INVESTIGATING THE MECHANISM OF ATP-DEPENDENT DEGRADATION
OF A BACTERIAL PROTEIN INVOLVED IN NUCLEIC ACID METABOLISM

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>λN</td>
<td>Lambda N protein: a λ phage protein that allows <em>E. coli</em> RNA polymerase to transcribe through termination signals in the early operons of the phage</td>
</tr>
<tr>
<td>λNC26</td>
<td>λN with a cysteine at the 26 position, all other cysteines mutated to leucine, and all tryptophans mutated to phenylalanine</td>
</tr>
<tr>
<td>λNC42</td>
<td>λN with a cysteine at the 26 position, all other cysteines mutated to leucine, and all tryptophans mutated to phenylalanine</td>
</tr>
<tr>
<td>λNC99</td>
<td>λN with a cysteine at the 26 position, all other cysteines mutated to leucine, and all tryptophans mutated to phenylalanine</td>
</tr>
<tr>
<td>8998Bz</td>
<td>YRGITCSGRQ-K(Bz)</td>
</tr>
<tr>
<td>A</td>
<td>Alanine, Ala</td>
</tr>
<tr>
<td>AAA+</td>
<td>ATPase associated with various cellular activities</td>
</tr>
<tr>
<td>Abz</td>
<td>anthranilamide, the fluorescent quencher in the fluorescent peptides</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AMPPNP</td>
<td>Adenyl 5-imidotriphosphate – a nonhydrolyzable ATP analogue</td>
</tr>
<tr>
<td>ATPase</td>
<td>adenosine triphosphatase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>BME</td>
<td>beta-mercaptoethanol</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BsLon</td>
<td><em>Bacillus subtilis</em> Lon</td>
</tr>
<tr>
<td>Bz</td>
<td>benzoic acid</td>
</tr>
<tr>
<td>C-His-λN</td>
<td>C-terminal his-tagged λN</td>
</tr>
</tbody>
</table>
Cam  Chloramphenicol
C  endpoint
CAPS  3-(Cyclohexylamino)-1-propanesulfonic acid
CcrM  *Caulobacter crescentus* cell cycle regulated DNA methyltransferase
ClpXP  an ATP-dependent protease belonging to the same family as Lon
CsFR\(\lambda\)N  fluorescent protein substrate of Lon developed by GenScript, full length \(\lambda\)N protein contain an Abz and nitrotryrosine at the carboxyl
CsFR\(\lambda\)NA  10 % CsFR\(\lambda\)N + 90 % C99 (this substrate mixture was used to correct for the inner filter effect otherwise observed at high peptide substrate concentration)
C terminal  carboxyl-terminus
Cys  C, Cystoine
DAM  *E. coli* DNA adenine methyltransferase
dansyl  5-(dimethylamino)naphthalene-1-sulfonyle
dansyl\(\lambda\)NC26  \(\lambda\)NC26 with a dansyl attached to the cysteine at the 26 position
dansyl\(\lambda\)NC42  \(\lambda\)NC42 with a dansyl attached to the cysteine at the 42 position
dansyl\(\lambda\)NC99  \(\lambda\)NC99 with a dansyl attached to the cysteine at the 99 position
DCM  *E. coli* DNA cytosine methyltransferase
DNA  deoxyribonucleic acid
DTT  dithiothreitol
*E. coli*  *Escherichia coli*
EDTA  Ethylenediaminetetraacetic acid
ELon  
*Escherichia coli* Lon

FRET  
fluorescence resonance energy transfer

sFR\(\lambda\)N  
\(\lambda\)N with a FRET pair attached

FR89-98  
a fluorescent model peptide substrate consisting of residues 89-98 of the \(\lambda\)N protein and containing the fluorescence donar Abz and the fluorescence quencher Y-NO\(_2\)

H\(_2\)O  
water

HCl  
hydrochloric acid

HEPES  
4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid

His  
histidine

hLon  
human Lon

HPLC  
high-performance liquid chromatography

Hsluv  
an ATP-dependent protease belonging to the same family as Lon

IPTG  
isopropyl B-D-1-thiogalactopyranoside, used to induce gene expression

\(k_{\text{cat}}\)  
\(V_{\text{max}}/[E]\)

\(k_{\text{cat}}/K_m\)  
Substrate specificity constant

\(K_i\)  
Inhibition constant

\(K_m\)  
Michaelis constant equal to [substrate] required to reach \(\frac{1}{2}\) \(V_{\text{max}}\)

\(k_{\text{obs}}\)  
rate/[E]

K  
lysine, Lys

Kan  
Kanamycin

KOAc  
potassium acetate

kDa  
kilodalton
KP\textsubscript{i} potassium phosphate
MANT 2'-(or 3') O-(N-methylantnraniloyl)
MgATP magnesium adenosine triphosphatase
Mg(OAc)\textsubscript{2} Magnesium acetate
MOPS 3-(N-morpholio)propanesulfonic acid
msec milliseconds
MsLon Mycobacterium smegmatis Lon
n hill coefficient
N-His-\&N N-terminal his-tagged \&N
Ni-NTA agarose agarose nickel nitriliotriacetic acid
nm nanometer
NsFR\&N fluorescent protein substrate of Lon developed by GenScript, full length \&N protein contain an Abz and nitrotryrosine at the amino terminal
NsFR\&N\&A 10 % NsFR\&N + 90 % C26 (this substrate mixture was used to correct for the inner filter effect otherwise observed at high peptide substrate concentration)
N terminal amine-terminus
NO\textsubscript{2} Nitro
OD Optical density
\textsuperscript{32}P radioactive isotope of phosphorus
P11 Phosphocellulose cation exchange resin used to purify Lon
PEI cellulose polyethylenimine-cellulose
P\textsubscript{i} inorganic phosphate
PMT Photomultiplier tube
PVDF Polyvinylidene difluoride membrane
S  Serine, Ser

[S]  substrate concentration

S1  fluorescent peptide substrate of Lon developed by our lab based on λN protein (3-NO₂)YRGITCSGRQK(Abz)

S2  nonfluorescent analogue of S1 YRGITCSGRQK(Bz)

S3  10% S1 + 90% S2 (this substrate mixture was used to correct for the inner filter effect otherwise observed at high peptide substrate concentration)

SB  superbroth, media for bacteria growth

SDS  Sodium Dodecyl Sulfate

SDS-PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sec  seconds

S. Typhimurium  *Salmonella enterica* subspecies enterica serovar Typhimurium

TCEP  tris(2-carboxyethyl)phosphine

TFA  trifluoroacetic acid

TLC  thin-layer chromatography

Tris  Tris(hydroxymethyl)aminomethane

Trp  tryptophan

Tyr  tyrosine

WT  wild type

Y- NO₂  nitrotyrosine, the fluorescence quencher used in the fluorescent peptides

UV  Ultraviolet

v  Rate

V_{max}  Maximal rate
Investigating the Mechanism of ATP-dependent Degradation of a Bacterial Protein Involved in Nucleic Acid Metabolism

Abstract

By

ITEEN CHENG

Lon is an ATP-dependent serine protease that degrades damaged, misfolded, and certain regulatory proteins in the cell in order to maintain proper cellular function. Lon exists as a homo-oligomer with one ATPase domain and one protease domain in each sub unit. Truncated ELon mutants were used to determine the functionality of the N-terminal domain. Through steady-state and pre-steady-state conditions it was found that despite the removal of ~30 % of the N-terminal, substrate degradation still takes place. A physiological substrate of Esherichia coli Lon (ELon) called lamda N (λN) was used to demonstrate that the directionality and rate of cleavage. Using fluorescent model substrates (dansylλN and sFRλN), as a tool, we have employed steady-state and pre-steady-state kinetic techniques to evaluate the rate of translocation and cleavage under ATP conditions. The results obtained from these experiments suggest that ELon delivers the C-terminal of λN to the active site first followed by the N-terminal. The translocation of λN in the presence of ATP shows an increasing in step curve; this could be a result of changes in a conformation change of Lon as the fluorescent substrate moves closer to the active site. After complete translocation, cleavage of all peptide cleavage sites takes place within a short time frame. The initial burst of ATPase activity suggests it is correlated to the initial stage of the
translocation step. This indicates that ATP hydrolysis occurs before complete substrate translocation. Collectively, these results can be taken and fit with a global explorer program to yield a possible mechanism for ELon. Using this information we can determine the differences it has with hLon (human Lon protease). Previously, ADP was found to inhibit ELon activity. However, ADP inhibition on hLon is unknown. The similarities and differences obtained from hLon can provide information in the development of new therapeutic approaches to cure certain bacterial diseases in human.
Chapter 1:

Introduction of Lon Protease
Lon (protease La) is an ATP-dependent serine protease that is found ubiquitously in nature. In eukaryotes, Lon is localized in the mitochondria and helps maintain proper cellular function, while in prokaryotes it is found in the cytosol.\textsuperscript{1-7} Lon, like other ATP-dependent proteases such as FtsH, ClpAP, ClpXP, and HslUV, belongs to the AAA+ (ATPase Associated with various cellular Activities) family of proteins. These proteins contain an ATPase domain, which is highly conserved and contains a Walker A and B motif where ATP binding and hydrolysis takes place.\textsuperscript{8, 9} Lon is considered to be one of the simplest proteases because it contains both the ATPase and protease domain in a single subunit (Figure 1.1).\textsuperscript{10-12} The quanterny structure of ELon has been found to be a hexamer using electron microscopy.\textsuperscript{13, 14}

1.1 Function of Lon

The main function of Lon is to degrade damaged, irregular and short-lived regulatory proteins in cells in order to maintain proper cellular function.\textsuperscript{3, 10, 11, 15-18} Lon protease has three activities: intrinsic ATPase, substrate stimulated ATPase, and ATP-dependent proteolysis. In bacteria, such as Escherichia coli (ELon), Lon is not only the major contributor to degrading damaged proteins but it is also regulates proper cellular function by degrading damaged and irregular proteins. In other words, Lon is therefore a critical protein for maintaining cellular homeostasis. For example, Lon degrades SulA protein, which inhibits cell division and the production of capsular polysaccharide by degrading the transcriptional activator RcsA.\textsuperscript{19, 20} Lon is also critical for maintaining the structure and integrity of mitochondria in humans.\textsuperscript{21} Oxidative stress damages
proteins which are accumulated in the mitochondria. Lon has been found to degrade oxidatively damaged proteins over their native counterpart and as a result, it has been suggested that Lon is linked to protecting the mitochondria.\textsuperscript{22, 23} Therefore, it has been implied that Lon is linked to age related mitochondrial dysfunction.

