using the fluorogenic peptide substrate, suc-LY-AMC (Bewley et al., 2006). Degradation of α -casein was measured by determining the change in fluorescence anisotropy of BODIPY-labeled α -casein during degradation. Standard ClpA and ATP-dependent α -casein degradation assays used a buffer containing 80 mM Tris-HCl, pH 8.0, 21 mM KCl, 10 mM MgCl₂, 1 mM DTT, 1 mM EDTA, 4 mM ATP, 100 μ g/mL α -casein (1 μ g/mL BODIPY α -casein-FL) and 100 nM ClpP₁₄ or deletion variant. For Michaelis-Menten kinetic analysis of wt-ClpAP, the α -casein concentration is given (Supplementary Fig. 3). For ATP-independent degradation experiments, $\Delta 14$ -ClpP was used at between 10 and 500 nM and the substrate concentrations were varied from 10 to 1000 μ g/mL in buffer containing 100 mM Tris-HCl pH 8.0, 0.05% Tween-20. The degradation of oxidized insulin β -chain, glucagon, and GFP-SsrA were carried out using published procedures (Thompson and Maurizi, 1994; Thompson et al., 1994; Weber-Ban et al., 1999).

2.5. Inactivation with covalent serine protease inhibitors

Wt-ClpP and the various deletion variants (2 mg/ml) were incubated alone (control) or with DFP (5 mM), suc-LY-CMK (2 mM) or PMSF (1 mM) at 25 °C for 1 h, followed by addition of a second aliquot of the appropriate inhibitor (Final concentrations 10 mM DFP, 4 mM suc-LY-CMK, 2 mM PMSF) and further incubation for 30 mins. The reaction was terminated by addition of one volume of isopropanol (−20 °C) followed by centrifugation (14,000g) at 4 °C. The pellets were resuspended in 100 μ L 25 mM Tris-HCl, pH 7.5, 200 mM NaCl and 0.1 mM EDTA and ClpP purified by analytical size exclusion chromatography (Superdex 200). The extent of covalent modification was determined by MALDI-TOF mass spectrometry and reverse phase (RP) chromatography. For reactions containing DFP and suc-LY-CMK, >95% of the ClpP proteins were modified with the

diisopropyl phosphate adduct (ΔM_r 164 $\pm$ 3 Da) and suc-LY-methyl ketone adduct (ΔM_r 427 $\pm$ 4 Da) while PMSF treatment of did not alter the mass obtained by MALI-TOF spectrometry or retention on a C18 RP column of any of the ClpP variants tested. Each sample was then assayed for peptidase and protease activity using the appropriate standard assay conditions.

2.6. Structure determination

Crystals of Δ 14-ClpP and Δ 17-ClpP were grown in sitting drops from 50% MPD, in 100 mM sodium citrate at pH 6.4 and protein at 10–15 mg/ml in 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA and 10 mM Tris-HCl at pH 7.5. Data collected at 99 K from single crystals on X12B, NSLS, NY using a Quantum 4 cell CCD detector were processed using HKL (Table 2) (Otwinowski and Minor, 1997). For Δ 14-ClpP, despite having angles that are close to 90°, the data only process in space group P1. The lack of symmetry was confirmed by inspection of diffraction images.

In each case, residues 20–193 from 1YG6 were used as a starting model (Bewley et al., 2006). All refinement steps used CNS (Brunger et al., 1998). For Δ 14-ClpP, rigid body refinement was followed by a simulated annealing protocol to 3000 K prior to initial map calculation. 56-fold, 14-fold averaged and unaveraged $2F_o - F_c$ and $F_o - F_c$ electron density maps were calculated. Initially NCS constraints and subsequently NCS restraints were applied throughout the refinement, as judged appropriate by monitoring the behavior of R_{free} . Refinement, punctuated by rounds of model building, was performed until the model could not be improved as judged by a reduction in R_{free} . The current model contains residues 17–193 for all monomers (Table 2). For Δ 17-ClpP, rigid body refinement was followed by torsion angle refinement at 5000 K. The free reflections were excluded in shells to prevent the effect of NCS-related reflections artificially lowering the R_{free} . The use of NCS constraints did not result in a substantial lowering of R_{free} , whereas use of very tight NCS restraints did. The current model contains residues 17–193 for all 14 monomers (Table 2).

