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Recent Developments inThe Mechanistic Enzymology of The ATP-Dependent Lon Protease from Escherichia Coli: Highlights From Kinetic Studies

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Recent developments in the mechanistic enzymology of the ATP-dependent Lon protease from *Escherichia coli*: highlights from kinetic studies

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Lon protease, also known as protease La, is one of the simplest ATP-dependent proteases that plays vital roles in maintaining cellular functions by selectively eliminating misfolded, damaged and certain short-lived regulatory proteins. Although Lon is a homo-oligomer, each subunit of Lon contains both an ATPase and a protease active site. This relatively simple architecture compared to other hetero-oligomeric ATP-dependent proteases such as the proteasome makes Lon a useful paradigm for studying the mechanism of ATP-dependent proteolysis. In this article, we survey some recent developments in the mechanistic characterization of Lon with an emphasis on the utilization of pre-steady-state enzyme kinetic techniques to determine the timing of the ATPase and peptidase activities of the enzyme.

Introduction

Lon (or protease La) is a serine protease that selectively degrades abnormal proteins and short lived regulatory proteins in various organisms.[1 4] In eubacteria and mitochon dria, the Lon holoenzyme is a soluble cytoplasmic or matrix localized complex respectively, whereas in archaea the pro tease is membrane bound. Lon derives its name from the phenotype of *Escherichia coli* that lack the *lon* gene, which exhibit a cell division defect and are *lon*ger than their wild type counterparts.[5] Since its discovery, studies have shown that Lon is essential for cellular homeostasis, mediating protein quality control and metabolic regulation in both bacteria and mitochondria.[6 8] Pathogenic bacteria such as *Brucella abortus* and *Salmonella typhimurium*, require Lon mediated proteolysis for the expression of virulence genes that promote mammalian cell infection.[9 11] Based upon sequence homology, Lon belongs to the family of AAA^+ proteins (ATPases Associated with a variety of cellular Activities), whose mem bers are involved in processes including DNA replication, transcription, membrane fusion, and proteolysis.[12 15] Unlike the other soluble ATP dependent proteases which contain separate oligomeric ATPase and protease components, Lon exists as a homo oligomer, with each polypeptide sub unit containing both an ATPase and a protease domain. Since the two hydrolytic activities are obligatorily coupled in the enzyme, Lon provides the simplest model for studying the mechanism of ATP dependent proteolysis.

Mechanistic implications from existing studies and questions to be answered

Electron microscopic imaging of *E. coli* Lon demonstrates a hexameric

ring shaped structure with a central cavity[16] whereas cryoelectron microscopy shows that *Saccharomyces cerevisiae* Lon (or Pim1p) is a heptameric ring shaped protease.[17] The X ray structure of intact Lon is currently unknown; however, structures of truncated Lon protease domains of several bacterial homologs reveal that the proteolytic active site contains at least a conserved catalytic Ser Lys dyad.[18 21] Mutation of either residue to Ala abolishes protease but not ATPase activity.[22,23] However, the kinetics of ATP hydrolysis can be affected by other mutations within the protease catalytic site.[20,22,23] Thus, it appears the two hydrolytic activities are perhaps loosely interconnected during catalysis.

The current paradigm of ATP dependent proteolysis is that a target substrate is initially engaged *via* an unstructured or loosely folded region after which it is unfolded and translocated to the proteolytic active site where it is cleaved in a sequential stepwise manner.[24 37] Therefore Lon may utilize a similar mechanism in degrading proteins. Cleavage site selection by Lon has been analyzed using both endogenous and reporter substrates.[38 48] Results show that all Lon proteases cleave substrates not at defined amino acid sequences but at sites where hydrophobic residues are adjacent to the scissile bond. For endogenous bacterial and mitochondrial substrates, Lon mediated cleavage occurs principally within or adjacent to α helices and β sheets. In addition, Lon appears to utilize more than one mechanism to initiate protein degradation. For example, initial cleavages within SulA, a bacterial cell division inhibitor are located at a central functional region that may rapidly inactivate the protein and trigger unfolding.[45] The major Lon cleavages sites within the bacterial S2 ribosomal protein are located at the interior of the molecule;[47] by contrast, human Lon initiates substrate cleavage at surface exposed sites of the mitochondrial processing peptidase α subunit (MPPα) and the steroidogenic acute regulatory protein (StAR), which mediates steroid hormone biosynthesis.[48] Interestingly, mitochondrial Lon degrades MPPα only when it is folded, which deviates from the normal "loosely structured protein substrate profiles" expected for substrates of Lon. These observations collectively suggest that there is more to learn about the rules and mechanism(s) by which folded and unfolded substrates are recognized, engaged and degraded by this protease family.