It has been found that certain pathogenic bacteria, such as \textit{Salmonella enterica} subspecies enterica serovar Typhimurium (S. Typhimurium) are responsible for causing a range of human diseases, such as gastroenteritis and typhoid fever. S. Typhimurium Lon protease is required for systemic infection in mice, this is a common study model for S. Typhimurium infection in humans.\textsuperscript{24} When Lon-deficient S. Typhimurium is administered as an oral vaccine in mice it has been shown to confer protection against subsequent infection by S. Typhimurium.\textsuperscript{25} ELon and S. Typhimurium Lon share > 99\% sequence identity.\textsuperscript{26} As opposed to the bacterial and human (hLon) enzymes, there is only 42\% sequence identity.\textsuperscript{26} Exploiting differences between hLon and bacterial Lon may be a possible way to find compounds that can selectively inhibit the bacterial version over the human version. Bacterial Lon has been studied, however details of how hLon works are still unknown. Therefore, identifying specific bacterial Lon inhibitors could be used as a novel therapeutic approach in treating certain bacterial infections.\textsuperscript{27, 28} Before we can exploit the differences between the two, we need to know how hLon works. Therefore, our lab is focused on designing experiments that will provide information about the mechanism of Lon.
Figure 1.1 Structure of Lon.
Lon oligomer is composed of an ATPase domain and a protease domain in each monomeric subunit. Six subunits form the hexameric structure of Lon.

Figure 1.2 Domain organization of ELon in the primary sequence. Numbers above the domain represent the amino acid numbers.
1.2 Structure of Lon

ELon has a molecular weight of approximately 88 kDa, while hLon has a molecular weight of approximately 98 kDa. ELon consists of three domains: the ATPase domain, the protease domain, and the N-terminal domain (Figure 1.2). Unlike most ATP-dependent proteases in which the ATPase and protease domains are separate and oligomerized to form a functional enzyme, Lon has been found to be a homo-oligomer with both its ATPase and protease domains in the same monomeric unit. The crystal structure of the N-terminal domain of ELon has been published but the function is unclear; however, it is thought to be involved in substrate recognition. Only parts of the crystal structure for the ATPase domain have been reported. Upon ATP binding, Lon, like other AAA proteases, has been shown to induce a conformational change. The crystal structure for the protease domain has been solved for both ELon and hLon. Based on the proposed crystal structure, Lon is revealed to oligomerize into a ring shaped structure with the proteolytic active site in the center.
1.3 General mechanism of Lon

Although Lon requires ATPase activity to degrade protein or peptide substrates, the ATPase activity is not entirely coupled to the peptidase activity, as the enzyme hydrolyzes ATP in the absence of peptide substrate.\textsuperscript{36} It has been found when the proteolytic active site S679 in ELon is mutated to an alanine (S679A ELon), proteolytic activity, but not ATPase activity, was abolished\textsuperscript{36, 37} The ATPase rate is enhanced in the presence of protein substrate and that enhancement is still observed with the mutant, indicating that mutant still binds protein substrates.
**Figure 1.3 Proposed Mechanism for ATP-Dependent Proteolysis.**

The protease recognizes and binds a protein substrate (step 1). Upon ATP hydrolysis, the protein is unfolded (step 2), and then translocates through the central cavity to the proteolytic site (step 3), where protein degradation takes place (step 4). If an unstructured substrate is used, step 2 can be bypassed.
A general mechanism has been proposed: first, the protease recognizes and binds a protein substrate. Upon ATP hydrolysis, the protein is unfolded, and then translocated through the central cavity to the proteolytic site, where proteolysis occurs. During ATP hydrolysis, ATP is hydrolyzed to from ADP and P\(_i\). Unstructured substrates can bypass the unfolding step and be directly translocated into the central cavity for protein degradation.\(^3\) Lon activity is dependent on the presence of ATP and Mg\(^{2+}\).\(^1\)\(^,\)\(^2\)\(^,\)\(^3\)\(^,\)\(^9\) Mn\(^{2+}\) and Ca\(^{2+}\) can replace Mg\(^{2+}\), but with less efficacy.\(^3\)\(^,\)\(^9\) The products of the enzyme's protease activity are typically five to fifteen amino acids long.\(^3\)\(^,\)\(^4\)\(^0\)\(^,\)\(^4\)\(^1\) It has been observed that another nucleotide, such as non-hydrolyzable analogue, AMPPNP (Figure 1.4), can support peptide bond cleavage but at a slower rate.\(^3\)\(^,\)\(^4\)\(^2\) The products are still the same, indicating that nucleotide binding but not hydrolysis is required for cleavage. This suggests that the binding of ATP has an effect on the activation of the peptidase activity of Lon. Figure 1.5 shows the mechanism for ATP-dependent protein breakdown by the protease Lon.\(^3\) The model indicates an “active” and an “inactive” form of Lon, where the binding of ADP causes Lon to become inactive over time. The binding of additional protein substrate promotes the release of ADP and allows another ATP molecule to bind, repeating the cycle.\(^3\)\(^,\)\(^4\)\(^3\)
Figure 1.4 Structure of ATP verses the non-hydrolyzable analogue AMPPNP. AMPPNP has an imido linker (boxed in red) between the β and γ phosphates instead of oxygen. This causes AMPPNP to be non-hydrolysable because the phosphate at γ position cannot be attacked.

Figure 1.5 Proposed mechanism for *E. coli* Lon protease. ATP binding activates the proteolytic site and the peptide bond is cleaved. Upon ATP hydrolysis, the enzyme is inactivated due to ADP being bound.
1.4 **Substrate of Lon**

Small synthetic peptides have previously been used to study the protease activity of Lon; however their hydrolysis requires only ATP binding. Small protein substrates do not mimic the physiological substrates of Lon, therefore they do not enhance ATP hydrolysis in the same way as protein substrates. The ATPase activity of Lon protease is also stimulated by the protein substrates. Some of these protein substrates of ELon include λN, RcsA, CcdA, and SulA. Not all of these substrates are specific to Lon. There has been no specific sequence tag to help predict substrate cleavage sites by Lon. It has been found that Lon generally cleaves between hydrophobic residues that are surface-exposed and surrounded by highly charged environment without apparent preference for secondary structure; however it does not cleave after all hydrophobic residues. The λN protein is a bacteriophage protein and is a physiological substrate of ELon that does not have a secondary structure in the absence of RNA. Thus, the protein does not need to be unfolded before translocation and cleavage, therefore skipping step 2 in Figure 1.3. The cleavage sites of λN have been determined by Maurizi, M.R., as seen in Figure 1.6.
Figure 1.6 λN protein sequence and cleavage profile.44

The λN protein has seven major Lon cleavage sites (red arrows).
1.5 Monitoring peptide cleavage

The presence of multiple cleavage sites makes it difficult to directly correlate the binding and hydrolysis of ATP to a specific cleavage event. In order to address this issue, a ten amino acid synthetic peptide mimic of λN protein that contains one single cleavage site was synthesized by Irene Lee. As seen in Figure 1.7, this substrate contains residues 89-98 of λN protein. This synthetic peptide, FR89-98 only contains one Lon cleavage site as well as residues 89-98 of λN (Figure 1.7a). Fluorescent resonant energy transfer (FRET) was utilized by using this peptide substrate. FR89-98 contains a fluorescent donor anthranilamide (Abz) on the C-terminal lysine residue and a fluorescent quencher, 3-nitrotryosine (Y-NO₂), on the N-terminal. The cleavage site is located between residues Cys93 and Ser94. When the quencher and the donor are in close proximity to each other, the emission of the donor is quenched. The cleavage site is located in between the two groups, therefore, when cleaved, the donor and quencher separate, and the two are no longer close enough to be quenched, leading to an increase in emission of the donor over time (Figure 1.8).
Figure 1.7 Structure of the fluorescent and non-fluorescent peptide substrate λN89-98.53