3. Results and discussion

3.1. ATP- and ATPase-independent degradation of α -casein by ClpP

To define the roles of the N-terminal region in ClpP, a series of N-terminally truncated ClpP variants (deletion variants) were constructed lacking 4 (Δ 4), 6 (Δ 6), 10 (Δ 10), 14 (Δ 14), 17 (Δ 17) or 24 (Δ 24) residues from the mature amino terminus, respectively, based their location in the structure (Fig. 1a). In the structure, the first 20 residues fold into two broad conformations, termed up and down, respectively. In each case, an antiparallel two-stranded β -sheet form and ‘up’ and ‘down’ are determined by the location of the turn. For the up conformation, removal of six residues eliminates upto the first β -strand, 14 eliminates this strand, 17 removes the second β -strand and 24 removes the entire N-terminus and the first helix. Δ 4-ClpP was selected based upon its behavior in the previous assays (Bewley et al., 2006) and Δ 10-ClpP was selected as it was the residue closest to the N-terminus most often visible in the down conformation. With the exception of Δ 24, all of the remaining variants were expressed at high levels in a soluble form, assembled into tetradecamers and exhibited peptidase activity similar to wild type (Supplementary Fig. 1). This is in contrast to the observations of Kang and coworkers who report that for C-terminally His-tagged human ClpP, deletions beyond Leu2 exhibit low peptidase activity (Kang et al., 2004). This difference could possibly be accounted for by differences in oligomer stability between human and *E. coli* ClpP (Kang et al., 2005). To test whether any of the *E. coli* deletion variants could cooperate with ClpA to degrade α -casein, a 27 kDa model unfolded substrate, we incubated 12.5 nM ClpA₆ with increasing concentrations of wt-ClpP, Δ 4-, Δ 6-, Δ 10-,

$\Delta 14$ - and $\Delta 17$ -ClpP in the presence of 5 mM ATP (Fig. 1b). As expected, ClpA and wt-ClpP associate in the presence of ATP ($K_{app} < 2$ nM) to form active ClpAP complexes that rapidly degrade α -casein with a k_{cat} of ~ 10.5 min⁻¹. Neither $\Delta 4$ -ClpP nor $\Delta 6$ -ClpP supported this activity, however, $\Delta 14$ -ClpP, and to a much lesser extent $\Delta 10$ -ClpP and $\Delta 17$ -ClpP, degraded this substrate. Interestingly, the rate of degradation with the $\Delta 14$ -ClpP alone increased linearly over the concentration range tested, suggesting that either it forms low affinity complexes with ClpA or that $\Delta 14$ -ClpP alone is the active species. To test whether the deletion variants form active complexes with ClpA, we examined whether they stimulated the basal ATPase activity of ClpA (Fig. 1c). Wt-ClpP stimulates this activity by ~ 3.5 -fold with K_{app} of 20 nM. The increased K_{app} compared with α -casein degradation is consistent with previous reports that substrate binding at the active site of ClpP enhances its affinity for ClpX and ClpA (Joshi et al., 2004). By contrast, none of these deletion variants affected ClpA ATPase, even when present at 400 nM, suggesting that they do not associate with ClpA. Furthermore, stable ClpAP complexes were not observed with any of the deletion variants in light scattering experiments (Supplementary Fig. 2). These results suggest that $\Delta 10$ -ClpP, $\Delta 14$ -ClpP and $\Delta 17$ -ClpP may degrade α -casein by a ClpA-independent mechanism.

Having established that the deletion variants do not physically interact (at least strongly) with ClpA, we sought to determine whether ClpA or any co-purifying ATPase contributed to the observed degradation of α -casein by the three deletion variants. The rate of α -casein degradation by wt-ClpP and $\Delta 14$ -ClpP was measured with and without added ClpA and ATP (Supplementary Fig. 3). As expected, degradation of this substrate by wt-ClpP is dependent on ClpA and hydrolyzable ATP. By contrast, these factors had no effect on degradation by $\Delta 14$ -ClpP (degradation by $\Delta 10$ - and $\Delta 17$ -ClpP were also ClpA-independent (data not shown)). Further, ATP γ S, ADP and EDTA, which are non-covalent inhibitors of many ATPases including ClpA and ClpX, had no measurable effect on degradation by $\Delta 10$ -, $\Delta 14$ - and $\Delta 17$ -ClpP but strongly inhibited wt-ClpAP (Table 1). Moreover α -casein degradation by $\Delta 10$ -, $\Delta 14$ - and $\Delta 17$ -ClpP is an intrinsic property of these proteases, since the serine protease inhibitors DFP and suc-LY-CMK form covalent adducts with active site residues and block degradation (Table 1). Mutation of the active site serine (S97) or histidine (H122) to alanine abolishes degradation by highly purified $\Delta 14$ -ClpP-S97A and $\Delta 14$ -ClpP-H122A. Taken together, these results indicate that removal of 10–17 residues from the N-terminus of *E. coli* ClpP activates it for degradation of a large unfolded protein substrate in the absence of its cognate ATPase.

