BIOCHEMISTRY

 Volume 47, Number 12 March 25, 2008

Current Topics

Resolving Individual Steps in the Operation of ATP-Dependent Proteolytic Molecular Machines: From Conformational Changes to Substrate Translocation and Processivity[†]

Stuart Licht*[,‡] and Irene Lee*[,§]

Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, and Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44106

Received January 5, 2008; Revised Manuscript Received February 11, 2008

ABSTRACT: Clp, Lon, and FtsH proteases are proteolytic molecular machines that use the free energy of ATP hydrolysis to unfold protein substrates and processively present them to protease active sites. Here we review recent biochemical and structural studies relevant to the mechanism of ATP-dependent processive proteolysis. Despite the significant structural differences among the Clp, Lon, and FtsH proteases, these enzymes share important mechanistic features. In these systems, mechanistic studies have provided evidence for ATP binding and hydrolysis-driven conformational changes that drive translocation of substrates, which has significant implications for the processive mechanism of proteolysis. These studies indicate that the nucleotide (ATP, ADP, or nonhydrolyzable ATP analogues) occupancy of the ATPase binding sites can influence the binding mode and/or binding affinity for protein substrates. A general mechanism is proposed in which the communication between ATPase active sites and protein substrate binding regions coordinates a processive cycle of substrate binding, translocation, proteolysis, and product release.

ATP-dependent proteases degrade abnormal or damaged proteins as well as short-lived regulatory proteins to maintain proper cellular function (*1*). On the basis of sequence homology, the ATP-dependent proteases are assigned to the AAA$^+$ (ATPases associated with a variety of cellular activities) family of proteins, the members of which are involved in processes that include DNA replication, transcription, membrane fusion, and proteolysis (*2*). These proteins all share a highly conserved ATPase module consisting of the Walker A and B motifs. Another common feature is the presence of sensor residues toward the C-terminus, which function to sense the presence of a γ-phosphate in the nucleotide and to catalyze nucleotide hydrolysis. The C-terminus also contains a domain proposed to interact with protein substrates, which has been termed the substrate sensor and discrimination (SSD) domain (*3*).

Like other AAA$^+$ proteins, ATP-dependent proteases are oligomeric enzymes which assemble into ring-like or barrel-like structures, forming a cavity within the center of the macromolecular structure (*4*). This central cavity is also known as the proteolytic chamber, where unfolded protein substrates are translcoated and subsequently degraded by the

[†] The work described in the Lee group was supported by a grant from the National Institutes of Health (GM067172). Work in the Licht group is supported in part by the Beckman Foundation (Beckman Young Investigator Award).

* Both authors contributed equally to the preparation of this paper; both authors are corresponding authors. S.L.: phone, (617) 452-3525; fax, (617) 258-7874; e-mail, lichts@mit.edu. I.L.: phone, (216) 368-6001; fax, (216) 368-3006; e-mail, Irene.Lee@case.edu.

[‡] Massachusetts Institute of Technology.

[§] Case Western Reserve University.

10.1021/bi800025g CCC: $40.75
Published on Web 03/01/2008

proteolytic site. Degradation of structured protein substrates requires the presence of ATP. During protein degradation, ATP is hydrolyzed; however, the cleavage of the peptide bond and the hydrolysis of ATP are not necessarily stoichiometrically coupled (*5*). In the degradation of unstructured peptides or proteins by some of the ATP-dependent proteases, binding alone of ATP is sufficient, but the efficiency of peptide bond cleavage is reduced compared to the efficiencies of the reactions accompanied by ATP hydrolysis (*6*). As the ATPase domains found in the ATP-dependent proteases have been shown to function as molecular chaperones or motors in other proteins that remodel substrates, it has been proposed that one of the functions of the ATPase domain in these proteases is to unfold and/or translocate protein substrates (*7*).

ATP-dependent proteases can be divided into two categories: the heterosubunit complexes that contain an ATPase component and a separate protease component and the homosubunit complexes that contain an ATPase and a protease component within the same polypeptide subunit (*4*). In bacteria, the heterosubunit class is composed of mainly the Clp-like proteases, while the homosubunit class is composed of Lon-like and FtsH-like proteases. Despite the difference in subunit organization, the two classes of ATP-dependent proteases share architectural and mechanistic similarities. Crystallographic studies of AAA^+ proteins, in conjunction with biochemical−mutagenesis experiments, support a general mechanistic theme for the ATPase module: that the ATPase module functions to unfold, translocate, and activate the proteolytic site through conformational changes induced by repetitive ATPase cycles (*8, 9*). In the presence of ATP, Clp, Lon, and FtsH proteases degrade protein substrates without generating partially digested protein intermediates (*10–12*). During the time course of the reactions, only undigested substrates and fully digested peptide products are produced, suggesting that proteolysis is processive. Since protein degradation is typically accompanied by repetitive cycles of ATP binding and hydrolysis, it is plausible that the ATPase activity contributes to processivity.

A general mechanistic scheme accounting for the role of the ATPase in the ATP-dependent proteolysis mechanism can be proposed (Figure 2). Crystallographic studies of the heterosubunit ATP-dependent protease HslUV (also known as ClpYQ) have provided structural insights into the conformational changes associated with the ATPase cycle (*9*), and structural along with biochemical studies performed on other Clp proteases (*10, 12–23*) have provided additional mechanistic information. The mechanistic model proposed for HslUV is often used as a reference point for comparison in mechanistic proposals for Lon and FtsH, which also draw on crystallographic studies. In the proposed model, the ATPase module of the ATP-dependent protease first binds or interacts with a specific recognition peptide element within a protein substrate in a manner independent of ATP (step 1). Although the substrate specificities are not well-defined for all ATP-dependent proteases, examining the degradation profiles of selected protein substrates reveals that translocation is initiated at a specific interaction site between the protein substrate and the ATPase module. In the Clp and FtsH proteases, an 11-amino acid residue sequence known as the ssrA tag (*7, 24*) and other short sequence motifs (*25, 26*) have been shown to serve as recognition tags. In Lon protease, protein degradation is also initiated by interaction of the enzyme with certain specific recognition elements within the substrate, but no consensus sequences have been identified (*27, 28*). The presence of ATP induces a series of conformational changes within the enzyme subunits that leads to unfolding (step 2), followed by internalization (also known as translocation) of the protein substrate into the central cavity (step 3). Polypeptide unfolding and translocation are proposed to constitute the rate-limiting step of the reaction (*18*). Both unfolding and translocation are hypothesized to be carried out by repetitive cycles of ATP hydrolysis that induce conformational changes within the ATP-dependent protease structure (*6, 9*). It is suggested that the ATPase module of the ATP-dependent proteases unfolds and translocates protein substrates into the central cavity of the protease structure via a threading mechanism (*10, 21*) (i.e., the polypeptide substrate is transported through the narrow central pore of the enzyme in a roughly linear conformation). Although the details of the kinetic coordination of the unfolding and translocation steps are not clear, the two processes can be experimentally separated and independently evaluated. By using unstructured substrates, the translocation and peptidase events can also be selectively examined. In step 4 of Figure 2, the unfolded and translocated polypeptide substrates are sequestered within the central cavity of the protease machinery (the proteolytic chamber), where peptide bond cleavage occurs. While this mechanism predicts the sequence of events occurring along the reaction pathway, it does not reveal how different nucleotide-liganded enzyme forms relate to specific steps. Mechanistic studies directed toward elucidating the functional roles of the various enzyme intermediates generated by the ATPase cycle are therefore needed.

Understanding the functional roles of the enzyme intermediates generated by microscopic events along the ATPase cycle is likely to be required to explain how ATP-dependent proteases maintain their processivity. Since the degradation of protein substrates generates only completely digested peptides ranging from approximately 5 to 20 amino acids without generation of partially digested protein intermediates, we conclude that ATP-dependent proteases degrade their substrates processively; however, it is not clear how the substrate translocation event is coordinated with peptide bond cleavage to accomplish the observed processivity. Processivity would seem to require coordination of reactions at the ATPase and peptidase sites to ensure that the translocating substrate was continuously fed into the proteolytic chamber. However, the details of this coordination might differ substantially between systems where the ATPase and protease active sites are covalently linked (e.g., Lon and FtsH proteases) and those where that association is noncovalent (e.g., the Clp proteases). In the remaining sections of this work, we will discuss existing data that provide support for the proposed general mechanism, as well as unique mechanistic features pertinent to the Clp, Lon, and FtsH systems.