λN89-98 consists of residues 89-98 of the λN protein. The fluorescent analog contains the fluorescent donor Abz and the fluorescent quencher Y-NO2 (A). The non-fluorescent analog contains Bz instead of Abz and Y instead of Y-NO2 (B). Both peptides are degraded identically by Lon. The arrow indicates the cleavage sites.
Figure 1.8 Peptidase activity assay. In the presence of Lon and ATP, the fluorescence peptide is hydrolyzed. Peptide cleavage is monitored by an increase in fluorescence over time, as the fluorescence donor (Abz) is separated from the quencher (Y-NO2) when the cleavage site between Alanine and Lysine is cleaved. In the absence of ATP, no increase in fluorescence signal is observed overtime. The rate of reaction is determined from the slope of the linear region of the time course. Plots were obtained from my WT ELon peptidase activity experiments.
The peptidase activity of Lon can be measured in real time at excitation 320 nm and emission 420 nm using fluorescence spectroscopy. Using this peptide model we could continuously monitor the peptide cleavage of Lon. However, at high concentrations (> 150 μM) of peptide, an inner filter effect was observed. The fluorescence due to the cleavage of the peptide deviates from the linear relationship between the signal and the amount of peptide hydrolyzed. Because nitrotyrosine quenches anthranilamide, the fluorescence is through an intermolecular mechanism rather than an intramolecular mechanism. In order to counteract the inner filter effect problem, a non-fluorescent (Figure 1.7b) version of λN89-98 was made. The non-fluorescent version had a benzoic acid amide instead of the Abz, and the Y-NO₂ was replaced with a tyrosine. By using a mixture of the two peptides (S3, 90% non-fluorescent and 10% fluorescent), the inner filter effect was corrected and high concentrations of peptide were monitored. Other peptides containing Lon cleavage sites were developed as well. Small peptide substrates have worked well thus far, however Lon degrades full length substrates that contain more than one cleavage site and there have been no tools to monitor this thus far. Full length λN sequence was made with a fluorescent acceptor and quencher. This will be described in a later chapter.

My project is concentrated on finding details that will explain the protease mechanism of ELon in order to help determine the mechanism of human Lon. I have used steady-state and pre-steady-state kinetic techniques in order to determine the kinetic mechanism of the proteolytic activity. These techniques helped me to explore the role of ATP binding and hydrolysis in peptide cleavage.
and explore whether Lon must recognize specific sequences in the substrate in order for degradation to occur.
Chapter 2

Contribution of the N-terminal of Lon Protease to Protein Degradation
2.1 Introduction

Lon protease is a homo-oligomeric serine protease that is activated by ATP.\textsuperscript{2,3} In previous studies, it has been determined that the enzyme shows intrinsic ATPase activity ($k_{cat} \sim 0.2 \text{ sec}^{-1}$), but when Lon is stimulated by peptide or protein substrates the $k_{cat}$ is $\sim 1 \text{ sec}^{-1}$.\textsuperscript{34, 46, 53} Being an oligomeric protease machine, Lon contains an amino-terminus, an ATPase and a proteolytic domain within each enzyme subunit.\textsuperscript{3} The ATPase and proteolytic domains have been known to share high sequence homology in most bacterial Lons, while the amino-terminal is diverse. For example, in a recent study, most of the crystal structure of BsLon (\textit{bacillus subtilis} lon) has been solved. BsLon residues 246-770 share about 70% sequence homology with residues 248-772 in ELon, which contains the ATPase domain and the proteolytic domain. While the N-terminal domain shows a 47% similarity. Comparing the sequences of BsLon and ELon reveals that the only structurally undefined region in the former consists of residues 210-245, which correspond to residues 212-247 in ELon.

The trypsin-sensitive lysine residues are found in the structurally unknown region (K235 and K239 in ELon\textsuperscript{34} and K233 and K237 in BsLon). Given the conserved nature of residues surrounding positions 200-240 of the two bacterial Lon and the lack of protection from limited tryptic digestion from nucleotide binding, this area may be important for enzyme function. In a publication by J. Patterson et al., the enzyme undergoes conformational change when nucleotides bind, and two prominent fragments with molecular masses of 67 and 26 kDa were found.\textsuperscript{34} Trypsin cleavage sites identified by peptide sequencing revealed
that the 67 kDa fragment belongs to the ATPase domain, while the 26 fragment belongs to the amino terminus domain. In order to evaluate the function of the N-terminal domain, a series of truncated proteins were generated. The truncated mutant Δ 232-252 was generated by removing residues 232-252 of WT ELon. From previous studies in our lab, performed by J. Patterson et al., K239 of ELon was found to be susceptible to limited tryptic digestion in the ATP bound enzyme form.\textsuperscript{34} Truncated mutant Δ1-239 was generated to evaluate if this part of the N-terminal is needed in order for enzymatic activity to occur. The third mutant, Δ1-252, was generated for comparison such that its functional profile could be compared to those determined for the other two mutants and to WT Lon in order to deduce the contribution of residues 239 to 252 towards enzyme activity. All mutants were cloned by a previous undergraduate Jon Huang. All mutants were sent out for sequencing to ensure that the mutations were correct.

It is known that Lon possesses intrinsic ATPase activity that is stimulated by protein or certain peptide substrates.\textsuperscript{3, 36, 56} In order to evaluate the effects of enzyme truncation, intrinsic and stimulated ATPase activity of truncated mutants were compared to WT ELon. In this chapter, protein degradation is also examined. Lon is a processive protease that unfolds and translocates multiple Lon cleavage sites during protein degradation; efficient proteolytic activity is dependent on full ATPase hydrolysis activity, and therefore the reduction in efficiency of ATPase in the truncated mutants may also be affected.\textsuperscript{3, 44} In the protein degradation studies, two different substrates were used. The degradation of endogenous unstructured substrate λN was compared with the putative
substrate *Caulobacter crescentus* CcrM (CcrM) (gift from Benkovic lab at Penn State University). *Caulobacter crescentus* CcrM is known as Cell Cycle Regulated DNA Methyltransferase and is an adenine DNA methyltransferase anticipated to adopt a globular structure like those found in certain DNA adenine methyltransferase. Using CcrM the degradation profile of a structured substrate versus an unstructured substrate was examined.

### 2.2 Methods and Material

#### 2.2.1 Materials

Restriction endonucleases were purchased from Promega and from New England Biolabs. Oligonucleotides were custom-synthesized by IDT, Inc. Solvents, buffers, chromatography resin, antibiotics, culture media and PEI cellulose TLC plates were purchased from Fisher Biotechnology and from Sigma/Aldrich. Plasmids used for protein expression and competent cells were purchased from Invitrogen and from Novagen. [α³²P]ATP was purchased from Perkin-Elmer Life Science.

#### 2.2.2 Lon purification

Lon was purified as previously described. In brief, *E. coli* cells hosting the Lon expression vector pet24c+ (previous student, Diana Vineyard provided the ELon plasmid) were grown in superbroth (SB, per L: 30 g bactotryptone, 20 g yeast extract, 10 g MOPS (3-(N-morpholio) propanesulfonic acid), pH 7, 30 µg/mL kan (kanamycin)) at 37 °C and induced with 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) at OD₆₀₀ = 1 for one additional hour. Cell lysate was loaded onto a phosphocellulose column pre-equilibrated with 50 mM KP₃
(potassium phosphate), pH 7, 1 mM DTT (Dithiothreitol), and 20 % glycerol, and then eluted with a linear gradient of KP$_i$ buffer (0.1-0.5 M KP$_i$, pH 7, 2 mM DTT, 20% glycerol). Lon positive fractions were pooled, concentrated by an amicon concentrator and then dialyzed into 65 mM KP$_i$, pH 7, 2 mM DTT, and 20 % glycerol. The dialyzed protein was loaded onto a DE 52 column equilibrated with the same buffer. The resulting column was eluted with a linear KP$_i$ gradient (0.085-0.3 M KP$_i$, pH 7, 2 mM DTT, 20% glycerol). Positive fractions were collected and run on a 12.5 % SDS-PAGE gel in order to confirm. The Lon positive fractions were pooled and precipitated with 100 % ammonium sulfate. The precipitate was recovered by centrifugation and then dissolved in a minimum amount of 75 mM KP$_i$, pH7, 75 mM KOAc, 2 mM DTT, 5 mM Mg(OAc)$_2$ (magnesium acetate), and 20% glycerol prior to purification by a Superose 6 (pharmacia) gel filtration column equilibrate with the same phosphate buffer. Lon recovered from gel filtration chromatography was concentrated and stored in aliquots at -80 °C.

2.2.3 Protein quantification

The concentrated Lon mutants were quantified using Bradford assay. Purified E. coli Lon of known concentration was used as a standard. The concentration of each mutant was calculated using their molecular weights, Δ 232-252 (85,000 g/mol), Δ1-252 (58,764 g/mol), and Δ1-239 (60,338 g/mol). The concentration of protein could be determined by using absorbance spectroscopy at A$_{280}$. The molar absorption coefficients of each mutant at 280nm were calculated based on equation 2.1.
\[ \varepsilon(280) (\text{M}^{-1}\text{cm}^{-1}) = (#\text{Tyr})(1490) + (#\text{Trp})(5500) + (#\text{cystine})(125) \]  \hspace{1cm} (2.1)

The molar absorption coefficients were determined for \( \Delta 232-252 \) (46300 M\(^{-1}\)cm\(^{-1}\)), \( \Delta 1-252 \) (37360 M\(^{-1}\)cm\(^{-1}\)), and \( \Delta 1-238 \) (37360 M\(^{-1}\)cm\(^{-1}\)). The concentration can be calculated using the Beer-Lambert law equation 2.2

\[ A = \varepsilon I C \]  \hspace{1cm} (2.2)

Where \( A \) is the absorbance at 280 nm, \( \varepsilon \) is the molar absorption coefficient (M\(^{-1}\)cm\(^{-1}\)), \( I \) is the pathlength (cm), and \( C \) is the protein concentration (M).