3.2. Rapid degradation of α -casein by $\Delta 14$ -ClpP

A more detailed kinetic analysis was performed to further characterize the ATPase- and ATP-independent reaction catalyzed by $\Delta 10$ -, $\Delta 14$ - and $\Delta 17$ -ClpP. Degradation of α -casein with ClpAP and ATP follows Michaelis-Menten kinetics with a K_m of 0.1 ± 0.05 μ M and a k_{cat} of $\sim 10.5 \pm 0.1$ min⁻¹ (data not shown). Consistent with a role for ClpA in substrate binding, initial experiments with $\Delta 14$ -ClpP suggest that the K_m for ClpA-independent degradation of α -casein is at least 10-fold higher with wt-ClpAP. $\Delta 14$ -ClpP degraded α -casein with a K_m of 1.8 ± 0.1 μ M, and a k_{cat} of 8.2 ± 0.1 min⁻¹ in low salt buffer (Fig. 1d). The large increase in K_m for degradation with $\Delta 14$ -ClpP is consistent with a role for the ATPase in substrate targeting. Remarkably, the k_{cat} for this reaction is similar to that for the ATP-dependent reaction with ClpAP indicating that active translocation of large unfolded substrates is not essential for this rapid entry and hydrolysis by ClpP. In this regard, the ATPase may be required to stabilize a translocation-competent conformation of the protease.

The activities of $\Delta 10$ - and $\Delta 17$ -ClpP were considerably lower than $\Delta 14$ -ClpP for this substrate and, due to their high K_m (>10 μ M) and low k_{cat} (<0.5 – 1 min⁻¹) values, the kinetic parameters could not be estimated reliably. However, based upon their relative activity with

1 mg/ml α -casein, $\Delta 10$ - and $\Delta 17$ -ClpP have between 5% and 10% activities compared to $\Delta 14$ -ClpP for this substrate. These data suggest that activation is not just a consequence of opening the pore since $\Delta 17$ -ClpP, which would have the largest pore, is inactive.

3.3. ATP-independent degradation by $\Delta 14$ -ClpP is processive

Complete digestion of α -casein with either $\Delta 14$ -ClpP alone or ClpAP and ATP together results in the production of short peptide products, as judged by the limiting anisotropy of these reactions. However, a hallmark of ATP-dependent degradation by ClpAP is that it occurs processively: substrates engaged by this protease are completely degraded to short (3–15) residue peptides during a single cycle of interaction with the enzyme (Thompson et al., 1994). By contrast, Goldberg demonstrated that wt-ClpP can cooperate with GroEL to degrade a model misfolded substrate, CRAG, via a non-processive mechanism involving multiple rounds of association and cleavage by wt-ClpP followed by rebinding and releases by GroEL (Kandror et al., 1994). To test whether degradation of α -casein by $\Delta 14$ -ClpP occurs processively, like ClpAP, or sequentially, as in the GroEL-dependent reaction, we analyzed the time course of degradation by reverse phase chromatography (Fig. 2a). The rate of accumulation of most products, as judged by a quantitative analysis of the changes in peak heights, was similar and each accumulated until nearly all of the substrate was depleted. The same pattern of product peaks was observed even when the substrate was present at >1000 fold excess over $\Delta 14$ -ClpP. The majority of products were small peptides and no significant accumulation of higher molecular weight products appeared, as judged by SDS-PAGE (data not shown). A small fraction of the α -casein (<5%) was cleaved by $\Delta 14$ -ClpP to yield a ~14kDa product that accumulated slowly during degradation and persisted after nearly all of the α -casein had been degraded. A similar sized product was also observed with ATP-dependent degradation by ClpAP in our studies and in a previous report (Thompson and Maurizi, 1994). This product does not appear to be an intermediate and, based upon our analysis, may correspond to a phosphorylated α -casein species, since phosphatase treatment reduces accumulation of this product (data not shown). If degradation had occurred by a non-processive mechanism, we would have seen large products released from the enzyme at early times followed at later times by smaller products, after the uncleaved substrate had become depleted.