ULTRASTRUCTURE OF CLP PROTEASES

The Clp proteases are complexes of two distinct oligomeric enzymes. The protease component is a heptameric serine or threonine protease; Clp family proteases include ClpP and

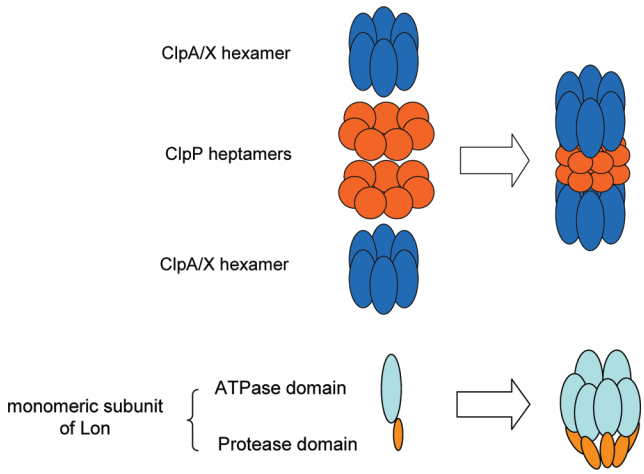

FIGURE 1: Ultrastructures of the ATP-dependent proteases Clp and Lon. In the top panel, the active complex in Clp proteases comprises two protease heptamers (ClpP, red) and two ATPase hexamers (ClpA or ClpX, blue). The two oligomeric components assemble into a cylindrical structure with a central pore that is sufficiently wide to accommodate a translocating polypeptide. In the bottom panel, each subunit of Lon is comprised of an ATPase component (gray) and a protease component (orange). The subunits self-assemble in the presence of Mg^{2+} to form an oligomeric structure with a central cavity that can presumably accommodate a translocating polypeptide.

HslV (*1, 4*). The ATPase component is a hexamer in its active form; Clp family ATPases include ClpA, ClpX, and HslU (*7*).

Binding of ATP to the Clp ATPases is required for them to assume their active, hexameric form (Figure 1). For ClpX, each subunit contains a single AAA^+ ATP binding module, including a Walker A and Walker B motif (*29*). This binding site is thus required both for hexamerization and for catalytic activity. In the case of ClpA, each subunit contains two AAA^+ ATP binding modules. One binding site appears to be mainly required for hexamerization (although it does catalyze ATP hydrolysis at a low rate), while the second site catalyzes rapid ATP hydrolysis and appears to be responsible for the bulk of the observed ATPase activity (*30*). Once formed, the hexameric complex is stable over multiple turnovers (*22*). An X-ray structure of hexameric HslU in complex with HslV has been determined (*31*); for ClpA (*15*) and ClpX (*19*), monomer structures have been determined, allowing construction of hexameric models (*8, 15, 32*) that agree with structural data from electron microscopy (*17*).

ClpP (*33*) is a serine protease and HslV (*31*) a threonine protease that form stable dimers of oligomers (hexamers for HslV, heptamers for ClpP). The tetradecameric assembly of ClpP forms a cylindrical structure with a channel running down the central axis (Figure 1) and a central cavity ~50 Å in diameter (*13*). Structural and functional studies of ClpP (*34*) suggest that protein substrates enter through the axial pores at the ends of the protease complex, while peptide products have two potential exit routes: through the axial pores or through a set of equatorial pores. Both of these routes appear to be conformationally gated. In the case of the equatorial pores, motions associated with the proposed gating can be detected by NMR, and disulfide cross-linking in the pore region results in trapping of small substrates in the complex (*35*). In the case of the axial pores, X-ray structures indicate that the N-terminus of the complex may either form a stable helix outside the axial pores or occupy the pore in disordered form (*36*).

Typically, the active complex in Clp proteases comprises two protease heptamers and two ATPase hexamers (*17, 37*) (Figure 1). Electron micrographs of the ClpAP complex indicate that the proteins assemble into a cylindrical structure with a central pore that is sufficiently wide to accommodate a translocating polypeptide (*17*). Each ATPase hexamer interacts directly with one protease heptamer, suggesting that protein substrates pass directly from the central pore of the ATPase to the central pore of the protease. In addition, the protease heptamers are stably bound to each other; for ClpP, the dissociation constant for the heptamer−heptamer interaction is subnanomolar (*22*). For ClpAP, the ATPase−protease complex is stable for multiple turnovers. However, the canonical complex is not necessarily the only active species in vivo: complexes with only a single ATPase are also likely to be catalytically active, as are complexes with two different ATPases (e.g., ClpA and ClpX).

One surprising consequence of the structural models for Clp proteases is that the 7-fold symmetry of the ATPase component is not matched to the 6-fold symmetry of the protease component. Conformational changes that establish pseudosymmetry have been proposed as likely intermediates in the catalytic cycle, on the theory that symmetry matching would allow favorable complementary interactions between the two components to be maximized (*36*). Structures of ClpP suggest that conformational changes in the N-terminus could induce pseudo-6-fold symmetry via removal of a single N-terminus from the axial pore (*36*). Further work will be necessary to ascertain the extent to which conformational changes of the complex induce pseudosymmetry.

Taken as a whole, the structural data suggest that although the ATPase and protease sites are on different polypeptides, the entire ATPase−protease complex acts as an integrated functional unit. However, these studies alone do not provide the details of how conformational changes in the two components allow the complex to carry out its functions of unfolding, translocating, and hydrolyzing protein substrates. Functional studies have begun to provide a full mechanistic description of these proteolytic machines.

STEADY-STATE KINETIC−FUNCTIONAL STUDIES OF CLP PROTEASES

Steady-state kinetic studies indicate that ATP hydrolysis is coupled to processive proteolysis of protein substrates in the Clp proteases. Although the rate of ATP hydrolysis in the absence of protein–substrates can be surprisingly high, addition of protein substrates increases the ATPase rate (*38*). In addition, ATP hydrolysis is required for the proteolysis of large protein substrates, whether they are folded or unfolded, while smaller peptides can be proteolyzed in an energy-independent fashion (*12, 38*). These results indicate that the Clp ATPases require the energy of ATP hydrolysis to unfold protein substrates and present them to the protease active sites (Figure 2).

Clp proteases appear to act as molecular machines, using the free energy of ATP hydrolysis to do the mechanical work of unfolding protein substrates (*16, 18, 39*). Many equivalents of ATP are hydrolyzed per protein unfolded, and the free energy expended is typically in great excess over the folding

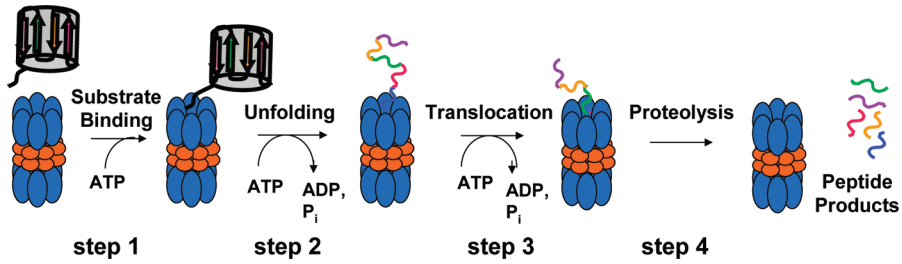

FIGURE 2: Proposed general mechanism for ATP-dependent proteases. The oligomeric structure of a heterosubunit ATP-dependent protease is used as an example to illustrate the general mechanism. For some processive proteases, step 2 can be bypassed by using an unstructured protein as a substrate. Step 4 can be removed from the reaction scheme when proteolytically inactive enzyme mutants possessing wild-type ATPase activities are used.

free energy of the substrate. The rate of protein unfolding is correlated not only with the global stability of the protein (*18*) but also with the local stability of the substrate at the substrate recognition site (e.g., the ssrA tag of ClpA/X substrates) (*10, 40*). Experiments with a ClpX construct in which the six subunits are fused into a single polypeptide indicate that a single active subunit is sufficient to unfold protein substrates. Taken together, these results support a model in which one or more ATP hydrolysis events are coupled to a conformational change in an ATPase subunit that exerts force on the folded protein substrate. In this model, each individual conformational change has a low probability of unfolding the protein, and multiple ATPase cycles involving multiple subunits are required to ensure a high probability of substrate unfolding.