### 2.2.4 \( \lambda N \) purification

The plasmid of \( \lambda N \) (pHF012, or \( \lambda \)NC99; gene was synthesized by Genescript), containing an N-terminal 6x His tag, was transformed into the BL21 DE3 and the cells were grown to \( \text{OD}_{600} = 0.6 \) in 30 \( \mu \)g/mL kan, in SB (superbroth), and induced for 2 hours with 1mM IPTG. The cells were pelleted by centrifugation, and lysed by adding 5 mL of lysis buffer per gram of cells (lysis buffer: 100 mM sodium phosphate pH 8.0, 10 mM Tris, 1 mM beta-mercaptoethanol, 8 M urea) and stirring for 45 minutes. All cell debris was pelleted by centrifugation. The cleared lysate was loaded onto a Ni-NTA agarose column (Qiagen) equilibrated with lysis buffer. The column was then washed with lysis buffer to remove all excess proteins. The \( \lambda N \) protein was eluted from the column with elution buffer in a step gradient fashion (elution buffers: a. (100mM sodium phosphate pH 6.3, 10 mM Tris, 1 mM beta-mercaptoethanol, 8 M urea), b. (100mM sodium phosphate pH 5.8, 10 mM Tris, 1 mM beta-mercaptoethanol, 8 M urea), c. (100mM sodium phosphate pH 4.5, 10 mM Tris, 1 mM beta-mercaptoethanol, 8 M urea)). The eluted protein was extensively dialyzed into \( \lambda N \)
storage buffer to remove urea (λN storage buffer: 20 mM Tris pH 7.6, 50 mM sodium chloride, 1 mM beta-mercaptoethanol, 20 % glycerol). A white precipitate formed during dialysis and was removed by centrifugation. The λN protein in λN storage buffer was analyzed by SDS-PAGE on a 12.5 % acrylamide gel and stained with Coomassie to assess purity. The protein was quantitated by using an extinction coefficient, at A280 (ε = 14,060 M⁻¹ cm⁻¹). To confirm accuracy, Bradford assay was also used to determine the concentration of λN (13,212 g/mol).

2.2.5 λN degradation assay

λN degradation assays contained 50 mM HEPES pH 8.0, 15 mM magnesium acetate, 5 mM DTT, 1 μM wild-type or E. coli Lon mutants (Δ232-252, Δ1-252, Δ1-238), and 20 μM wild-type λN protein; the reaction was initiated with 5mM ATP at 37°C. At various time points, reaction aliquots (10 μL) were quenched with 5 x SDS-PAGE loading buffer (2 μL). The aliquots were loaded and run on 17.5% SDS-PAGE gel and stained with Coomassie to detect the protein. The gel was viewed using the Biorad Gel Doc 2000 and Quantity One quantitation software.

2.2.6 His-CcrM degradation assay

λN degradation assays contained 50 mM HEPES pH 8.0, 15 mM Mg(OAc)₂, 5 mM DTT, 1 μM wild-type or E. coli Lon mutants (Δ232-252, Δ1-252, Δ1-238), and 1 μM λN or His-CCRM (gift from Benkovic laboratory) protein; reaction was initiated with 4 mM ATP at 37°C. At various time points, reaction aliquots (5 μL) were quenched with 5 x SDS-PAGE loading buffer (2 μL). The aliquots were
loaded and run on 15 % SDS-PAGE gel and stained with Coomassie to detect the protein. The gel was viewed using the Biorad Gel Doc 2000 and Quantity One quantitation software.

2.3 Results and discussion

2.3.1 Overexpression and purification of ELon mutants

Based on the amino acid sequencing results of ELon, the predicted molecular weights of the purified Lon mutants were: Δ 232-252 (85,000 g/mol), Δ1-252 (58,764 g/mol), and Δ1-239 (60,338 g/mol), respectively. Figure 2.1 shows the 12.5 % SDS-PAGE result of the purified proteins, including WT ELon for comparison. The estimated molecular weight of each mutant agrees well with the predicted molecular weight. To confirm the concentrations of each protein construct, both UV absorption and Bradford assay were performed as described in the material and methods sections.
Figure 2.1 Purified wild-type and mutant ELon.

12.5 % SDS-PAGE gel of purified WT and Lon mutants. Purified WT and Lon mutants were visualized by Coomassie Brilliant Blue staining of a denaturing gel containing each protein. Lane 2-4, in order, shows: WT, ∆232-252, ∆1-252, ∆1-239. \(^{67}\)
2.3.2 ATPase activity of mutant Lon versus WT

In previous studies, Lon has been shown to possess intrinsic ATPase activity which can be stimulated by protein or by certain peptide substrates.\(^3, 36, 56\) In order to evaluate the effects of the enzyme truncation, a previous graduate student, Natalie Mikita, used a substrate called λN to stimulate the ATPase activity of WT and Lon mutants.\(^44\) ATPase activity of Lon was measured by a radiometric ATPase assay, which was used to characterize the mechanism of WT ELon.\(^34\) The relative ATPase activity was measured at 10-fold the \(K_m\) of ATP in WT ELon, in the absence and presence of 2-fold the \(K_m\) of λN. All kinetic parameter values are summarized in Table 2.1. Based on the kinetic parameters that I obtained, neither the \(K_m\) of ATP nor the ability of λN to stimulate ATP hydrolysis in each protein is altered by any of the mutants; however, the \(k_{cat}\) of ATPase is reduced by 3.5 to 10-fold in Δ232-252 and in Δ1-252, respectively compared to WT and to Δ1-239 ELon. From previous studies, residues 232-252 are not part of the AAA+ domain, but results show a reduction in \(k_{cat}\) in the truncated mutant. One possible explanation is that the region containing residues 232-252 plays a role in modulating the ATPase activity. Another explanation is that this region is responsible for the folding/structural integrity of the hexameric enzyme that affects ATPase function.
Table 2.1 Comparison of the steady-state kinetic parameters for ATP hydrolysis activity for WT ELon and mutant ELon constructs.

<table>
<thead>
<tr>
<th></th>
<th>$k_{\text{cat}}$ (sec$^{-1}$)</th>
<th>$K_{\text{m}}$ (µM)</th>
<th>$k_{\text{cat}} + \lambda N$ (sec$^{-1}$)</th>
<th>$K_{\lambda N}$ (µM)</th>
<th>ATPase enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.43 ± 0.12</td>
<td>47 ± 10</td>
<td>1.32 ± 0.15</td>
<td>1.4 ± 0.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Δ232-252</td>
<td>0.12 ± 0.01</td>
<td>72 ± 15</td>
<td>0.18 ± 0.02</td>
<td>5.3 ± 2.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Δ1-252</td>
<td>0.06 ± 0.01</td>
<td>53 ± 12</td>
<td>0.12 ± 0.01</td>
<td>4.8 ± 1.7</td>
<td>1.9</td>
</tr>
<tr>
<td>Δ1-239</td>
<td>0.65 ± 0.16</td>
<td>50 ± 12</td>
<td>1.25 ± 0.20</td>
<td>1.6 ± 0.9</td>
<td>1.9</td>
</tr>
</tbody>
</table>
We have demonstrated ATPase stimulation of WT with 89-98 peptide, however we have not studied the ATPase stimulation with full length. Since Lon degrades full length substrates, we studied the ATPase stimulation with full length substrate with the mutants and WT for comparison. Stimulated ATPase activity of ELon mutants was also performed (Table 2.1). This provides the apparent affinity of various Lon constructs for λN ($K_{\lambda N}$). Based on Table 2.1, the $k_{cat}$ values of λN-stimulated ATPase are lowered in the mutants that lack the region 232-252. A slight variation in the $K_{\lambda N}$ values is detected among the enzymes. When standard error is taken into consideration the data suggest that $K_{\lambda N}$ values are comparable. 232-252 constitutes the putative coiled-coil region in Lon that may be involved in substrate recognition; the contribution of this region toward λN binding is minimal. When all the data are taken together, the loss in ATPase activity is the most pronounced effect associated with the removal of 232-252 in ELon. Since Δ1-239 displays WT-like ATPase activity, it is concluded that the region within 240-252 most likely contributes to ATPase activity.