Comparison of the chromatograms from complete digestion of α -casein degraded in an ATP/ATPase-independent manner by $\Delta 14$ -ClpP and ATP-dependent degradation by ClpAP suggest that both enzymes have a similar preference for peptide bonds in the substrate (Fig. 2b). To further explore this idea, MALDI-TOF/MS fingerprints were obtained for six of the common product peaks identified in the C_{18} reverse phase chromatograms from ATP-dependent degradation with ClpAP and ATP-independent degradation with $\Delta 14$ -ClpP (Fig. 2c). The strong correspondence between the masses of the peptides observed in both peaks is clearly evident; a similar level of correspondence was observed for the five other sample pairs (data not shown). These data demonstrate that $\Delta 14$ -ClpP degrades α -casein, processively, in an ATP/ATPase-independent fashion, with a similar pattern of cleavage site preferences to the ATP-dependent ClpAP. They also provide additional support for the model in which the ClpP architecture is sufficient for processive degradation of unfolded protein substrates (Thompson et al., 1994). Finally, they suggest that active translocation of protein substrates is not required for controlling the size of peptide products as previously proposed (Choi and Licht, 2005). Instead, even for relatively large unfolded substrates, once the substrate is engaged by the protease it is retained until it is fully degraded.

3.4. $\Delta 14$ -ClpP is activated for degradation of other model substrates

To further characterize the ability of the deletion variants to degrade ClpAP substrates, we examined their activity with two oligopeptide substrates, oxidized insulin β -chain and

glucagon (Thompson and Maurizi, 1994), and a folded substrate, GFP-SsrA (Singh et al., 2000). Oxidized β -insulin and glucagon are degraded rapidly by ClpAP but only very slowly by ClpP alone (~5% of wt-ClpP). $\Delta 17$ -, $\Delta 14$ - and $\Delta 10$ -ClpP degraded this substrate at varying levels. $\Delta 14$ -ClpP was the most active (500% of wt-ClpP, 25% of ClpAP), while $\Delta 10$ - and $\Delta 17$ -ClpP had 100% and 50% the activity of wt-ClpP, respectively, and $\Delta 4$ - and $\Delta 6$ -ClpP were inactive (Supplementary Fig. 4). Each active variant cleaved this substrate at the same sites as ClpAP and ClpP alone (Thompson and Maurizi, 1994). Similar results were obtained using glucagon as a substrate (data not shown). To test whether any of the deletion variants could degrade a folded substrate, we examined their activity on GFP-SsrA. This substrate cannot enter the proteolytic chamber of ClpP unaided and requires ATP-dependent unfolding by ClpA or ClpX for degradation (Kim et al., 2000; Singh et al., 2000). As expected, only wt-ClpAP degraded this substrate even when $\Delta 10$ -, $\Delta 14$ -, and $\Delta 17$ -ClpP were assayed in the presence of ClpA and ATP to generate a small pool of unfolded GFP-SsrA. The inability of the deletion variants to degrade GFP-SsrA, even when unfolded by ClpA, may be due to the low affinity of these variants for this substrate and/or the relatively low concentrations of unfolded GFP-SsrA that accumulates under these conditions.

The pattern of activity of the deletion variants ($\Delta 14$ -ClpP > $\Delta 10$ -ClpP > $\Delta 17$ -ClpP) for degradation of the oligopeptide substrates parallel their activity in α -casein degradation. The fact that only $\Delta 14$ -ClpP was more active in degrading the oligopeptide substrates than wt-ClpP suggests that the increased activity of $\Delta 14$ -ClpP is not simply the result of opening up the pore and may point toward an as yet undefined role for portions of the N-terminal region in facilitating substrate entry into ClpP. However, the inability of all of the variants to degrade folded GFP-SsrA reinforces the essential role of the ATPases in delivering folded substrates in an appropriately unfolded form to ClpP.