Efficient translocation of unfolded protein substrates also requires ATP hydrolysis. Natively unfolded substrates such as casein and carboxymethylated titin require ATP hydrolysis to be proteolyzed at their maximal rates (*12, 18*). Analysis of the stoichiometry of ATP hydrolysis by ClpX (*18*) suggests that several ATP molecules are hydrolyzed for each substrate residue translocated through the ClpX complex. These studies support the idea that the ClpA/X ATPases actively drive unfolded polypeptides through the central pore of the complex and into the central chamber of the protease. In the most parsimonious model, the ATPase conformational change that exerts force on folded substrates to unfold them is the same as the one that exerts force to translocate the substrate after it is unfolded. The high number of ATPs hydrolyzed per substrate translocated ($\sim 10^2$) suggests that multiple conformational change cycles take place during each translocation event and/or that ATP hydrolysis is very inefficiently coupled to the conformational change.

CHARACTERIZING DISCRETE INTERMEDIATES IN SUBSTRATE TRANSLOCATION

Steady-state kinetic experiments support a model in which protein translocation is coupled to repetitive cycling of conformational changes in ClpA/X. To test this model, efforts have been made to characterize microscopic steps within a single turnover using transient-state kinetics and related approaches. Stopped-flow kinetic experiments have been carried out in which fluorescence resonance energy transfer between a ClpP-bound fluorophore and a substrate-bound fluorophore reports on substrate translocation (*21*). Full development of the FRET signal requires ~10 s, suggesting that translocation takes place on this time scale. In addition, the FRET signal exhibits a lag phase (~1 s), consistent with translocation occurring in multiple steps; as originally shown for DNA helicases, a lag phase is observed when multiple irreversible substeps are required to take place before the signal of interest can be observed (*41*).

Analysis of ClpAP peptide products also supports the idea that translocation of protein substrates takes place in multiple discrete steps. The distribution of peptide product sizes exhibits a peak at seven to eight amino acid residues (*14*). If translocation occurred continuously, the distribution of product sizes would be expected to be exponential (i.e., exhibiting a peak at zero residues). The observation of a non-zero peak in the product size distribution thus suggests that ClpA translocates protein substrates in discrete steps. In addition, perturbations that change the rate of ATP hydrolysis and/or amide bond hydrolysis do not affect the product size distribution, which would be expected if ClpA and ClpP activities are coordinated, with a translocation step triggering activation of amide bond hydrolysis by ClpP. Kinetic simulations of several alternative mechanisms for size control, such as the exit pores acting as a filter that excludes large peptides, do not account for a peak in the size distribution that is not affected ATPase or protease rate.

Recent work has investigated the structural and functional properties of proposed intermediates in protein translocation. Using protein substrates engineered to include a photoreactive group, Horwich and co-workers demonstrated that the translocating substrate contacts the D2 loop of ClpA (*8*). Site-directed mutagenesis of the residues that react in the photolabeling experiments confirms the functional importance of this region of the protein, a loop that occupies the central pore of the ClpA hexamer in the model derived from crystallographic studies of the monomer (*15*). On the basis of these experiments, Horwich and co-workers propose a mechanism in which the D2 loop binds to protein substrates at the mouth of the ClpA pore, and ATP hydrolysis initiates a conformational change that brings the D2 loop and the bound substrate through the pore. This proposed conformational change would thus move the D2 loop from the initial substrate binding site at one of the axial openings of ClpA (D2 in the "up" conformation) to the other axial opening (D2 in the "down" conformation), which faces ClpP, positioning the substrate to exit ClpA and enter ClpP. Recent mutational studies in a homologous loop in the ATPase component of HslUV support a similar role for the Tyr residue in mediating substrate translocation (*20*).

Mechanisms in which ClpA binds, translocates, and releases a protein substrate imply that the conformational changes that mediate translocation also cause a change in

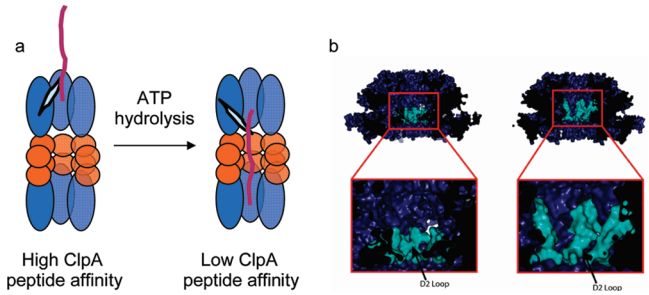

FIGURE 3: (a) Working model for coordination of conformational changes in energy-dependent substrate translocation by ClpA. Substrate (purple) binds to the D2 loop (cyan), which carries it into ClpP and releases it. (b) Structural model for the proposed conformational change [(based on the previously proposed hexameric models (*8, 15*)].

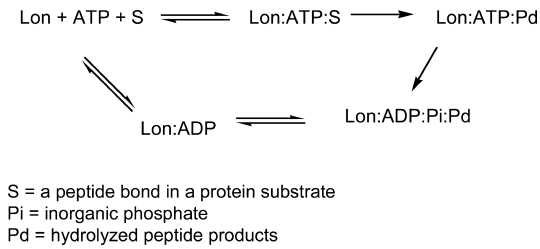

FIGURE 4: Proposed ATP−ADP exchange proteolytic mechanism for Lon.

substrate binding affinity. Recent single-molecule fluorescence studies support this view (*42*). Total internal reflection microscopy allows individual ClpA binding events to be detected for a fluorescently tagged peptide substrate. The time that the substrate spends bound to ClpA depends on what nucleotide is present. When the poorly hydrolyzable ATP analogue ATPγS is present, at least two distinct residence times are observed: one short (~100 ms) and the other long (~10 s). However, in the presence of ATP, which ClpA hydrolyzes rapidly to ADP, only one distinct residence time is observed, at an intermediate duration (~1 s). These results are consistent with a model in which nucleotide hydrolysis causes changes in substrate affinity. In addition, the results support the hypothesis that the prehydrolytic state of the enzyme exhibits the highest substrate binding affinity (although short, nonproductive events are also observed), while the posthydrolytic state exhibits lower affinity. These results, in combination with the observations about the function of the D2 loop (*20*), support a mechanism in which ATP hydrolysis drives both the motion of the D2 loop through the central pore and a switch to a low-affinity binding conformation, allowing release of the translocated peptide into ClpP (Figure 3).

STRUCTURE AND FUNCTION OF LON

Unlike the Clp protease complexes that contain separate oligomeric subunits for ATP hydrolysis and proteolysis, Lon is a homo-oligomer composed of identical subunits, which carry both ATPase and protease domains. Each Lon subunit has four domains (*43*): the amino-terminal (N) domain that is implicated in the binding of some protein substrates, the ATPase (A) domain containing Walker A and B motifs mediating ATP binding and hydrolysis, the proposed substrate sensor and discriminatory domain (SSD), and the proteolytic domain (P-domain) located at the carboxyl terminus. As a significant degree of sequence homology is shared between the ATPase domains of Lon and the other ATP-dependent proteases, it is proposed that the function of ATP binding and hydrolysis in Lon is to unfold and translcoate substrates.

Structural studies demonstrate that the Lon holoenzyme is a large homo-oligomeric ring-shaped complex. Unlike the Clp proteases, oligomerization of enzyme subunits requires the presence of Mg^{2+} but is independent of ATP. As demonstrated by analytical ultracentrifugation and electron microscopy studies, bacterial Lon proteases are hexameric ring-shaped structures containing a central cavity (*44, 45*), whereas *Saccharomyces cerevisiae* Lon (ScLon)[1] is a heptameric ring-shaped complex with flexible subunits (*46*). In the absence of nucleotide, the ring-shaped complexes of ScLon contain asymmetric enzyme subunits. However, in the presence of ATP or nonhydrolyzable ATP analogues, the enzyme subunits form symmetric ring-shaped complexes, suggesting that ATP promotes conformational changes within the enzyme subunits. Limited proteolytic footprinting studies of EcLon also reveal that adenine nucleotide protects Lon from nonspecific proteolysis, thereby providing support for the existence of at least one conformational change occurring in Lon upon binding to ATP, as observed in the crystallographic studies of HslU (*47, 48*).