### 2.3.3 Gel degradation of λN versus His-CCRM substrate

It is known that protein degradation is dependent on the ATPase activity of ELon. The reduction in the efficiency of ATPase in Δ1-252 and Δ232-252 mutants may also affect proteolytic activity. In order to qualitatively evaluate such possibility, a time course of WT and Lon mutant degrading the endogenous unstructured protein λN was obtained and compared using SDS-PAGE. The concentration of λN used was 20 μM, which was 2-fold the $K_m$ based on the value from the Maurizi study. At 1 μM enzyme concentration, only WT and
Δ1-239 show λN degradation after 30 min; however, WT Lon was seen to be more efficient than Δ1-239 in degrading λN (Figure 2.2 A-B). In contrast, mutants Δ1-252 and Δ232-252 showed no degradation (Figure 2.2 C-D). When the concentration of the enzyme was increased to 3 μM, λN degradation was also detected in Δ232-252 and Δ1-252 mutants; however, the efficiency was reduced compared to WT and Δ1-239. (Figure 2.2 E-H) Taking these results into account, the data reveal that despite the removal of ~30% of the protein from the N-terminal, Lon still remains functional but not as efficient as an ATP-dependent protease in processing the unstructured substrate λN. The putative structured protein substrate *Caulobacter crescentus* CcrM was obtained and compared using SDS-PAGE. CcrM, which is an adenine DNA methyltransferase, is anticipated to adopt a globular structure like those found in certain DNA adenine methyltransferase\(^ {57, 58}\) and was found to be poor substrate of WT ELon (Figure 2.3 A-D). Under equal stoichiometric amounts of enzyme and substrate concentrations, only slight CcrM degradation was observed in the reaction containing WT protease. When comparing these results with Figure 2.2, CcrM degradation results suggest that residues 1-239 contribute to the processing of a structured substrate such as CcrM. This is because Δ232-252 also fails to degrade CcrM, which suggests that the residues flanking 232 to 239 are likely involved in mediating CcrM degradation.
Figure 2.2 ATP-dependent degradation of λN protein by WT and Lon mutants.

Reaction aliquots containing ELon or mutant (1 μM, panels a-d; 3 μM, panels e-h), λN (20 μM) and 5 mM ATP in reaction buffers were quenched at the indicated times. The quenched time points were resolved by 17.5 % SDS-PAGE and visualized by Coomassie Brilliant Blue. Lanes 1-6 (in order) contain the time points when the reaction was quenched at 0, 1, 2, 5, 10, and 30 min. WT (a, e) and Δ 1-239 (b, f) degrade λN protein more efficiently than Δ 232-252 (c, g) and Δ 1-252 (d, h).
Figure 2.3 ATP-dependent degradation of His-CCR M by WT and Lon mutants.

Reaction time points containing 1 μM ELon or mutant digested with 1 μM CcrM in the presence of 5 mM ATP were quenched at various time points and then resolved by 15% SDS-PAGE. Lanes 2-8, in order, show the degradations of His-CcrM at 0, 1, 2, 5, 10, 30, 60 and 120 min. WT ELon (a) shows a slight degradation when compared to Δ 1-239 (b), Δ 232-252 (c), and Δ 1-252 (d) after 120 min.61
2.4 Conclusion

Lon possess intrinsic ATPase activity that is stimulated by protein or by certain substrates. In order to examine the effects of the truncated enzyme on the degradation of protein substrate, radiometric ATPase assays were performed. All of the truncated mutants possessed intrinsic ATPase activity stimulated by concentrations of λN corresponding to 3 to 5-fold the $K_m$ of λN. The $k_{obs}$ values for WT and Δ1-239 are comparable but Δ1-252 and Δ232-252 are ~4-fold lower. The values are similar for intrinsic and for stimulated (Table 2.1). The $K_m$ values for both intrinsic and stimulated have been altered due to the mutations.

In order to investigate whether the reduced efficiency of ATPase in Δ1-252 and Δ232-252 would affect the proteolytic activity, a time course of WT and mutant degradation of unstructured and structured proteins was performed. After 30 min, WT and Δ1-239 showed degradation of about half, but Δ1-252 and Δ232-252 showed no signs of degradation. Once the enzyme concentration was increased to 3 μM almost all λN was degraded for WT and Δ1-239 at 10 min. Even Δ1-252 and Δ232-252 showed degradations after 10 min, although still not as efficient as WT and Δ1-239. After observing the results on an unstructured substrate, a structured protein substrate, Caulobacter crescentus CcrM, was also studied. This experiment was performed under equal stoichiometric amounts of enzyme and substrate concentrations. After viewing the results on an SDS-PAGE, degradations were seen after 120 min for WT ELon; however, no degradation was seen for the three truncated mutants.
Taking the results from the ATPase and degradation studies, it is concluded that the region encompassing residues 232 to 252 is primarily accountable for the observed reduction in ATPase activity of the Lon mutants. Since residues 232 to 252 are not part of the ATPase domain, the effect of this region on the ATPase activity of ELon is thus allosteric. Comparing the activity profiles of WT with all three truncated mutants reveals that the degradation of the structured protein CcrM requires residues 232-239 (amino acid sequence: EQMKAIQK), since only WT possesses those residues. Therefore, residues within 232-239 are needed to process CcrM. Furthermore, λN degradation was observed in the reaction containing 3 μM, but not 1 μM of Δ232-252 or Δ1-252. A lack of protein degradation with 1 μM concentration of the mutants could be due to subsaturation of protease for substrate with reduced affinity. At 3 μM enzyme, there are more enzyme:substrate complexes formed, and therefore substrate degradation becomes apparent. This is supported by the observation that the $K_{\lambda N}$ values of WT and Δ1-239 are approximately 3 times lower than the other two mutants. This supports the idea that the region containing residues 240-252 (amino acid sequence: ELGEMDDAPDENE) contribute to substrate interaction. It has been proposed in previous studies that ELon binds protein substrate in multiple sites,\textsuperscript{62,63} and residues 232-239 and residues 240-352 are likely two of those spots. Another possible explanation for the apparent lack of CcrM degradation is that residues found in position 232-239 are needed to unfold the substrate in order for degradation to occur. Since λN lacks a defined structure,\textsuperscript{51}
unfolding is not necessary for protein translocation to occur before the degradation process.
Chapter 3

Steady State and Pre-steady State of Mutant Lon
3.1 Introduction

In order for peptide bond cleavage to occur, Lon unfolds and delivers multiple scissile bonds within the unfolded substrate to the proteolytic site. In chapter two, a qualitative representation of the truncated mutants degrading λN was examined. In this chapter, peptide bond cleavage in a quantitative manner was examined by kinetic techniques. In order to quantitatively examine peptide bond cleavage in each truncated mutant, a previously discovered fluorogenic peptide stated in Chapter 1, FR89-98, was used to monitor the kinetics.

The important aspects of the kinetic mechanism of an enzyme are ascertained from studying steady-state kinetic analysis. These studies allowed the determination of WT ELon previously. In contrast, the kinetics of the mutants is unknown, therefore, the techniques used to characterize WT can be used to characterize the mutants and compare them to WT. This allowed the determination of the importance of the N-terminal region of Lon. When steady-state studies were used together with pre-steady-state studies, the mechanism of Lon were closely examined. Stopped-flow spectroscopy was used to perform pre-steady-state studies. This technique rapidly mixes the reactants and monitors the reaction on the millisecond timescale, the time for a single enzyme turnover. Pre-steady-state cleavage has been used to monitor WT ELon and a lag phase was observed. The experiment provided information about the rate limiting step of the reaction, as well as the rate constant of each event under pre-steady state conditions. The lag phase in the pre-steady-state time course indicates that the step before cleavage is rate limiting. In contrast, we do not
know if the lag phase is still present with the ELon mutants; therefore, we wanted to use the same technique to monitor the pre-steady-state kinetics of the mutants and compare them to WT ELon.

3.2 Methods and Material

3.2.1 General materials

Plasmid construction, protein expression and purification were previously described in Chapter 2.

3.2.2 Peptidase of Mutant ELon

Peptidase activity was monitored on the Fluoromax-3 spectrofluorimeter (Horiba Group) as described previously. Assays contained 50 mM HEPES at pH 8.0, 5 mM Mg(OAc)$_2$, 2 mM DTT, varied FR89-98 or S3 peptide concentration (excitation at 320 nm and emission at 420 nm) and 150 nM Lon (WT and mutant); the reaction was incubated for 1 minute at 37 °C before being initiated with 1 mM ATP. FR89-98 is 100% fluorescently labeled peptide $\lambda$N89-98 (Y(NO$_2$)RGITCSGRQK(Abz)), and S3 is 10% fluorescently labeled peptide $\lambda$N89-98 with 90% non-fluorescent analog of BZ89-98 (YRGITCSGRQK(Bz)). S3 is a substrate mixture used to correct for the inner filter effect otherwise observed at high peptide substrate concentration. All assays were performed at least in triplicate, and the averaged value of the rates determined for each set of mutants was fitted to equation 3.1 as previously described.

$$k_{obs} = \frac{k_{cat}[S]^n}{(k_m[S]^m)} \quad (3.1)$$
Where $k_{obs}$ is the observed rate, $k_{cat}$ is the rate constant of product formation at saturating substrate concentration, $K_m$ is the Michaelis-Menton constant, $[S]$ is the substrate concentration, and $n$ is the hill coefficient.