3.5. The structures of $\Delta 14$ -ClpP and $\Delta 17$ -ClpP pore character is conserved

To gain insight into how removing 14 residues from the N-terminus of ClpP activates it for ATPase-independent degradation of α -casein, we determined the structures of $\Delta 14$ -ClpP and $\Delta 17$ -ClpP and compared them to our structures of wt-ClpP and the V6A ($\Delta 6$ -ClpP) variant (Bewley et al., 2006). In the up conformation of wt-ClpP, the backbone atoms of F17 form hydrogen bonds to those of V6 and the side chain is sandwiched between those of S21 and I7. In the down conformation, the backbone atoms of F17 hydrogen bond to those of E14 whereas the side chain adopts a range of rotomers. This results in a closed pore in the up conformation and hydrophilic pore of ~12 Å in the down conformation with a 15 Å-long channel of mixed character. By contrast, in the structure of $\Delta 6$ -ClpP (Bewley et al., 2006), residues 15–17 are in a different conformation; R15 lies on the ATPase-binding surface, S16 forms mixed interactions with L24 and F49, and the side chain of F17 is oriented to the center of the pore, which is open (~15 Å diameter). Residues 7–14 are disordered (Bewley et al., 2006).

In the structure of $\Delta 14$ -ClpP, determined here at 2.6 Å resolution, the interactions thought to stabilize the up and down conformations observed in the structure of the wild type protein have been removed. The aromatic ring of F17 adopts a new position relative to the structures of the wt ClpP and $\Delta 6$ -ClpP described above, tucking into a hydrophobic pocket formed by the highly conserved residues I19 from its monomer and L24 and F49 from an adjacent monomer (Fig. 3). These residues are critical to ClpP function, since the variants I19A, L24A, F49A in wt-ClpP have normal peptidase activity but are unable to degrade substrate proteins or bind to ClpA ((Bewley et al., 2006) and MCB, JMF, unpublished observations). The pore, created by the backbone atoms of F17, is ~15 Å in diameter, and although density is not interpretable for residues 15–16, modeling their potential positions in an extended conformation would still result in an open pore (~5 Å) and a short channel. If an open pore is sufficient for α -casein degradation, then it would follow that the $\Delta 17$ -ClpP variant should

be similarly active, however, this was not the case. Indeed it is less active than wt-ClpP alone in degrading the oligopeptide substrates insulin and glucagon, suggesting that the first few residues in $\Delta 14$ -ClpP must play some role either through stabilizing incoming substrates or facilitating their entry in substrate degradation. In the structure of $\Delta 17$ -ClpP, determined here to 3.0 Å resolution, the removal of 17 authentic residues results in a pore that is ~15 Å in diameter. This construct begins with an initiator methionine immediately prior to the authentic E18; density for the side chain of the methionine is visible and it occupies a position similar to F17 in the structure of $\Delta 14$ -ClpP, in that it tucks into the hydrophobic pocket described above. In this case, the channel is somewhat shorter than observed in the structure of the wild type protein (~3–5 Å). The character of the pore is similar to that observed in both $\Delta 14$ -ClpP and wild-type ClpP.

So far, we have demonstrated that removal of between 10 and 17 residues from the N-terminal region of ClpP results in an oligomer that is able to degrade large model substrates in an ATP- and ATPase-independent manner. We have further found that this is not simply due to an increased pore size, since removal of 14 residues results in a faster rate of degradation of model substrate relative to removal of 17 residues. Comparison of structures of the wild type ClpP and $\Delta 6$ -ClpP, with those of $\Delta 14$ -ClpP and $\Delta 17$ -ClpP show differences in the position residue 17 and in the character of the pore and channel. Based on all biochemical and structural data, maintenance of a mixed character pore and the positioning of R15 on the surface of ClpP seem to be essential characteristics of the mechanism.

3.6. Is any of this kinetic and structural data relevant to the biological function of ClpP and the mechanism of substrate translocation?

In most orthologs, the N-terminal 20 amino acids are highly conserved. However, there are a few sequences, largely from bacteriophage, that have truncated N-termini relative to the consensus. Specifically, they lack the first 12–14 residues corresponding to the first strand in *E. coli* ClpP and thus, in sequence, look more like $\Delta 14$ -ClpP. Based upon sequence analysis of their N-terminus and the surrounding residues, these prohead maturation serine proteases from phage likely do not interact with a ClpA/X-like ATPase and due to their truncated amino terminus, may be constitutively active for their proteolytic substrates.