At present, only structures of the truncated N-terminal domain, the carboxyl terminus of the ATPase module including the substrate sensor and discriminatory domain (SSD), and the proteolytic domain of several bacterial Lon homologues have been determined (*3, 43*). The structures of the proteolytic domains of bacterial Lons are hexameric rings containing a central cavity, and the presence of a proteolytic dyad consisting of a conserved Ser and Lys has been implicated; however, these proteins are inactive. Replacement of the Ser or Lys residue in the catalytic dyad with an Ala abolishes the proteolytic activity but not the ATPase activity (*5, 49, 50*). Mutations in the vicinity of the proteolytic site Ser 679 lead to reduction in the ATPase activity of EcLon, but the mechanism for this reduction in activity is yet to be determined (*50*). In contrast, mutation of the ATP-binding site abolishes both the ATPase and protease proteolytic activities of EcLon. Interestingly, mixed oligomeric complexes composed of wild-type EcLon and the inactive EcLon E614K mutant are inactive (*51*). Collectively, these results indicate that the catalytic activity of Lon is significantly affected by the communication between the ATPase and the proteolytic sites as well as by intersubunit interactions with the enzyme, a characteristic mechanistic feature found in the AAA^+ proteins.

FUNCTIONAL STUDIES OF LON

As the degradation of unstructured polypeptides by Lon can be sustained by nonhydrolyzable analogues of ATP such as AMPPNP with identical cleavage specificity, it has been proposed that ATP hydrolysis is needed for unfolding the

[1] Abbreviations: AMPPNP, adenylyl 5-imidodiphosphate; Abz, anthranilamide; Bz, benzoic acid; Mant, 2′(3′)-*O*-(*N*-methylanthraniloyl); dansyl, 5-(dimethylamino)-1-naphthalenesulfonyl; EcLon, *Escherichia coli* Lon protease; ScLon, *S. cerevisiae* Lon protease; NSF D2, *N*-ethylmaleimide-sensitive factor second ATPase domain; SRH, second region of homology.

substrates, whereas ATP binding alone is minimally needed for activating peptide bond cleavage (*6*). This proposal fits the general reaction scheme depicted in Figure 2 in which an unfolded protein substrate is translocated into the central cavity in Lon where it is sequestered and degraded. An important aspect in the enzymology of Lon is thus to elucidate the functional roles of the enzyme intermediates generated in Lon during each ATPase cycle.

As protein substrates are cleaved by Lon in the presence of ATP at multiple sites to yield small peptide products, it is proposed that Lon utilizes a repetitive reaction cycle to sequentially process each scissile peptide bond in a protein substrate (*6*). According to this model, the activity of Lon is modulated by an ATP−ADP exchange mechanism such that the binding of ATP and ADP to Lon activates and inactivates the enzyme, respectively. Equilibrium binding studies performed by Menon and Goldberg demonstrated that ADP binds to Lon with a much higher affinity than inorganic phosphate and ATP. It is thus proposed that ADP release limits enzyme turnover (*6*). Protein or polypeptide substrates that stimulate the ATPase activity of Lon can bind to an allosteric site in Lon to promote the release of ADP. Because the proposed active form of the enzyme is the ATP-bound form, it is implied that ATP hydrolysis occurs after peptide cleavage. While this model accounts for the ATP requirement in Lon, it does not explain why ATP, which is hydrolyzed during peptide cleavage to yield ADP, is a better activator compared to the nonhydrolyzable ATP analogues. A more detailed analysis of the kinetic mechanism of the ATP-dependent peptidase mechanism is therefore needed.

Because of the complexity of the reaction sequence, the choice of a substrate is crucial for elucidating the microscopic events occurring along the Lon reaction pathway. Relatively large proteins are not optimal substrates for mechanistic analysis because they contain multiple and diverse Lon cleavage sites, which will not allow for a clear correlation between ATP binding and hydrolysis and a single peptide cleavage event. The hydrophobic tetrapeptides that have been used as substrates in characterizing Lon are also suboptimal because they inhibit the ATPase activity of the enzyme and are degraded at a significantly slower rate as compared to protein substrates (*6*). A fluorogenic peptide substrate (FRETN 89–98) containing residues 89–98 of the λ N protein is cleaved via a mechanism similar to that of the full-length protein (*52*). Most importantly, this peptide substrate stimulates the ATPase activity of Lon as protein substrates do, and its degradation proceeds at a faster rate when ATP is hydrolyzed. Therefore, this peptide has been used to determine the timing of ATP hydrolysis and peptide bond cleavage in one ATPase cycle (*52*). Kinetic studies with this model substrate could potentially be used as a testing ground to evaluate how the cleavage of the different peptide bonds in an unfolded protein substrate is coordinated with the substrate translocation step.

Using the hydrolyzed products of ATP and FRETN 89–98 as inhibitors, steady-state kinetic analyses were used to construct a kinetic model that contains the minimum number of steps involved in the ATP-dependent peptide hydrolysis reaction by EcLon (*52*). Furthermore, ADP has been shown to act as a competitive and a noncompetitive inhibitor against the ATPase and the peptidase activity of EcLon, respectively. The apparent K_i of ADP increases with the concentration of

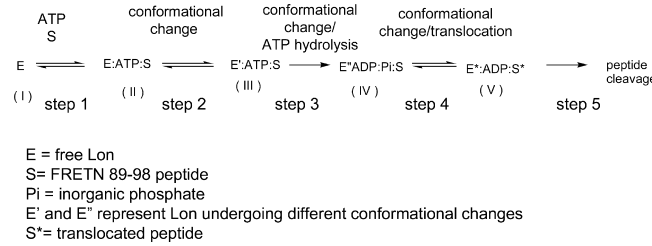

FIGURE 5: Proposed kinetic mechanism for the first turnover of an ATP-dependent peptide cleavage reaction in EcLon.

peptide substrate, thereby providing kinetic evidence to support the possibility that protein or peptide substrates bind to an allsoteric site in Lon to promote the release of ADP. To measure the kinetics of ADP interacting with EcLon, the fluorescently labeled nucleotide Mant-ADP has been used. Transient kinetic experiments performed to test the hypothesis that ADP release is rate-limiting in turnover reveal that Mant-ADP release matches the k_{cat} of Mant-ATPase turnover in Lon in the absence but not in the presence of peptide substrate (*53*). Further studies will thus be required to define the molecular nature of the rate-limiting step in the ATPase turnover reaction.

Menon and Goldberg utilized a radioactive nucleotide binding assay to demonstrate that despite being a homohexamer, EcLon exhibits two distinct affinities for ATP (K_d < 1 and 10 μM, respectively) (*6*). Rapid quench experiments further demonstrate that the two kinds of ATPase sites differ in their rates of catalyzing ATP hydrolysis, which suggests that they exhibit different functions in mediating peptide cleavage (*54*). The low-affinity sites catalyze ATP hydrolysis prior to peptide bond cleavage and are 3 orders of magnitude faster in hydrolyzing ATP than the high-affinity sites. During the time regime when the peptidase reactions were monitored, only the low-affinity sites hydrolyzed ATP. Therefore, it is conceivable that the low-affinity ATPase sites function to promote peptide translocation, whereas the high-affinity sites, which remain ATP-bound, allow the enzyme to cleave the translocated peptide. In fact, although the high-affinity sites hydrolyze ATP at a much reduced rate compared to the low-affinity sites, selective occupancy of ATP at the high-affinity sites supports multiple rounds of peptide cleavage at reduced efficiency, thereby confirming that the proteolytic site of Lon can be minimally activated by ATP bound to the high-affinity sites. Nevertheless, ATP binding and hydrolysis at both the high- and low-affinity sites are necessary for optimal cleavage.