3.2.3 Pseudo-first order time course of S3 cleavage by fluorescent stopped flow

Pre-steady-state experiments were performed on a KinTek Stopped Flow instrument controlled by the data collection software Stop Flow version 7.50 β. The sample syringes were maintained at 37 °C by a circulating water bath. Syringe A contained 1 μM Lon (mutant or WT), with 1 mM peptide substrate S3, 5 mM Mg(OAc)$_2$, 50 mM Tris-HCl (pH 8.1), 5 mM DTT, 30 mM KOAc, and 30 mM KPi. Syringe B contained 1 mM peptide substrate S3, 5 mM Mg(OAc)$_2$, 50 mM Tris-HCl (pH 8.1), 5 mM DTT, 30 mM KOAc, 30 mM KPi, and 1 mM ATP. Peptide cleavage was detected by an increase in fluorescence (excitation of 320 nm and emission with a 400 nm long-pass filter) following rapid mixing of the syringe contents in the sample cell over 120 sec (600 points for the first 20 sec and 400 points from 21 to 120 sec). The baseline of the fluorescence was normalized to zero, and the data shown are the results of averaging at least four traces. The concentration of the hydrolyzed peptide was calibrated by determining the maximum fluorescence generated per micromolar peptide due to complete digestion by trypsin under identical conditions on the stopped-flow. The lag equation was fitted to the averaged time courses:

$$ F = A \exp^{-k_{lag}t} + v_{ss}t + C $$

(3.2)
where $Y$ is the micromolar concentration of the hydrolyzed peptide S3, $A$ is the amplitude of the reaction, $k_{\text{lag}}$ is the pre-steady-state rate constant in seconds, $t$ is the time in seconds, $v_{ss}$ is the steady-state rate in units of micromolar product per second, and $C$ is the endpoint. The $v_{ss}$ value can be converted to a first-order rate constant ($k_{ss}$ in the unit of per second) by dividing by the enzyme concentration. The $v_{ss}$ values were obtained from those reported for the respective enzyme in Table 3.1. Since equation 3.2 is the general function that quantifies a biphasic time course, when $Y = 0$ at $t = 0$ and $C = A$, the equation changes to

$$Y = -A + A \exp^{-k_{\text{lag}}t} + v_{ss}t + C \quad (3.3)$$

Such that

$$Y = -A \left( \exp^{-k_{\text{lag}}t} \right) + v_{ss}t \quad (3.4)$$

When $A$ is defined as $v_{ss} - v_i/k_{\text{lag}}$, equation 3.2 becomes equivalent to the equation defining hysteresis, where $v_i$ is the rate corresponding to the initial phase of the time course.

For WT and Δ1-239, only the first 20 sec were used to obtain the pre-steady-state rate constant, while for Δ232-252 only the first 60 sec were used, and for Δ1-252 all 120 sec were used. Δ232-252 and Δ1-252 has a longer lag phase, therefore, a longer time duration was needed to observe the lag phase for these two mutants.
3.3 Results and discussion

3.3.1 Peptidase rate of WT versus mutant ELon

Based on the proposed general mechanism of Lon, Lon needs to unfold and deliver/translocate multiple scissile bonds within the unfolded substrate to the proteolytic site for peptide bond cleavage to occur.\textsuperscript{63} In the previous chapter, it was shown that deficiency in protein degradation does not necessarily reflect the competency of the proteolytic site in catalyzing peptide bond cleavage. In order to quantitatively assess the functionality of the proteolytic site, a peptide substrate whose degradation allowed the quantification of the proteolytic activity was used. In order to make a quantitative assessment, a fluorogenic peptide substrate (FR89-98) containing residues 89-98 of λN and a Lon cleavage site was used to quantify the ATP-dependent peptidase activity of Lon.\textsuperscript{53} Translocation and cleavage of the peptide bond in this substrate exhibited ATP-dependency.\textsuperscript{67} Therefore, comparing the activity profile of Lon degrading λN versus FR 89-98 revealed the extent to which the truncated region in the respective mutant affected multiple peptide bond cleavage versus single peptide bond cleavage. The amount of S3 hydrolyzed was calculated from the fluorescence signal generation as stated in the materials and methods section. Figure 3.1 shows the steady-state kinetics of ATP-dependent peptidase activity of Lon mutants. The curve was fitted using equation 3.4, and the steady-state results are summarized in Table 3.1. The peptidase results reveal that the Δ1-239 and Δ232-252 Lon mutants catalyzed peptide cleavage with $k_{cat}$ and $n$ values similar to WT Lon. For Δ1-252, the $n$ value is similar to WT Lon, but the
$k_{\text{cat}}$ value is ~1.8-fold lower. $K_m$ for all the mutants is ~2.5-fold higher than that of WT Lon. Taking the collective results into account, it appears that the mutants possess intact ATP-dependent peptidase activity despite the reduced ATPase activity that was observed in Δ232-252 and Δ1-252 Lon.
Figure 3.1 Steady-state kinetics of ATP dependent S3 cleavage by Lon mutants.61

The ATP dependent peptidase activities of Δ1-239 (▲), Δ1-252 (●) and Δ232-252 (■) were monitored using a modified FRET assay where the cleavage of the S3 peptide containing a fluorescent donor and quencher results in donor/quencher separation, and an increase in fluorescence over time can be detected. The initial steady-state rates of S3 cleavage were obtained from the time courses of peptide cleavage at varying [S3] and converted to steady-state rate constants by dividing by protein concentration. Error bars show the experimental standard deviations of at least three independent trials from the averaged values. The sigmoidal curves were fit with equation 3.1 to provide the kinetic parameters summarized in Table 3.1. Kinetic results were compared with previously published WT ELon data.34

Table 3.1 Kinetic parameters of the ATP-dependent peptidase activity of WT and ELon mutants.61

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$ (sec$^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}/K_m$ (sec$^{-1}$)</th>
<th>Hill coefficient (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>9.0 ± 0.5</td>
<td>102 ± 30</td>
<td>0.158 ± 0.009</td>
<td>1.60</td>
</tr>
<tr>
<td>Δ232-252</td>
<td>8.65 ± 0.52</td>
<td>261 ± 27</td>
<td>0.0287 ± 0.0001</td>
<td>1.67 ± 0.16</td>
</tr>
<tr>
<td>Δ1-252</td>
<td>5.97 ± 0.35</td>
<td>254 ± 24</td>
<td>0.0263 ± 0.0052</td>
<td>1.95 ± 0.21</td>
</tr>
<tr>
<td>Δ1-239</td>
<td>9.40 ± 0.68</td>
<td>280 ± 20</td>
<td>0.262 ± 0.042</td>
<td>1.20 ± 0.15</td>
</tr>
</tbody>
</table>

*WT ELon values were previously published34
3.3.2 Stopped flow of WT versus mutant Lon

In the peptidase study, two out of the three mutants showed $k_{\text{cat}}$ values similar to WT ELon, while all three mutants showed ATP-dependent peptidase activity despite the removal of 30% of the amino-terminal. In previous studies, it was shown that the translocation and cleavage of the peptide bond in the S3 substrate exhibited ATP dependency.\textsuperscript{67} Using the fluorescent stopped-flow kinetic technique, the ATP-dependent peptide translocation step occurring in the pre-steady-state phase of the peptidase reaction catalyzed by WT ELon was identified.\textsuperscript{66, 67} The substrate translocation step was detected as a lag phase in the peptidase time course. In order to study the step prior to peptidase action and gain insight into the rate-limiting step in the ATP-dependent degradation of FR89-98, the cleavage site FR89-98 was monitored under excess substrate concentration over [Lon] in the presence of 1 mM ATP by a stopped-flow apparatus. Under this pseudo-first-order reaction condition, the enzyme was predominantly bound with ATP and S3, and therefore, the substrate binding should not be partially rate limiting and should be detected in the lag phase.

The lag kinetic time courses shown in Figure 3.2 reveal the conversion of the enzyme:ATP:S3 complex to enzyme:ADP:P$_i$:hydrolyzed peptides. Lag kinetics were determined for each Lon mutant by using equation 3.2. The $k_{\text{obs}}$ values correspond to the steady-state rate of the cleavage of the labeled site at 1 mM of FR89-98. The $k_{\text{lag}}$ value reflects the rate constant for the transition of the slow to the fast phase of the time course. From previous studies on WT ELon, $k_{\text{lag}}$ is assigned to the ATP-dependent substrate translocation step occurring prior
to peptide bond cleavage in the substrate. The fact that the $k_{cat}$ values of S3 hydrolysis among WT and Lon mutants are comparable but their $k_{lag}$ values differ suggests that the efficiencies of the ATP-dependent substrate translocation vary in these enzymes. Δ232-252 and Δ1-252 show a ~10-fold reduction in the $k_{lag}$ of ATPase dependent S3 cleavage compared to Δ1-238 and WT ELon, which are comparable. All kinetic parameters are summarized in Table 3.1.
Figure 3.2 Stopped-flow analysis of 1 mM S3 cleavage by ELon and mutants.\textsuperscript{61}

The ATP-dependent cleavages of 1 mM S3 by 1 μM WT (X), Δ1-239 (▲), Δ1-252 (●) and Δ232-252 (■) Lon were monitored by fluorescent stopped-flow spectroscopy. The time course of S3 cleavage catalyzed by WT and Δ1-239 (▲) Lon (A), where compared to the time courses catalyzed by Δ1-239 (▲), Δ232-252 (■) and Δ1-252 (●) Lon (B). The pre-steady state lag rate constants were determined by the fitting of each time course with equation 3.2. The fluorescence changes associated with peptide cleavage were converted to product concentrations. Each time course shown is an average of at least four traces.
3.4 Conclusion

Quantitative assessment of peptide bond cleavage by truncated mutants was determined using a fluorogenic substrate. The peptidase results reveal that Δ1-239 and Δ232-252 ELon mutants catalyze cleavage of FR89-98 with steady-state kinetic parameters that are comparable to those of WT Lon, with the $k_{cat}$ and $n$ values matching the ones determined for WT Lon. Δ1-252 has a $k_{cat}$ that is ~1.8-fold lower than WT ELon, but the $n$ value is comparable to WT ELon. All the mutants have a $K_m$ value that is ~2.5-fold higher than in WT Lon. Taken together, all the mutants possess intact ATP-dependent peptidase activity despite the reduced ATPase activity observed in Δ232-252 and Δ1-252 Lon.