4. Conclusions

The studies described above demonstrate that the structural and architectural features of the ClpP oligomer are sufficient to promote entry of appropriately unfolded substrates into the proteolytic chamber and ensure their processive degradation to short peptides. In the context of the intact ClpAP and ClpXP proteases, the role of the ATPase component is likely to be in substrate recognition, unfolding and delivery to ClpP in a form suitable for degradation. The structures of $\Delta 14$ -ClpP and $\Delta 17$ -ClpP described here show a novel conformation for residue 17 that places it in a hydrophobic pocket formed by conserved residues I19, L24 and F49. Based on analysis of all of the structures, it would seem that preservation of the character of the pore and protection of the hydrophobic pocket has implications for ClpP function. The ATPase components also play a key regulatory role in activating ClpP, perhaps via a swinging gate mechanism to convert it into the state required for substrate access. However, all of the necessary elements required for activation, substrate entry and processive degradation are contained in ClpP itself. In addition, the novel conformation of the short N-termini in ClpP observed in the structure of $\Delta 14$ -ClpP may have important implications for the function of the “ClpP-like” prohead maturation proteases found in some bacteriophages. Our analysis suggests that they may exist in an activated state and thus not require an ATPase for function or that they serve some additional yet uncharacterized role.

Note added in proof: At the time of submission of this paper, two papers (Jennings et al., 2008a; Jennings et al., 2008b) appeared that describe the ability of ClpP to degrade substrate in the absence of ClpP and implicate the N-termini in substrate entry into ClpP. The results of our study are broadly in agreement with these, however, there are several key differences. First, $\Delta 14$ -ClpP degrades α -casein with a k_{cat} similar to that of ClpAP, while wt-ClpP alone is at least 2000 to 5000-fold slower under the conditions used in our study. Moreover, the high activity of $\Delta 14$ -ClpP, which lacks most of the N-terminal region and cannot reach into the active site is, on the face of it, at odds with the model proposed by Jennings and coworkers (Jennings et al., 2008a) that suggests that the N-terminus directly coordinates the proteolytic active sites. This discrepancy warrants further investigations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH RO1-GM57390 and the OBER US-DOE DE-AC0298-CH10886.

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Fig. 1.

Deletion of 10–17 residues from the N-terminus of ClpP activates the oligomer for ClpA- and ATP-independent degradation of α -casein. (a) Ribbon diagram showing the relative location of the deletions in the structure of the ClpP monomer as black spheres. The first 24 amino acid residues of the mature N-terminus are shown with the positions of the deletion variants highlighted in red lettering. The secondary structure elements of the up conformation are shown above the sequence and those for the down conformation below the sequence; solid red arrows represents β -strands and cylinders represent α -helices. (b) Ability of wt-ClpP and the N-terminal deletion variants to support α -casein degradation. Titration of ClpA₆ in the presence of ATP with wt-ClpP fits to a hyperbolic activity curve in which the proteolytically active species is a 1:1 ClpAP complex. Titrations with wt-ClpP alone, $\Delta 4$ - or $\Delta 6$ -ClpP do not degrade α -casein at a measurable level. $\Delta 14$ -ClpP, and to a much lesser extent $\Delta 10$ - and $\Delta 17$ -ClpP, support degradation but do not fit to a 1:1 stoichiometry with ClpA for the active species. (c) Only wt-ClpP stimulates the intrinsic basal ATPase activity of ClpA₆. Titration of ClpA₆ ATPase activity with increasing concentrations of wt-ClpP, $\Delta 4$ -, $\Delta 6$ -, $\Delta 10$ -, $\Delta 14$ - and $\Delta 17$ -ClpP. (d) Initial rate of degradation of α -casein with $\Delta 14$ -ClpP alone. In the ATP-dependent reaction buffer, α -casein tended to aggregate at high concentrations (70 fold higher anisotropy). This prevented

an accurate determination of K_m for the ATP-independent reaction by $\Delta 14$ -ClpP in this buffer. Eliminating the Mg^{2+} and KCl in the reaction buffer (low salt buffer) minimized aggregation allowing characterization of the $\Delta 14$ -ClpP.