A PROPOSED KINETIC MODEL FOR THE ATP-DEPENDENT PEPTIDE BOND CLEAVAGE REACTION

The mechanism shown in Figure 5 accounts for the microscopic events detected in the first turnover of ATP-dependent FRETN 89–98 cleavage. The binding of peptide and that of ATP are independently measured by fluorescence spectroscopic techniques (*55*). A fluorescence anisotropy study was used to demonstrate that the peptide substrate containing residues 89–98 of λ N and a dansyl moiety (dansylN 89–98) binds to Lon (enzyme form I) in a manner independent of ATP. The K_d of peptide binding is comparable to the K_m of FRETN 89–98 cleavage, indicating that the presence of ATP does not alter the affinity of Lon for the

peptide, and the binding of peptide undergoes a rapid equilibrium mechanism. To monitor ATP binding, Mant-ATP was used as a probe (*53*). Stopped-flow fluorescence kinetic experiments reveal that the ATP binding to Lon (enzyme form I) proceeds through a two-step mechanism composed of initial ligand binding (to produce enzyme form II) followed by a conformational change step (to produce enzyme form III). The kinetic profiles of Mant-ATP and Mant-AMPPNP (a nonhydrolyzable analogue of Mant-ATP) binding are identical and independent of the presence of peptide. Collectively, these results suggest that peptide and ATP bind to Lon in a manner independent of one another to form the ternary complex as represented by enzyme form III in Figure 5. Furthermore, the intermediate that distinguishes the ATP- and AMPPNP-activated forms occurs after the formation of enzyme form III.

Using [α-^{32}P]ATP and FRETN 89–98 as reagents, the timing of ATP hydrolysis and peptide bond cleavage was determined by transient kinetic experiments. The first turnover of ATP hydrolysis displays burst kinetics with a rate constant of 10 s^{-1}, while the hydrolysis of FRETN 89–98 under identical conditions displays lag kinetics with a rate constant of ~1 s^{-1} (*52*). On the basis of these findings, it is concluded that ATP hydrolysis occurs before peptide bond cleavage, and the formation of a proteolytically competent enzyme intermediate depends on the buildup of an intermediate generated by ATP binding and/or hydrolysis.

To characterize the "ATP-dependent intermediate" that was proposed on the basis of the kinetics of ATP and FRETN 89–98 hydrolysis, the kinetics of dansylN 89–98 interacting with two proteolytically inactive Lon mutants (S679W and S679A) were determined (*55*). The dansyl moiety in dansylN 89–98 interacts with the Trp residues in each Lon mutant (three Trp residues in S679A and four in S679W) to generate fluorescence resonance energy transfer signals that were detected by stopped-flow kinetic techniques. In the two mutants, the proteolytic residue Ser 679 was replaced with Ala and Trp, respectively. As both mutants displayed wild-type ATPase activity, their interactions with dansylN 89–98 during the binding and hydrolysis of ATP could be kinetically characterized. A first-order rate constant of ~10 s^{-1} was detected for the peptide interacting with S679W. This rate constant agrees with the burst rate constant detected for the first turnover of ATP hydrolysis. Therefore, the formation of enzyme form IV is likely facilitated by the coupled hydrolysis of ATP. In accord with this proposal is the detection of an ~10-fold lower rate constant for the formation of enzyme form IV when ATP is replaced with the nonhydrolyzable analogue AMPPNP. Further support comes from the observation that the lag rate constant for peptide bond cleavage in the presence of AMPPNP is also ~10-fold lower compared to that of the ATP-mediated peptidase reaction.

Another enzyme intermediate, designated enzyme form V in Figure 5, was detected when dansylN 89–98 was mixed with the mutant S679A or S679W in the presence of ATP (*55*). The rate constant for the formation of enzyme form V (~0.8 s^{-1}) approximates the pre-steady-state lag rate constant for cleavage of FRETN 89–98 by wild-type Lon (~1 s^{-1}). Therefore, it is proposed that step 4 contributes to the rate-limiting step in the first turnover of Lon-mediated peptide bond cleavage in the presence of ATP. Since the formation of enzyme form V occurs before peptide bond cleavage and exhibits the same ATP dependency as the substrate translocation found in ATP-dependent proteases (step 3 in Figure 2), it has been proposed that step 4 of Figure 5 constitutes the substrate translocation step.

Given the variety of methods that have been used to characterize the substrate translocation step in the Clp proteases, the ATP dependency of the proposed substrate translocation in Lon should be testable by comparable techniques. Moreover, in considering the degradation of the full-length λN protein, one should be able use the kinetic model shown in Figure 5 as a starting point for evaluating whether the cleavage of each site undergoes the same repetitive catalytic cycle. An alternative model in which some or all of the potential cleavage sites are first translocated to the central cavity prior to peptide bond cleavage (a mechanism similar to that proposed for the Clp proteases) can also be tested.

STRUCTURE AND FUNCTION OF FTsH

Of the three ATP-dependent proteases discussed in this work, FtsH is the only integral membrane protein (*26*). The amino terminus of each FtsH subunit consists of two transmembrane domains flanked by a periplasmic (intermembrane) domain (~20% of the full-length polypeptide subunit). As in the case of bacterial Lon, FtsH is a homohexameric protein containing an ATPase module and a protease domain. The protease domain contains a conserved Zn(II) binding motif, which is defined by the sequence HEXXH, where X is any amino acid residue. The proteolytic activity of FtsH is stimulated by Zn(II) (*26*). Crystal structures of truncated FtsH containing the protease domain and the soluble cytosolic subunits lacking the transmembrane domains reveal that the Zn(II) is coordinated with two His residues and an Asp in the proteolytic site (*56, 57*). Because of the presence of the SRH (second region of homology) motif toward the carboxyl end of the ATPase module, FtsH is often termed an AAA protease, which constitutes a subfamily of the AAA^+ protein (*2*). Electron microscopy analysis (*58*) and crystallographic studies of different truncated constructs of the bacterial homologues (*56, 57*) suggest a ring-shaped structure for FtsH. Like Lon protease, the six enzyme subunits self-assemble to form a central pore leading into a cavity where the proteolytic sites are located.

An intriguing property of FtsH is its ability to extract protein substrates from the membrane lipid bilayer while conducting ATP-dependent protein degradation (*26*). As a membrane bound ATP-dependent protease, FtsH degrades both regulatory cytoplasmic proteins such as heat shock protein transcriptional regulatory σ32 and uncomplexed membrane protein subunits such as the F_0a subunit of the ATP synthetase. Like those of other ATP-dependent proteases, the substrate specificity of FtsH is not well defined. The enzyme exhibits preferences toward positively charged and hydrophobic residues at the cleavage site (*59*). The initiation of protein degradation also requires interaction between FtsH and a recognition peptide sequence such as the ssrA tag within a protein substrate (*26*).

MECHANISTIC CHARACTERIZATION OF FTsH

Evaluation of the ATP dependence of FtsH reveals that ATP binding alone supports cleavage of the fluorogenic

tetrapeptide Suc-LLVY-AMC, whereas the degradation of protein substrates requires both ATP binding and hydrolysis (*60*). FtsH is identified as a processive protease on the basis of the observation that only undigested substrate and fully digested peptide products of <3 kDa were observed during degradation of σ32 by FtsH (*61*). The lack of partially digested intermediates is similar to the results obtained for Clp and Lon proteases, and indicative of processivity in proteolytic cleavage.

The majority of the mechanistic characterization of FtsH has been directed toward elucidating the mechanisms of ATP hydrolysis and substrate translocation using molecular modeling, mutagenesis, and crystallography (*56, 57, 62, 63*). The results generated from these studies are consistent with the general mechanism proposed for the ATP-dependent proteases as depicted in Figure 2. However, FtsH has very limited unfolding activity compared to the other ATP-dependent proteases, exhibiting a preference for degrading proteins with relatively low thermostabilities (*64*). It is proposed that this property serves as a substrate selection mechanism in FtsH. A homology structural model for the ATPase domain of FtsH has been constructed on the basis of the crystal structure of NSF-D2, and site-directed mutagenesis has been used to identify a conserved arginine residue important for mediating ATP hydrolysis in the SRH (*60, 62, 63, 65*). Additional mutagenesis experiments further identify residues lining the central pore of the protease complex that may participate in directional translocation of the peptide substrate to the protease chamber, also proposed to exist in the Clp proteases. Mutation of the Phe or Gly residue within the MFVG motif in *E. coli* FtsH affects ATP hydrolysis, and consequently protein degradation. On the basis of sequence homology, it is further proposed that the Phe residue in the MFVG motif adopts the same function as the Tyr residue in the D2 loop of ClpA or HslUV in mediating substrate translocation.