Lon mediated protein turnover *in vivo* is likely modulated or regulated by factors that affect the enzymatic activity of Lon and/or the conformational state of substrates. For example, unfolded protein substrates stimulate both the peptidase and ATPase activities of Lon.[4,49] In addition, the binding of inorganic polyphosphate (polyP) within the ATPase domain of *E. coli* Lon has been shown to promote the specific association and degradation of free ribosomal proteins.[47,50,51] On the other hand, conformational sensitivity of protein substrates to Lon mediated proteolysis is influenced by their protein protein interactions or binding to cellular factors. For example, mitochondrial MPPα is degraded by Lon only when it is unassembled since heterodimeric MPPα complexed with MPPβ is stable.[48] Lon also belongs to a unique group of proteases that also bind to DNA and RNA.[50,52 59] Bacterial Lon binds to double stranded DNA with little sequence specificity whereas mitochondrial Lon binds preferentially to single stranded DNA and RNA in a sequence dependent manner. Further experiments are required to elucidate the physiological importance of DNA and RNA binding by Lon and the functional relationship between nucleic binding and enzymatic activity.

Despite efforts to elucidate the reaction mechanism of Lon by the aforementioned techniques, fundamental questions remain as to how ATP binding and hydrolysis, as well as the product,

ADP, affect the kinetic activity of Lon protease. Since polypeptide cleavage by Lon is accompanied by ATP hydrolysis, understanding the kinetic connection between the two hydrolytic activities will provide unique mechanistic insights into Lon and other ATP dependent proteases.

Utilization of a kinetic approach to study Lon protease

Lon belongs to a diverse group of enzymes that transform the chemical energy derived from ATP hydrolysis into mechanical force associated with changes in protein conformation, translocation and/or movement along biopolymers such as polypeptides or nucleic acids.[12 15] Pre steady state enzyme kinetic techniques are often used to decipher the kinetic mechan ism that coordinates nucleotide binding and hydrolysis with the execution of physical work. For example, kinetic char acterization of T4 DNA replication in the presence of the clamp loader protein (gp 45) and polymerase (gp 44/62) reveal how ATP consumption is used to sustain processive DNA replication.[60 62] In these studies, a defined sequence of DNA primer/template is used as substrate for monitoring enzyme activities such that the rate constants associated with substrate binding, chemical conversion and product release could be determined from the reaction time courses. Using these kinetic constants, one can establish the sequence of events existing along the enzymatic reaction pathway and identify rate limit ing steps. Since Lon possesses both ATPase and peptidase activities that are functionally connected, a similar kinetic approach should benefit the mechanistic characterization of this protease. The fluorogenic peptide substrate system described below provides a key experi mental tool for studying the mechanism of Lon.

A fluorescence peptidase assay developed to study the ATP-dependent peptidase reaction mechanism of lon

Kinetic characterization of *E. coli* Lon was initially performed using either large proteins or hydrophobic fluorogenic tetrapeptides as substrates.[42,63 65] These studies reveal that while ATP binding minimally supports cleavage of tetrapep tides, protein degradation requires ATP hydrolysis. Interestingly, the degradation of proteins stimulates the ATPase activ ity of Lon whereas tetrapeptides do not.[4] The difference in degradation profiles towards various polypeptides using var ious adenine nucleotides lead to the proposal that ATP hydrolysis is used to unfold and/or translocate polypeptide substrates prior to their degradation.[30] Since ADP, the nucleotide product of the reaction, inhibits the proteolytic activity of Lon, it is proposed that polypeptide substrate promotes ADP release to cause an overall stimulation in the ATP hydrolysis cycle.[49,66,67]