Even though a majority of the amino-terminus was removed, Δ232-252 behaves like Δ1-252 ELon, which shows deficiency in catalyzing ATP hydrolysis in the absence and presence of substrate. In pre-steady-state experiments, the $k_{lag}$ values of Δ232-252 and Δ1-252 show a ~10-fold reduction compared to Δ1-239 and WT. As the lag phase of the ATP-dependent cleavage of S3 measures the kinetics of the first pass of substrate translocation, the mutants Δ1-252 and Δ232-252, lacking residues 232-252, have smaller $k_{lag}$ values because of the impaired substrate translocation capability. Smaller $k_{lag}$ values equals slower translocation of substrate, this could be due to impaired substrate translocation in the enzyme if the $k_{lag}$ is smaller in the mutant compared to WT ELon. As the degradation of λN also requires substrate translocation, the reduction of λN degradation activity detected in Δ1-252 and Δ232-252 is consistent with the
proposal. It can be concluded that residues 240-252 in ELon facilitate substrate translocation by maintaining optimal ATPase turnover.
Chapter 4

Characterization of Truncated $\lambda N$ and the Kinetic Parameters of $N_{s\lambda N}$ and $C_{s\lambda N}$
4.1 Introduction

The proposed general mechanism for ATP-dependent proteolysis of Lon coordinates repeated cycles of ATPase and peptidase activity in order to completely degrade the protein substrate containing multiple cleavage sites. This process is referred to as processive degradation.\(^\text{11, 12, 68}\) The process of unfolding and translocating the substrate into the proteolytic site is suggested to be the rate-limiting step, which is also dependent on the ATPase activity.\(^\text{69}\) However, it is unclear how the substrate translocation event is coordinated with peptide bond cleavage to completely digest the protein substrate during the reaction time course. Thus far, there has been no specific sequence tag to help predict substrate cleavage sites by Lon. It has been found that Lon generally cleaves between hydrophobic residues that are surface-exposed and surrounded by highly charged environment.\(^\text{50}\)

The coordination between ATPase and peptidase activities to generate completely digested peptides is unknown. Depending on the timing by which the different scissile sites in the protein substrate gain access to the proteolytic site and when peptide cleavage occurs, there can be two different mechanisms. Figure 4.1 shows a scheme that has two proposed models. In model 1, the protein substrate is completely translocated before peptide bond cleavage occurs. In this model, fully digested peptide products are generated at the end. The delivery of all Lon cleavage sites to the proteolytic chamber occurs before peptide bond cleavage commences. In model 2, the cleavage of each scissile peptide bond is preceded by a substrate translocation event, while the partially
digested substrate is still bound to Lon during the reaction. In this model, a cycle of translocation and peptide cleavage event occurs for each Lon cleavage site. These cause different sets of specific rate constants for the translocation and peptide bond cleavage events for each cleavage site. If the peptide bonds in each protein substrate are cleaved with comparable rates then the results from the kinetic parameters would support model 1 over model 2. In model 1, it is anticipated that the rate constants for all the scissile sites entering the proteolytic chamber (rate of translocation) to be faster than the rate constant for the peptide bond cleavage.
Figure 4.1 Two possible models for λN degradation by Lon.\textsuperscript{70}

In model 1, the protein substrate is completely translocated before peptide bond cleavage occurs, resulting in fully digested products. In model 2, the cleavage of each scissile peptide bond is preceded by a substrate translocation event, while the partially digested substrate is still bound to Lon during the reaction. In this model a cycle of translocation and peptide cleavage events occurs for each Lon cleavage site. Lon N-terminal domain is indicated in blue, ATPase domain in red and proteolytic domain in green.
This chapter is focused on determining the timing by which ELon catalyzes ATP- and AMPPNP-dependent cleavage of λN near the N- versus the C-terminal. \textit{In vitro}, ELon degrades purified λN protein in the presence of ATP.\textsuperscript{44}

Due to its lack of structure and its physiological relevance to Lon as a substrate, λN was used for the study. Since λN is unstructured, unfolding of the substrate is not necessary; therefore, translocation and cleavage of the multiple peptide bonds within the substrate during degradation can be considered. In order to study peptide bond cleavage, a fluorescent protein was made by GenScript and LifeTein. Using the same concepts as stated in Chapter 2, a fluorescent acceptor (Abz) and quencher (nitrotyrosine) were placed at the amino end (NsFRλN) or at the carboxyl end (CsFRλN); the model design of these substrates is shown in Figure 4.2. Figure 4.2A shows a representation of where the fluorescent acceptor and quencher of NsFRλN and CsFRλN are located. Figure 4.2B shows the sequence for both sFRλN.
A. Design of fluorescent λN protein used to monitor peptide bond cleavage by ELon. B. Amino acid sequences of the λN protein used to monitor peptide bond cleavage by WT ELon. A FRET pair of abz/nitrotyrosine was used in both λN proteins (blue for NsFRλN, and red for CsFRλN). ELon cleavage sites between the fluorescent pair are underlined. C. The amino acid sequence of the non-fluorescent His-tag λN protein used to counteract the inner filter effect.

Figure 4.2 λN model and sequence of sFRλN.  
A. Design of fluorescent λN protein used to monitor peptide bond cleavage by ELon. B. Amino acid sequences of the λN protein used to monitor peptide bond cleavage by WT ELon. A FRET pair of abz/nitrotyrosine was used in both λN proteins (blue for NsFRλN, and red for CsFRλN). ELon cleavage sites between the fluorescent pair are underlined. C. The amino acid sequence of the non-fluorescent His-tag λN protein used to counteract the inner filter effect.
In order to counteract the inner filter effect, non-fluorescent full length λNC26 (for NsFRλN) and λNC99 (for CsFRλN) were made; their sequences are indicated in Figure 4.2 C. All cysteines were substituted with leucines except at positions 26 and 99. By using a mixture of the two proteins (90 % non-fluorescent and 10 % fluorescent, NsFRλNA and CsFRλNA), the inner filter effect was corrected and high concentrations of peptide were monitored. Using the synthetic peptide, I was able to determine the rate of peptide bond cleavage for each terminal.

4.2 Methods and Materials

4.2.1 Materials

Restriction endonucleases were purchased from Promega or New England Biolabs. Oligonucleotides were custom-synthesized by IDT, Inc. Solvents, buffers, chromatography resin, antibiotics, culture media and PEI cellulose TLC plates were purchased from Fisher Biotechnology or Sigma/Aldrich. Plasmids used for protein expression and competent cells were purchased from Invitrogen and Novagen. [α\(^{32}\)P]ATP was purchased from Perkin-Elmer Life Science. NsFRλN was synthesized by GenScript, and CsFRλN was synthesized by LifeTein. Sequence of synthetic peptide was confirmed by the companies using mass spectrometry.

4.2.2 General Methods

ELon and λN purification procedures were performed as described in Chapter 2.\(^{53}\) Experiments were performed at least in triplicate. In all cases,
comparable results were obtained. Either representative data or averaged data are shown.

4.2.3 Characterization of the ATP- and AMPPNP- dependent degradation of N-his-λN

λN degradation assays contained 50 mM HEPES (pH 8), 75 mM KOAc, 15 mM Mg(OAc)_2, 5 mM DTT, 65 μM N-his-λN, and 5 mM nucleotide; and the reaction was initiated with 6 μM WT ELon at 37°C. At 0, 5, 10, and 20 min, reactions with N-his-λN and ATP or AMPPNP were quenched with 5 x SDS-PAGE loading buffer and incubated at 100 °C for 1 min. N-his-λN aliquots were then run on a 12.5 % SDS-PAGE gel and stained with Coomassie Brilliant Blue to detect the undigested substrates.

4.2.4 Edman sequencing of particle digested bands from AMPPNP

λN degradation assays were run with 5 mM AMPPNP using C-his-λN and N-his-λN, 20 μL was quenched at 60 min into 5 x loading buffer. Reactions were run on a 12.5 % gel and transferred to a PVDF membrane for 1 hour and 40 min. Prior to transfer, the PVDF membrane was treated with 100 % methanol for 15 min and equilibrated in transfer buffer (4.4 g CAPS (3-(Cyclohexylamino)-1-propanesulfonic acid) pH 11, 10 % methanol in 2 L) for 15 min. The membrane was stained with 7 % acetic acid, 50 % methanol and 0.2 % Coomassie Brilliant Blue R-250 for 2 min, washed with 50 % methanol and 7 % acetic acid for 10 min, and given a final wash of 90 % methanol and 10 % acetic acid. Bands were cut out and subjected to Edman sequencing. Edman sequencing was done by Leslie
Revoredo in Dr. Thomas Gerken’s Lab in the department of Biochemistry at Case Western Reserve University.