As in the case of Lon, the binding of ATP by FtsH induces a more compact conformational change within the enzyme that can be detected by limited tryptic digestion studies (*63*). Suno and co-workers recently discovered that the ATPase cycle may be coupled with an "open and close" motion of the hexameric enzyme complex (*57*). These results collectively suggest that the catalytic activity of FtsH is also dependent on the conformational changes in the enzyme driven by repeated cycles of ATP hydrolysis. Recently, two crystal structures of the cytosolic portion of bacterial (*Thermotoga maritima* and *Thermus thermophilus*) FtsH have been determined (*56, 57*). Both cytosolic FtsH constructs exhibit ATP-dependent proteolytic activities and display a hexameric structure containing a central pore leading to a proteolytic cavity. Interestingly, C6 symmetry is found only among the proteolytic domains. When bound to ADP, the ATPase modules in the structure of *T. maritima* FtsH and *Th. thermophilus* FtsH exhibit C2 and C3 symmetry, respectively.

To account for the observed mismatches in symmetry between the ATPase modules and the proteolytic domains, distinct substrate translocation mechanisms have been proposed for *T. maritima* and *Th. thermophilus*. In *T. maritima*, ATP hydrolysis may generate conformational changes within the enzyme complex that alter the symmetry among the enzyme subunits. The polypeptide substrate may therefore be "pulled" through the central pore via interaction with the conserved central pore residues in a manner similar to the proposed ClpAP or HslUV mechanisms (Figure 3). In *Th. thermophilus* FtsH, despite the occupancy of ADP in each enzyme subunit, an alternate arrangement of open and closed subunits was observed. Comparison of the ADP-bound enzyme form with the free enzyme reveals that the subunits undergo significant conformational changes upon nucleotide binding, suggesting that the enzymatic activity of FtsH is also coupled to conformational changes driven by the ATPase cycle, as observed in Clp and Lon proteases. To accommodate the observed mismatch in symmetry between the ATPase modules and the protease domains in the *Th. thermophilus* FtsH complex, Suno and colleagues proposed a mechanism in which the open enzyme subunit forms a narrow channel that translocates polypeptide substrate to the proteolytic site of the neighboring closed enzyme subunit. In their proposal, substrate is not translocated through the central pore.

Compared to Clp and Lon proteases, FtsH has been kinetically characterized only sparsely. Steady-state kinetic analyses have been performed in the ATP-dependent degradation of the endogenous protein substrate σ32 and a heptamer peptide containing a defined FtsH cleavage site (*61*). On the basis of the deduced kinetic parameters, a minimal kinetic mechanism has been proposed to account for the ATP-dependent peptide cleavage reaction. However, studies directed toward correlating the ATPase-induced conformational changes in FtsH with specific functions have not yet been reported. The same techniques that have been used to characterize the reaction intermediates generated along the Clp and Lon reaction pathways may also help elucidate mechanistic details of FtsH.

HOW DO ATP-DEPENDENT PROTEASES EXECUTE PROCESSIVE PEPTIDE BOND CLEAVAGE?

At the structural level, Clp, Lon, and FtsH proteases have significant differences. Clp proteases have their protease active sites shielded within a cylindrical cavity, while Lon is likely to have its active sites exposed to solvent. In FtsH, a Zn-dependent proteolytic site is found within the central cavity of the oligomeric enzyme complex. Therefore, it is likely that at the molecular level, these proteases utilize distinct mechanisms to coordinate substrate translocation with processive peptide bond cleavage. Despite these differences, however, Clp, Lon, and FtsH proteases may share several common mechanistic features that account for their processive enzymatic behavior.

One mechanistic feature shared by Clp, Lon, and FtsH proteases is that substrate translocation appears to be mediated by switches among a relatively small number of discrete conformations. For Clp, Lon, and FtsH proteases, the discrete conformational changes appear to be coupled to nucleotide hydrolysis. Kinetically distinct states that can be assigned as prehydrolytic and posthydrolytic conformations are observed in these systems. More work will be required to confirm the roles of these conformational changes in substrate translocation. However, the existing evidence strongly suggests that the nucleotide-dependent conformational changes do mediate substrate translocation. Amide

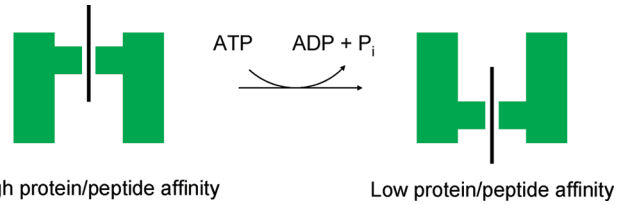

FIGURE 6: Proposed general mechanism for translocation by Lon/Clp proteases. The protease complex (green) initially binds the protein or peptide substrate with high affinity. Nucleotide hydrolysis induces a conformational change that shifts the position of the bound substrate and decreases the substrate affinity.

bond hydrolysis, the other irreversible covalent reaction catalyzed by these proteases, might also play a part; ClpP has been reported to cleave peptide substrates processively in the absence of ClpA (*12*).

Nucleotide-dependent conformational changes and translocation appear to be linked to changes in the location and/or affinity of peptide binding and, presumably, protein binding as well. For Lon, peptide product binding in the posthydrolytic isomerized form is noncompetitive with peptide substrate binding in the prehydrolytic state (*66*). For Clp proteases, the proposed posthydrolytic conformation exhibits a lower affinity for peptide substrates (*42*). These data are consistent with the idea that a general mechanism for Lon/Clp processive proteolysis will include both changes in substrate position and changes in substrate affinity as a consequence of nucleotide hydrolysis. Changes in substrate affinity are likely to be an essential element in the translocation mechanism, since the translocating substrate must bind with high affinity before and/or during translocation to ensure processivity, but with lower affinity after translocation to maintain a high rate of turnover.

On the basis of the common mechanistic features of these ATP-dependent proteases, it is possible to speculate about a general mechanism for processive proteolysis (Figure 6). In this mechanism, translocation is initiated by a conformational change that shifts the protein or peptide substrate's position in the protease. This conformational change is coupled to a thermodynamically favorable covalent reaction: ATP hydrolysis and/or amide bond hydrolysis. Translocation can occur because forward progress of the substrate is thermodynamically coupled to an irreversible reaction. The conformational change is also associated with a change in substrate affinity that results in substrate release after translocation. Binding of the substrate to another subunit in the high-affinity conformation allows another cycle of translocation to occur. Further mechanistic and structural work will be necessary to determine whether this general mechanism can account for processive proteolysis in Lon and Clp proteases.

ACKNOWLEDGMENT

We thank Christine Phillips (Massachusetts Institute of Technology) for assembling the structural model (Figure 3B).