Lon degrades polypeptides to yield products that are at least 4 to 5 amino acids long.[4,47] A decapeptide (also known as S1) containing the amino acid residues 89 98 of the bacteriophage λN transcription anti termination factor, and a fluorescence donor acceptor pair, was therefore developed to study the kinetics of Lon.[68] In intact S1, the fluorescence signal generated by anthra nilamide (donor) is internally quenched by nitrotyrosine (acceptor). However, upon cleavage at Cys Ser, the donor is separated from the acceptor such that an increase in fluorescence is detected (Fig. 1). This increase in fluorescence signal can be correlated with the absolute concentration of peptide cleavage to yield time courses that provide rates of the hydrolytic reactions. As S1 also stimulates the ATPase activity of Lon, it is considered a substrate mimic of λN, and has been used to study the kinetic coordination between ATP binding and hydrolysis with peptide bond cleavage. It should be noted that while S1 provides a means to monitor the activity of Lon, its use suffers from a technical limitation known as the inner filter effect. Specifically, at >150 μM S1, the fluores cence generated from peptide cleavage deviates from linearity because nitrotyr osine absorbs the fluorescence signal from anthranilamide *via* an inter rather than an intra molecular mechanism. The usable range of this peptide substrate however, can be expanded by a "spike in" technique that is commonly used in radioactive assays in which a small percentage of labeled substrate is mixed with unlabeled substrate to monitor a reaction. As shown in Fig. 2, the utiliza tion of a mixed peptide substrate pool (5% S1 and 95% S2), collectively known

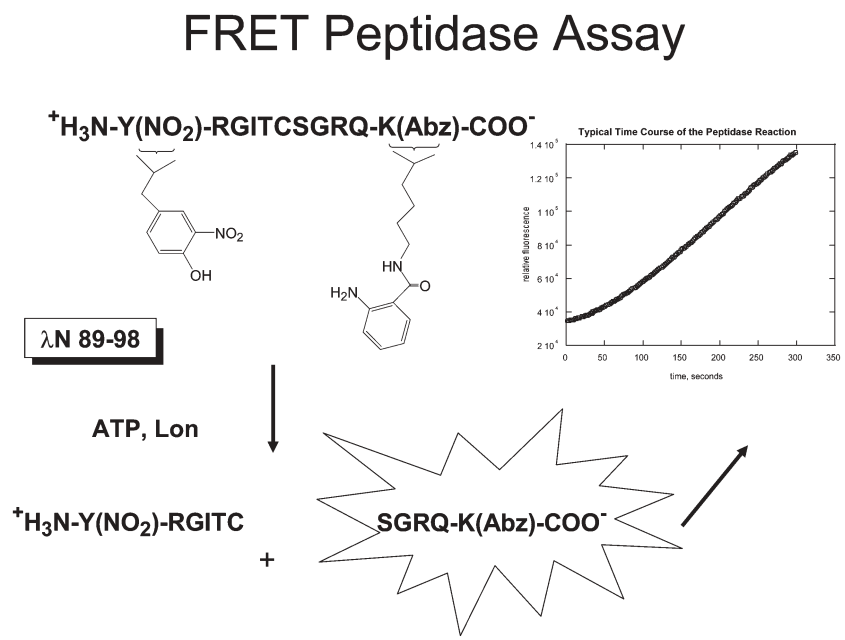


Fig. 1 The S1 peptide contains the anthranilamide fluorophore at the carboxyl terminal of S1 whose signal is absorbed by nitrotyrosine located at the amino terminal. Cleavage of S1 at Cys Ser by Lon separates the anthranilamide peptide product from nitrotyrosine such that the time course of peptide hydrolysis can be continuously monitored by fluorescence spectroscopy. The utilization of anthranilamide and nitrotyrosine as a fluorescence donor acceptor pair in internally quenched peptide substrates was developed by Meldal and Breddam.[76]