Fig. 2.

ATPase-Independent degradation of α -casein with $\Delta 14$ -ClpP occurs processively and generates a similar spectrum of peptide products as ATP-dependent degradation by wt-ClpAP. (a) Time course of α -casein degradation with $\Delta 14$ -ClpP. (b) Reverse phase chromatograms of α -casein degradation products from overnight energy-dependent ClpAP digestion and energy-independent digestion by $\Delta 14$ -ClpP. *denote equivalent pairs of peaks. (c) Mass spectrum of one pair of peaks observed in the product profiles from both $\Delta 14$ -ClpP and ClpAP of this substrate.

Fig. 3.

The conformation of F17 in the structure of $\Delta 14$ -ClpP is different to that observed in the structure of the wild type protein. (a) $2F_o - F_c$ electron density map centered on F17 of chain A. The electron density map is shown as blue mesh, contoured at 1.0σ . The atoms, (ball-and-stick), are colored: carbon, yellow; oxygen, red; nitrogen, cyan. Residue labeling is red for chain A and black for adjacent chains. (b) Superposition of the structure of wild-type ClpP onto $\Delta 14$ -ClpP. Adjacent chains are colored blue and cyan for $\Delta 14$ -ClpP and red and magenta for wild-type ClpP. In the structure of the wild type protein, V6 occupies the hydrophobic pocket formed by F49, L24 and L23, which in the structure of $\Delta 14$ -ClpP, is occupied by the side chain of F17.

Table 1
Inhibitors of ClpA alter the protease activity of wt-ClpP but not the protease activity of the deletion variants.

ClpA inhibitors		ClpP inhibitors			
ATP- γ S ^a	EDTA	PMSF	suc-LY-CMK		DFP
Protease ^b	Protease ^b	Peptidase ^{b,c}	Protease	Peptidase	Protease
wt-ClpP	<1	11 $\pm$ 4	101 $\pm$ 3	97 $\pm$ 3	<1
Δ 10	95 $\pm$ 9	98 $\pm$ 8	98 $\pm$ 4	108 $\pm$ 9	<1
Δ 14	99 $\pm$ 4	100 $\pm$ 3	110 $\pm$ 6	102 $\pm$ 4	<1
Δ 17	91 $\pm$ 9	89 $\pm$ 9	93 $\pm$ 3	105 $\pm$ 6	<1

^a 5 mM ATP- γ S in the α -casein degradation assay and 12.5 nM ClpA6, 100 nM wt-ClpP, 100 nM Δ 14-ClpP, 500 nM Δ 10- or Δ 17-ClpP.

^b In triplicate. Activity: percent activity remaining relative to equivalent untreated samples.

^c 2 mM suc-LY-AMC.

Table 2

Data collection and refinement statistics.

	Δ14-ClpP	Δ17-ClpP
<i>Data collection</i>		
Space group	<i>P1</i>	<i>C2</i>
Cell <i>a</i> , <i>b</i> , <i>c</i> (Å)	94.3, 161.7, 183.3	151.8, 108.6, 173.3
<i>α</i> , <i>β</i> , <i>γ</i> (°)	90.1, 90.0, 90.0	90.0, 113.9, 90.0
Resolution (Å)	30.0–2.5 (2.6–2.5) ^a	30.0–3.0 (2.1–3.0)
R_{merge}^b	6.3 (21.6)	7.7 (19.5)
$\langle I/\sigma I \rangle$	11.7 (2.0)	8.8 (3.8)
Completeness (%)	90.4 (58.8)	88.7 (90.5)
Redundancy	1.9 (1.7)	2.9 (2.8)
<i>Refinement^c</i>		
No. reflections/free	338254/1365	42769/1725
$R_{\text{work}}/R_{\text{free}}$	23.7/28.2	24.0/26.3
No atoms: protein	78624	19264
Water	48 per monomer	0
<i>B</i> factor protein	19.8	31.97
Water	19.8	n/a
Rmsd bond length (Å)	0.013	0.011
Bond angle (°)	1.6	1.5

^aHighest resolution shell in parenthesis.^b $R_{\text{merge}} = R_{\text{merge}} = \sum ||I_{\text{obs}}| - |I_{\text{avg}}|| / \sum |I_{\text{avg}}|$.^cResolution as for data collection.