REFERENCES

1. Gottesman, S., and Maurizi, M. R. (1992) Regulation by proteolysis: Energy-dependent proteases and their targets. *Microbiol. Rev. 56*, 592–621.
2. Ogura, T., and Wilkinson, A. J. (2001) AAA+ superfamily ATPases: Common structure−diverse function. *Genes Cells 6*, 575–597.
3. Smith, C. K., Wohnert, J., Sauer, R. T., and Schwalbe, H. (2001) Assignments of the ^{1}H, ^{13}C, and ^{15}N resonances of the substrate-binding SSD domain from Lon protease. *J. Biomol. NMR 21*, 387–388.
4. Lupas, A., Flanagan, J. M., Tamura, T., and Baumeister, W. (1997) Self-compartmentalizing proteases. *Trends Biochem. Sci. 22*, 399–404.
5. Fischer, H., and Glockshuber, R. (1993) ATP hydrolysis is not stoichiometrically linked with proteolysis in the ATP-dependent protease La from *Escherichia coli*. *J. Biol. Chem. 268*, 22502–22507.
6. Goldberg, A. L., Moerschell, R. P., Chung, C. H., and Maurizi, M. R. (1994) ATP-dependent protease La (lon) from *Escherichia coli*. *Methods Enzymol. 244*, 350–375.
7. Baker, T. A., and Sauer, R. T. (2006) ATP-dependent proteases of bacteria: Recognition logic and operating principles. *Trends Biochem. Sci. 31*, 647–653.
8. Hinnerwisch, J., Fenton, W. A., Furtak, K. J., Farr, G. W., and Horwich, A. L. (2005) Loops in the central channel of ClpA chaperone mediate protein binding, unfolding, and translocation. *Cell 121*, 1029–1041.
9. Wang, J., Song, J. J., Franklin, M. C., Kamtekar, S., Im, Y. J., Rho, S. H., Seong, I. S., Lee, C. S., Chung, C. H., and Eom, S. H. (2001) Crystal structures of the HslVU peptidase-ATPase complex reveal an ATP-dependent proteolysis mechanism. *Structure 9*, 177–184.
10. Lee, C., Schwartz, M. P., Prakash, S., Iwakura, M., and Matouschek, A. (2001) ATP-dependent proteases degrade their substrates by processively unraveling them from the degradation signal. *Mol. Cell 7*, 627–637.
11. Nishii, W., Suzuki, T., Nakada, M., Kim, Y. T., Muramatsu, T., and Takahashi, K. (2005) Cleavage mechanism of ATP-dependent Lon protease toward ribosomal S2 protein. *FEBS Lett. 579*, 6846–6850.
12. Thompson, M. W., Singh, S. K., and Maurizi, M. R. (1994) Processive degradation of proteins by the ATP-dependent Clp protease from *Escherichia coli*. Requirement for the multiple array of active sites in ClpP but not ATP hydrolysis. *J. Biol. Chem. 269*, 18209–18215.
13. Beuron, F., Maurizi, M. R., Belnap, D. M., Kocsis, E., Booy, F. P., Kessel, M., and Steven, A. C. (1998) At sixes and sevens: Characterization of the symmetry mismatch of the ClpAP chaperone-assisted protease. *J. Struct. Biol. 123*, 248–259.
14. Choi, K. H., and Licht, S. (2005) Control of peptide product sizes by the energy-dependent protease ClpAP. *Biochemistry 44*, 13921–13931.
15. Guo, F., Maurizi, M. R., Esser, L., and Xia, D. (2002) Crystal structure of ClpA, an Hsp100 chaperone and regulator of ClpAP protease. *J. Biol. Chem. 277*, 46743–46752.
16. Hoskins, J. R., Pak, M., Maurizi, M. R., and Wickner, S. (1998) The role of the ClpA chaperone in proteolysis by ClpAP. *Proc. Natl. Acad. Sci. U.S.A. 95*, 12135–12140.
17. Ishikawa, T., Beuron, F., Kessel, M., Wickner, S., Maurizi, M. R., and Steven, A. C. (2001) Translocation pathway of protein substrates in ClpAP protease. *Proc. Natl. Acad. Sci. U.S.A. 98*, 4328–4333.
18. Kenniston, J. A., Baker, T. A., Fernandez, J. M., and Sauer, R. T. (2003) Linkage between ATP consumption and mechanical unfolding during the protein processing reactions of an AAA+ degradation machine. *Cell 114*, 511–520.
19. Kim, D. Y., and Kim, K. K. (2003) Crystal structure of ClpX molecular chaperone from *Helicobacter pylori*. *J. Biol. Chem. 278*, 50664–50670.
20. Park, E., Rho, Y. M., Koh, O. J., Ahn, S. W., Seong, I. S., Song, J. J., Bang, O., Seol, J. H., Wang, J., Eom, S. H., and Chung, C. H. (2005) Role of the GYVG pore motif of HslU ATPase in protein unfolding and translocation for degradation by HslV peptidase. *J. Biol. Chem. 280*, 22892–22898.
21. Reid, B. G., Fenton, W. A., Horwich, A. L., and Weber-Ban, E. U. (2001) ClpA mediates directional translocation of substrate proteins into the ClpP protease. *Proc. Natl. Acad. Sci. U.S.A. 98*, 3768–3772.
22. Singh, S. K., Guo, F., and Maurizi, M. R. (1999) ClpA and ClpP remain associated during multiple rounds of ATP-dependent protein degradation by ClpAP protease. *Biochemistry 38*, 14906–14915.