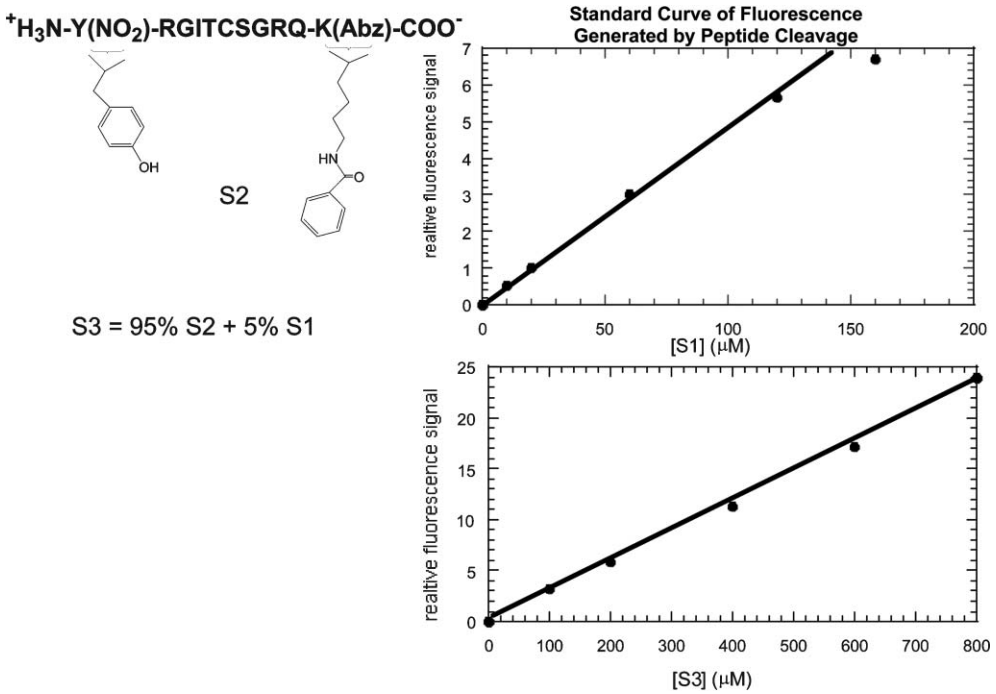


Fig. 2 The S2 peptide contains the same core sequence as S1, except nitrotyrosine is replaced with tyrosine and anthranilamide is replaced with benzoic acid. The S2 peptide is cleaved by Lon with identical kinetic parameters as S1. Due to inner filter effect, proteolytic cleavage of the S1 peptide generates fluorescence signal that correlates linearly with peptide concentration up to ~150 μM. However, the S3 peptide substrate system that contains 5% of S1 and 95% of S2 extends the linear range of the substrate.

as S3, generates linear fluorescence signals at substrate concentrations that are much higher than using the fluorescent peptide S1 alone.[67]

Kinetics of peptide hydrolysis

As a serine protease, Lon is activated by ATP or some non hydrolyzable ATP analogs such as AMPPNP, with the former being a more effective activator. During peptide cleavage, ATP is hydrolyzed to yield inorganic phosphate and ADP, which inhibits enzyme activity.[4,49,63] In the degradation of the S3 peptide, the steady state turnover of the reaction (k_{cat}) is seven fold higher in the presence of ATP than AMPPNP. This result demonstrates that although ATP binding supports peptide cleavage, ATP hydrolysis optimizes the reaction efficiency. Plotting the steady state rates of peptidase turnover as a function of [peptide] yields a sigmodial velocity plot that is best fit with a Hill equation, suggesting that the identical subunits of Lon may act cooperatively during catalysis.[67,68]

To better characterize the reaction intermediates along the peptidase pathway, we employed stopped flow fluorescence spectroscopy to monitor the first turnover of S3 peptide by Lon in the presence of ATP or AMPPNP, where the rates of peptide cleavage were quantified by the increase in fluorescence generated from the separation of the fluorescence quencher from donor in S3 (see above). In both cases, lag kinetic time courses are detected, indicating that S3 hydrolysis depends on the build up of a reaction intermediate (Fig. 3). The respective time courses were fit with the appropriate equation to yield a rate constant that represents the lag phase of the reactions. The lag rate constant for the ATP dependent peptidase reaction is seven fold higher than that obtained in the presence of AMPPNP, which further

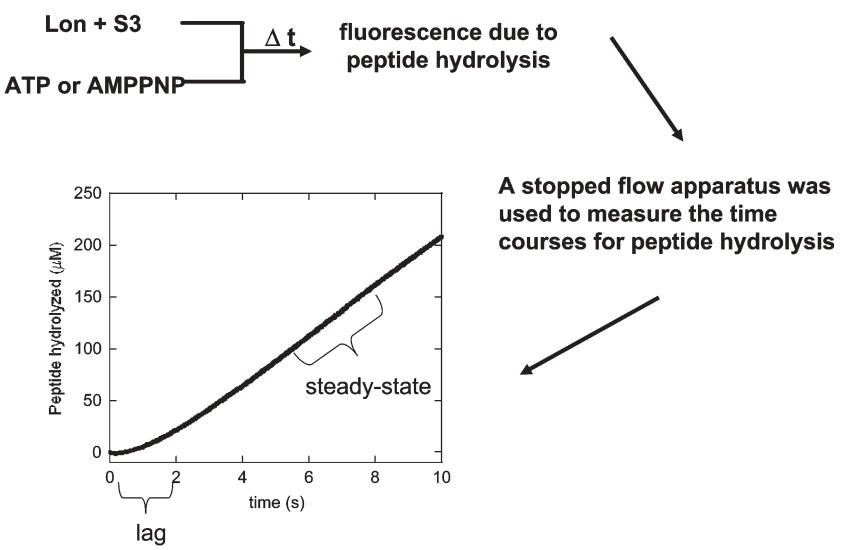


Fig. 3 The nucleotide dependent hydrolysis of the peptide substrate S3 can be monitored by stopped flow fluorescence spectroscopy.

confirms that ATP hydrolysis contri butes to the peptidase pathway in Lon. One possibility is that ATP binding and hydrolysis is used to generate an active peptidase form in Lon.[69]

Although ADP inhibits peptide hydro lysis by Lon, it induces the same con formational change in Lon as ATP or AMPPNP upon binding to the enzyme.[49,66,67,70] The mechanism of ADP inhibition was investigated by determining the inhibition constant of ADP (K_i) at various concentrations of ATP, S3 and ADP. On the basis that the K_i of ADP increases as a function of [S3] at constant [ATP], it is concluded that peptide substrates can alleviate ADP inhibition through allosteric interac tion.[67] Therefore it is possible that *in vivo*, Lon is ''turned off'' by binding to ADP and is allosterically ''turned on'' by protein substrates.

Kinetics of ATP hydrolysis by Lon

Lon possesses intrinsic ATPase activity that is stimulated by polypeptide sub strates.[4,49,63] Equilibrium binding experi ments revealed that despite being a homo oligomer, Lon has a set of high and low affinity sites for ATP, suggesting functional non equivalency among the subunits in Lon.[66] The functional non equivalency in the ATPase sites of Lon was characterized by chemical quench flow techniques, which monitor initial turnover of ATP hydrolysis at the different ATPase sites in Lon.[69,71] Under conditions of excess [ATP] over [enzyme], when all the ATPase sites are bound with ATP, the time course of [α^{32}P] ATP hydrolysis (Fig. 4) displays a burst in substrate consumption followed by a steady state production of ADP. The rate constant for the burst phase is 10 12 s^{-1} and is >10 fold faster than the steady state turnover number (k_{cat}) of ATP hydrolysis. The amplitude of the burst in ADP production, which gener ally reflects the concentration of active enzyme, was only 50% of the amount of Lon present in the reactions. This 50% reactivity is assigned to ''half sites reactivity'' in which the identical ATPase active sites on each Lon subunit possess two different affinities for ATP.[69,71] This conclusion is drawn according to three pieces of kinetic data. First, a filter binding study shows that all the enzyme subunits bind ADP. Secondly, a full burst in ADP production (with respect to total enzyme present) is observed when the reaction is chased with unlabeled ATP in a pulse chase experiment, indicating all the enzyme subunits can hydrolyze ATP. Finally, under single turnover conditions, when only the high affinity ATPase sites of Lon are occupied by ATP (*i.e.* 50% of the total enzyme concentration), Lon hydro lyzes ATP with a rate constant of 0.019 s^{-1}, which is >500 fold slower than that observed when all the ATPase sites are bound with ATP.[71] Taken together, the data indicate that the burst in ATP hydrolysis is generated by the low affinity sites, which constitute 50% of the total enzyme concentration, and the two set of ATPase sites hydrolyze ATP at different rates.