23. Wang, J. M., Hartling, J. A., and Flanagan, J. M. (1997) The structure of ClpP at 2.3 angstrom resolution suggests a model for ATP-dependent proteolysis. *Cell 91*, 447–456.
24. Gottesman, S., Roche, E., Zhou, Y. N., and Sauer, R. T. (1998) The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. *Genes Dev. 12*, 1338–1347.
25. Flynn, J. M., Levchenko, I., Seidel, M., Wickner, S. H., Sauer, R. T., and Baker, T. A. (2001) Overlapping recognition determinants within the ssrA degradation tag allow modulation of proteolysis. *Proc. Natl. Acad. Sci. U.S.A. 98*, 10584–10589.
26. Ito, K., and Akiyama, Y. (2005) Cellular functions, mechanism of action, and regulation of FtsH protease. *Annu. Rev. Microbiol. 59*, 211–231.
27. Nishii, W., Maruyama, T., Matsuoka, R., Muramatsu, T., and Takahashi, K. (2002) The unique sites in SulA protein preferentially cleaved by ATP-dependent Lon protease from *Escherichia coli*. *Eur. J. Biochem. 269*, 451–457.
28. Ondrovicova, G., Liu, T., Singh, K., Tian, B., Li, H., Gakh, O., Perecko, D., Janata, J., Granot, Z., Orly, J., Kutejova, E., and Suzuki, C. K. (2005) Cleavage site selection within a folded substrate by the ATP-dependent lon protease. *J. Biol. Chem. 280*, 25103–25110.
29. Gottesman, S., Clark, W. P., de Crécy-Lagard, V., and Maurizi, M. R. (1993) ClpX, an alternative subunit for the ATP-dependent Clp protease of *Escherichia coli*: Sequence and in vivo activities. *J. Biol. Chem. 268*, 22618–22626.
30. Singh, S. K., and Maurizi, M. R. (1994) Mutational analysis demonstrates different functional roles for the two ATP-binding sites in ClpAP protease from *Escherichia coli*. *J. Biol. Chem. 269*, 29537–29545.
31. Bochtler, M., Hartmann, C., Song, H. K., Bourenkov, G. P., Bartunik, H. D., and Huber, R. (2000) The structures of HsIU and the ATP-dependent protease HsIU-HsIV. *Nature 403*, 800–805.
32. Diemand, A. V., and Lupas, A. N. (2006) Modeling AAA+ ring complexes from monomeric structures. *J. Struct. Biol. 156*, 230–243.
33. Shin, D. H., Lee, C. S., Chung, C. H., and Suh, S. W. (1996) Molecular symmetry of the ClpP component of the ATP-dependent Clp protease, an *Escherichia coli* homolog of 20 S proteasome. *J. Mol. Biol. 262*, 71–76.
34. Gribun, A., Kimber, M. S., Ching, R., Sprangers, R., Fiebig, K. M., and Houry, W. A. (2005) The ClpP double ring tetradecameric protease exhibits plastic ring-ring interactions, and the N termini of its subunits form flexible loops that are essential for ClpXP and ClpAP complex formation. *J. Biol. Chem. 280*, 16185–16196.
35. Sprangers, R., Gribun, A., Hwang, P. M., Houry, W. A., and Kay, L. E. (2005) Quantitative NMR spectroscopy of supramolecular complexes: Dynamic side pores in ClpP are important for product release. *Proc. Natl. Acad. Sci. U.S.A. 102*, 16678–16683.
36. Bewley, M. C., Graziano, V., Griffin, K., and Flanagan, J. M. (2006) The asymmetry in the mature amino-terminus of ClpP facilitates a local symmetry match in ClpAP and ClpXP complexes. *J. Struct. Biol. 153*, 113–128.
37. Ortega, J., Lee, H. S., Maurizi, M. R., and Steven, A. C. (2004) ClpA and ClpX ATPases bind simultaneously to opposite ends of ClpP peptidase to form active hybrid complexes. *J. Struct. Biol. 146*, 217–226.
38. Thompson, M. W., and Maurizi, M. R. (1994) Activity and specificity of *Escherichia coli* ClpAP protease in cleaving model peptide substrates. *J. Biol. Chem. 269*, 18201–18208.
39. Weber-Ban, E. U., Reid, B. G., Miranker, A. D., and Horwich, A. L. (1999) Global unfolding of a substrate protein by the Hsp100 chaperone ClpA. *Nature 401*, 90–93.
40. Kenniston, J. A., Burton, R. E., Siddiqui, S. M., Baker, T. A., and Sauer, R. T. (2004) Effects of local protein stability and the geometric position of the substrate degradation tag on the efficiency of ClpXP denaturation and degradation. *J. Struct. Biol. 146*, 130–140.
41. Ali, J. A., and Lohman, T. M. (1997) Kinetic measurement of the step size of DNA unwinding by *Escherichia coli* UvrD helicase. *Science 275*, 377–380.
42. Farbman, M. E., Gershenson, A., and Licht, S. (2007) Single-molecule analysis of nucleotide-dependent substrate binding by the protein unfoldase ClpA. *J. Am. Chem. Soc. 129*, 12378–12379.
43. Rotanova, T. V., Botos, I., Melnikov, E. E., Rasulova, F., Gustchina, A., Maurizi, M. R., and Wlodawer, A. (2006) Slicing a protease: Structural features of the ATP-dependent Lon proteases gleaned from investigations of isolated domains. *Protein Sci. 15*, 1815–1828.
44. Park, S. C., Jia, B., Yang, J. K., Van, D. L., Shao, Y. G., Han, S. W., Jeon, Y. J., Chung, C. H., and Cheong, G. W. (2006) Oligomeric structure of the ATP-dependent protease La (Lon) of *Escherichia coli*. *Mol. Cells 21*, 129–134.
45. Rudyak, S. G., Brenowitz, M., and Shrader, T. E. (2001) Mg^{2+}-linked oligomerization modulates the catalytic activity of the Lon (La) protease from *Mycobacterium smegmatis*. *Biochemistry 40*, 9317–9323.
46. Stahlberg, H., Kutejova, E., Suda, K., Wolpensinger, B., Lustig, A., Schatz, G., Engel, A., and Suzuki, C. K. (1999) Mitochondrial Lon of *Saccharomyces cerevisiae* is a ring-shaped protease with seven flexible subunits. *Proc. Natl. Acad. Sci. U.S.A. 96*, 6787–6790.
47. Patterson, J., Vineyard, D., Thomas-Wohlever, J., Behshad, R., Burke, M., and Lee, I. (2004) Correlation of an adenine-specific conformational change with the ATP-dependent peptidase activity of *Escherichia coli* Lon. *Biochemistry 43*, 7432–7442.
48. Vasilyeva, O. V., Kolygo, K. B., Leonova, Y. F., Potapenko, N. A., and Ovchinnikova, T. V. (2002) Domain structure and ATP-induced conformational changes in *Escherichia coli* protease Lon revealed by limited proteolysis and autolysis. *FEBS Lett. 526*, 66–70.
49. Rotanova, T. V., Melnikov, E. E., Khalatova, A. G., Makhovskaya, O. V., Botos, I., Wlodawer, A., and Gustchina, A. (2004) Classification of ATP-dependent proteases Lon and comparison of the active sites of their proteolytic domains. *Eur. J. Biochem. 271*, 4865–4871.
50. Starkova, N. N., Koroleva, E. P., Rumsh, L. D., Ginodman, L. M., and Rotanova, T. V. (1998) Mutations in the proteolytic domain of *Escherichia coli* protease Lon impair the ATPase activity of the enzyme. *FEBS Lett. 422*, 218–220.
51. Oh, J. Y., Eun, Y. M., Yoo, S. J., Seol, J. H., Seong, I. S., Lee, C. S., and Chung, C. H. (1998) LonR9 carrying a single Glu614 to Lys mutation inhibits the ATP-dependent protease La (Lon) by forming mixed oligomeric complexes. *Biochem. Biophys. Res. Commun. 250*, 32–35.
52. Lee, I., Berdis, A. J., and Suzuki, C. K. (2006) Recent developments in the mechanistic enzymology of the ATP-dependent Lon protease from *Escherichia coli*: Highlights from kinetic studies. *Mol. Biosyst. 2*, 477–483.
53. Vineyard, D., Zhang, X., and Lee, I. (2006) Transient kinetic experiments demonstrate the existence of a unique catalytic enzyme form in the peptide-stimulated ATPase mechanism of *Escherichia coli* Lon protease. *Biochemistry 45*, 11432–11443.
54. Vineyard, D., Patterson-Ward, J., and Lee, I. (2006) Single-turnover kinetic experiments confirm the existence of high- and low-affinity ATPase Sites in *Escherichia coli* Lon protease. *Biochemistry 45*, 4602–4610.
55. Patterson-Ward, J., Huang, J., and Lee, I. (2007) Detection and characterization of two ATP-dependent conformational changes in proteolytically inactive *Escherichia coli* Lon mutants by stopped flow kinetic techniques. *Biochemistry 46*, 13593–13605.
56. Bieniossek, C., Schalch, T., Bumann, M., Meister, M., Meier, R., and Baumann, U. (2006) The molecular architecture of the metalloprotease FtsH. *Proc. Natl. Acad. Sci. U.S.A. 103*, 3066–3071.
57. Suno, R., Niwa, H., Tsuchiya, D., Zhang, X., Yoshida, M., and Morikawa, K. (2006) Structure of the whole cytosolic region of ATP-dependent protease FtsH. *Mol. Cell 22*, 575–585.
58. Shotland, Y., Koby, S., Teff, D., Mansur, N., Oren, D. A., Tatematsu, K., Tomoyasu, T., Kessel, M., Bukau, B., Ogura, T., and Oppenheim, A. B. (1997) Proteolysis of the phage lambda CII regulatory protein by FtsH (HflB) of *Escherichia coli*. *Mol. Microbiol. 24*, 1303–1310.
59. Akiyama, Y. (1999) Self-processing of FtsH and its implication for the cleavage specificity of this protease. *Biochemistry 38*, 11693–11699.
60. Yamada-Inagawa, T., Okuno, T., Karata, K., Yamanaka, K., and Ogura, T. (2003) Conserved pore residues in the AAA protease FtsH are important for proteolysis and its coupling to ATP hydrolysis. *J. Biol. Chem. 278*, 50182–50187.
61. Bruckner, R. C., Gunyuzlu, P. L., and Stein, R. L. (2003) Coupled kinetics of ATP and peptide hydrolysis by *Escherichia coli* FtsH protease. *Biochemistry 42*, 10843–10852.
62. Karata, K., Verma, C. S., Wilkinson, A. J., and Ogura, T. (2001) Probing the mechanism of ATP hydrolysis and substrate translo-

cation in the AAA protease FtsH by modeling and mutagenesis. *Mol. Microbiol. 39*, 890–903.
63. Karata, K., Inagawa, T., Wilkinson, A. J., Tatsuta, T., and Ogura, T. (1999) Dissecting the role of a conserved motif (the second region of homology) in the AAA family of ATPases. Site-directed mutagenesis of the ATP-dependent protease FtsH. *J. Biol. Chem. 274*, 26225–26232.
64. Herman, C., Prakash, S., Lu, C. Z., Matouschek, A., and Gross, C. A. (2003) Lack of a robust unfoldase activity confers a unique level of substrate specificity to the universal AAA protease FtsH. *Mol. Cell 11*, 659–669.
65. Okuno, T., Yamanaka, K., and Ogura, T. (2006) Characterization of mutants of the *Escherichia coli* AAA protease, FtsH, carrying a mutation in the central pore region. *J. Struct. Biol. 156*, 109–114.
66. Thomas-Wohlever, J., and Lee, I. (2002) Kinetic characterization of the peptidase activity of *Escherichia coli* Lon reveals the mechanistic similarities in ATP-dependent hydrolysis of peptide and protein substrates. *Biochemistry 41*, 9418–9425.

BI800025G