The importance of ''half sites reactiv ity'' is that both peptide cleavage and ATP hydrolysis at the low affinity sites reach steady state turnover prior to ATP hydrolysis at the high affinity sites. These data indicate that while the high affinity sites hydrolyze ATP slowly, they can support peptide cleavage with reduced catalytic efficiency compared to enzyme

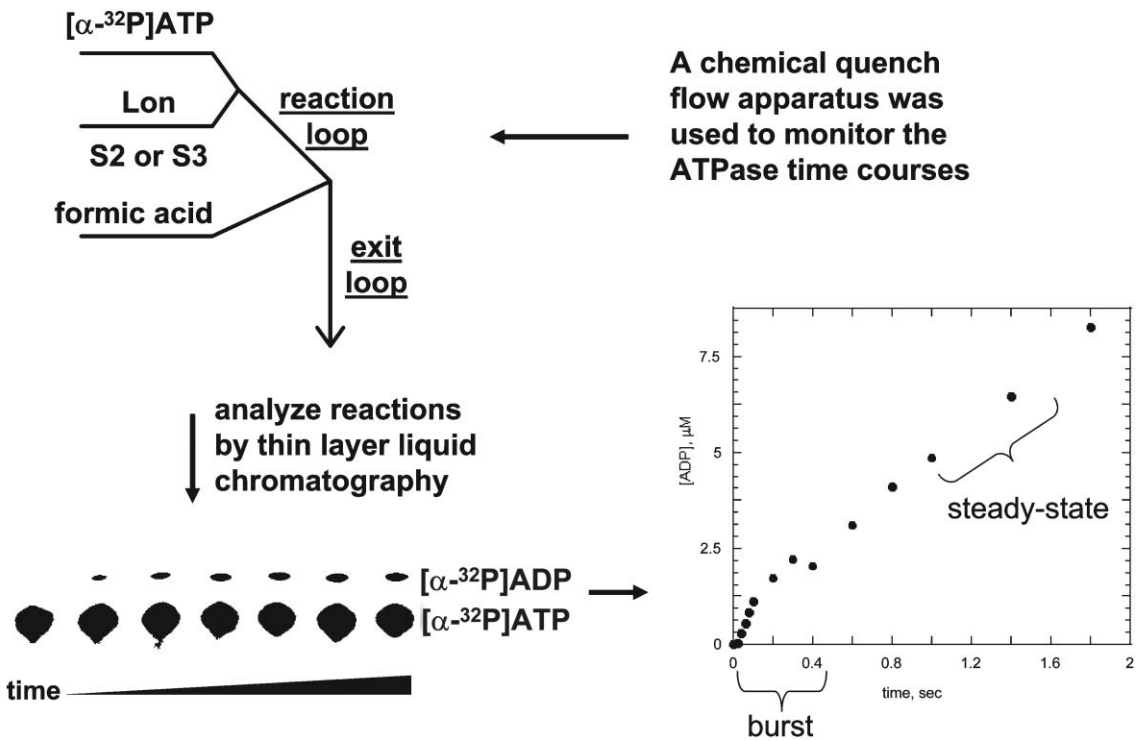


Fig. 4 The ATPase activity of Lon can be monitored by quantifying the amount of [α^{32}P]ADP generated from [α^{32}P]ATP at various time points using a chemical quench flow apparatus. Plotting the concentrations of ADP generated against the corresponding time points yields the time courses of the reactions.

with full ATP occupancy in both sets of sites. This result supports previous arguments that ATP hydrolysis and peptide cleavage are not stoichiometrically linked and implies that the two sets of ATPase sites may play different roles in mediating peptide cleavage.

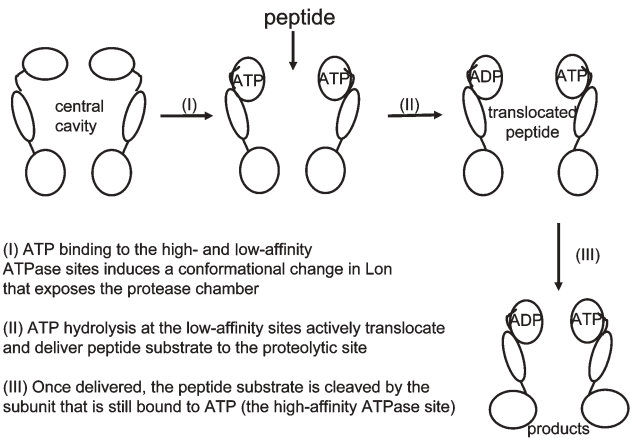
Functional connection between ATP hydrolysis and peptide cleavage

Under identical reaction conditions, the pre steady state time courses for ATP hydrolysis and peptide cleavage are mirror images of one another as peptide hydrolysis shows a lag preceding steady state turnover while ATP consumption shows a burst followed by steady state turnover.[69] The biphasic time course in ATP hydrolysis indicates that a step after nucleotide hydrolysis is rate limiting for Lon turnover. ATP hydrolysis must occur before peptide cleavage since the lag rate constant of $\sim 1\ s^{-1}$ for peptide is significantly slower than the burst rate constant of $\sim 10\ s^{-1}$ for ADP production. Furthermore, the k_{cat} of ATP hydrolysis ($1\ s^{-1}$) is comparable to the lag rate constant of peptide cleavage and suggests that the two hydrolytic reactions are coordinated with each other during the first enzyme turnover. As S3 peptide and λN protein both lack defined secondary structure, the ATPase dependency observed here may reflect the energy requirement to deliver peptide to the proteolytic site, which is likely to be distal from the ATPase domain.

These data were collectively used to develop the kinetic model relating the ATPase and peptidase activity of Lon as proposed in Scheme 1. Although Lon exists as a hexamer, for aesthetic purposes we represent it as a dimer containing one high and one low affinity ATPase site. The different kinetic behavior exhibited by the high and low affinity ATPase sites suggests that both sites function synergistically to cleave the peptide substrate.[69,71] As our model peptide substrates lack defined secondary structures, the observed ATPase dependency is attributed to the minimum energy requirement for Lon degrading an unfolded substrate containing one cleavage site. As indicated, ATP hydrolysis at the low affinity sites may function to translocate peptide substrate to the protease site in a manner that is similar to those observed in helicases.[72–74] The generation of ADP will inhibit further ATP hydrolysis at those sites, thereby preventing further peptide translocation. The "translocated peptide" however, will be hydrolyzed by the enzyme subunit containing the high affinity ATPase site. The proposed mechanism should be testable by pre steady state kinetic techniques.

The notion that ATP hydrolysis is used to deliver peptide substrate to the active site of Lon to increase substrate cleavage efficiency is consistent with observations that the non hydrolyzable ATP analog, AMPPNP, supports peptide cleavage albeit with reduced catalytic efficiency.[67,70] In fact, both ATP and AMPNP mediated peptide cleavage reactions display lag kinetics although they differ in the duration of the lag phase and k_{cat} for peptide hydrolysis. It is likely that without hydrolysis, AMPPNP is an inferior Lon activator because it cannot effectively deliver peptide to the protease active site. Therefore, it is predicted that ATP hydrolysis at the low affinity sites will be required to unfold as well as to deliver the scissile peptide bond to the protease site.

In summary, we have demonstrated that despite its complex proteolytic machinery, Lon can be characterized by classical enzyme kinetics techniques. The functional connection between the ATPase and peptidase activities of Lon is revealed by establishing the sequence of events occurring along the reaction pathway through enzymatic characterization of the respective hydrolytic activities under identical reaction conditions. In addition to investigating the kinetic mechanism of Lon, we have also used the fluorescence peptidase system to screen lead inhibitors against *Salmonella typhimurium* Lon[75] and learned that the proteasome inhibitor, MG 262, is a potent peptide based inhibitor against Lon. As bacterial and human Lon exhibit differences in the k_{cat}/K_m value in cleaving S3, a possible strategy to develop specific inhibitors against the bacterial enzyme will be to exploit its substrate specificity. However, the molecular mechanism by which Lon selects peptide or protein substrates is still not clear. At present, we are examining the kinetics of *E. coli* Lon degrading a panel of peptides of varying sequence in order to obtain mechanistic insights into how the primary amino acid sequences of polypeptide substrates affect Lon activity. In addition, we shall utilize the kinetic approach described in this article to study the processive degradation mechanism of Lon.[3,4,47,48]



Scheme 1

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