4.2.5 Monitoring of the ATP-dependent degradation of fluorescently labeled λN (FRλN) by steady state kinetics

The cleavage of fluorescently labeled sites in λN was measured using a Fluoromax 4 spectrofluorimeter (Horiba Group) as described previously. Reactions contained 50 mM HEPES pH 8.0, 5 mM Mg(OAc)$_2$, 2 mM DTT, varied concentrations of NsFRλNA or CsFRλNA (0.5, 1, 5, 10, 20, and 30 μM), (excitation at 320 nm and emission at 420 nm), and 150 nM WT ELon; the reaction was incubated for 1 min at 37°C before being initiated by 1 mM ATP. All assays were performed at least in triplicate, and the averaged value of the rates determined for each substrate was fit to equation (4.1).

\[
k_{obs,\lambda N,ATP} = \frac{k_{cat,\lambda N,ATP}[S]}{K_m + [S]} \quad (4.1)
\]

Where $k_{obs,\lambda N,ATP}$ is the observed rate, $k_{cat,\lambda N,ATP}$ is the max rate constant of product formation at the saturating substrate, $K_m$ is the Michaelis-Menten constant, and [S] is the substrate concentration.

4.2.6 Acid-Quench Peptidase Assay

Assays were performed at 37°C and contained 50 mM Tris-HCl (pH 8.1), 5 mM Mg(OAc)$_2$, 5 mM DTT, 8 μM CsFRλN, and 1 and 5 μM WT ELon. The reaction was initiated by adding 1 mM ATP, and 20 μL aliquots were quenched with 80 μL of 2 N HCl at 0, 5, 10, 20, 40, 60, and 90 sec. after trichloroacetic acid precipitation, which removed Lon. The reaction mixtures were neutralized with 1
M Tris/2 N NaOH at pH 8 and the fluorescence (excitation of 320 nm and the emission of 420 nm) of the solution was measured using a Fluoromax 4 spectrofluorimeter (Horiba Group). This method was previously worked out and used by Diana Vineyard.66

**4.2.7 Pseudo-first order time course of sFR\(\lambda\)N cleavage by fluorescent stopped flow**

Pre-steady-state experiments were performed on a KinTek Stopped Flow instrument, controlled by the data collection software Stop Flow v7.50 β. The sample syringes were maintained at 37°C by a circulation water bath. Syringe A contained 5 µM Lon monomer, varied concentrations of NsFR\(\lambda\)N or CsFR\(\lambda\)N (0.1-8 µM) peptide substrate, 50 mM Tris-HCl (pH 8.1), 5 mM Mg(OAc)\(_2\), 5 mM DTT, 30 mM KP\(_i\), 30 mM KOAc, and 0.01% Tween 20. Syringe B contained varied concentrations of NsFR\(\lambda\)N or CsFR\(\lambda\)N (0.1-8 µM) peptide substrate, 1 mM ATP, 50 mM Tris-HCl (pH 8.1), 5 mM Mg(OAc)\(_2\), 5 mM DTT, 30 mM KP\(_i\), 30 mM KOAc, and 0.01% Tween 20. Peptide cleavage was detected by an increase in fluorescence (excitation of 320 nm and emission of 420 nm) following rapid mixing of the syringe contents in the sample cell. The baseline of the fluorescence was normalized to zero, and the data shown are the results of averaging at least four traces. The concentration of hydrolyzed peptide was calibrated by determining the maximum fluorescence generated per micromolar peptide, which was done by complete digestion by trypsin under identical conditions on the stopped flow. The average time course was fit with equation 4.2
where $t$ is the time in seconds, $Y$ is the concentration of hydrolyzed peptide in micromolar, $A$ is the amplitude of the reaction, $k_{lag}$ is the pre-steady-state rate constant in seconds, $v_{ss}$ is the steady-state rate in units of micromolar product per second, and $C$ is the endpoint. The $v_{ss}$ value can be converted to a first-order rate constant ($k_{ss}$ in the unit per second) by diving by the enzyme concentration. Equation 7.1 is the general function that quantifies a biphasic time course. When $Y=0$ at $t=0$, $C=-A$, and equation 4.2 becomes

$$Y = -A + A e^{-k_{lag}t} + v_{ss}t \quad (4.3)$$

such that

$$Y = -A(1 - e^{-k_{lag}t}) + v_{ss}t \quad (4.4)$$

when $A$ is defined as $v_{ss} - v_i/k_{lag}$, equation 4.2 becomes equivalent to the equation defining hysteresis, where $v_i$ is the rate corresponding to the initial phase of the time course.

Varied concentrations of ATP (10-500 μM in syringe B) with 8 μM FRλN were also performed and fit to the same conditions as stated above. CsFRλNA and NsFRλNA represent 10 % of the FRλN protein and 90 % of the corresponding non-fluorescent λN protein. Plots displaying a sigmoidal behavior were fitted with equation 4.5

$$k = \frac{k_{max}[S]^n}{K^'+[S]^n} \quad (4.5)$$
where \( k \) is the observed rate constant, \( k_{\text{max}} \) is the maximum rate constant, \([S]\) is the variable substrate, \( K' \) is the Michaelis constant for \( S \), and \( n \) is the Hill coefficient.

Alternatively, peptide bond cleavage was also monitored at varying ATP concentrations and fit with equation 7.6

\[
k = \frac{k_{\text{max}}[S]}{K_s + [S]} \quad (4.6)
\]

where \( k_{\text{max}} \) is the maximum rate constant (same as \( k_{\text{cat,} \lambda N}, k_{\text{burst amp, ATP}}, k_{\text{ss, ATP}}, k_{\text{burst amp, } \lambda N}, \) and \( k_{\text{ss, } \lambda N} \)), \([S]\) is the variable substrate, and \( K_s \) is the \([S]\) required to obtain 50% of the maximal rate constant of the reaction. The \( K_s \) is also referred to as \( K_{\text{burst amp, } \lambda N}, K_{\text{ss, } \lambda N}, K_{\text{burst amp, ATP}}, K_{\text{ss, ATP}}, \) and \( K_{\lambda N} \).

### 4.3 Results and discussion

#### 4.3.1 Substrate degradation in the presence of AMPPNP versus ATP

\( \lambda N \) was degraded by Lon in the presence of the non-hydrolyzable ATP analog, AMPPNP.\(^{42,44} \) As discussed earlier, AMPPNP is non-hydrolyzable, it was used to slow down the degradation process. It has been shown that ATP and AMPPNP have the same peptide cleavage profile; therefore, we used AMPPNP in a degradation study.\(^{44} \) As seen in Figure 4.3, comparing the intensity of intact substrate of ATP- versus AMPPNP-dependent degradation, AMPPNP slows down the rate of degradation. To evaluate this possibility, \( \lambda N \) was degraded by Lon in the presence of 1 mM ATP versus AMPPNP, from which the reaction time courses were monitored by SDS-PAGE as shown in Figure 4.3. Comparing the intensities of the intact substrate band of the ATP- versus AMPPNP-dependent reaction at the 5, 10 and 20 min time points reveals that \( \lambda N \) is degraded faster in
the presence of ATP. Furthermore, a small amount of partially digested λN was
detected at the 10- and 20-min time points in the AMPPNP-dependent reaction.
This result supports the idea that AMPPNP slows down the reaction rate for
protein degradation. The AMPPNP results from this experiment were further
examined by Edman sequencing and western blot.
Figure 4.3 The degradation of \( \lambda N \) by ELon in the presence of AMPPNP or ATP.\textsuperscript{61}

Purified N-his-\( \lambda N \) (65 \( \mu \)M) was digested by 6 \( \mu \)M ELon in the presence of 5 mM ATP or AMPPNP, and quenched at the times indicated. The degradations were monitored by 12.5 \% SDS-PAGE. Arrows point to fully and partially digested products with ATP and AMPPNP.
4.3.2 *Edman sequencing of partially digested substrate λN*

In order to characterize the band seen in Figure 4.3, Edman sequencing was attempted using two constructs of λN, one with a C-terminal His tag and one with an N-terminal His tag. To identify the cleavage site of the band seen in Figure 4.3, degradation products with AMPPNP were electroeluted on to a PVDF membrane. The PVDF membrane was then subjected to Edman degradation (red box in Figure 4.4). The first 5 amino acid residues of the C-His-λN were KPVNR (residues 31-35), indicating cleavage between residues A30-K31 at the N-terminal. Attempts to identify the cleavage site in the band from the N-terminal His-tag λN by the same approach were unsuccessful. It is possible that the variant still has the N-terminal attached, indicating that the band represents the C-terminal being cleaved. Since attempts to sequence the product from the N-His-λN were unsuccessful, a previous lab member, Natalie Mikita, preformed western blot on the partially digested product for both N-His-λN and C-His-λN to examine if there is a preference in cleavage directionality. The results show a band containing the His tag in both λN constructs, suggesting that there is no directionality in cleavage.\(^7\) If there were directionality in protein cleavage then the N- or C-terminal his tag would not be detected by western blot, only one λN construct would be positive for His-tag detection in western blot. There is a possibility that both sites are cleaved without preference; however, further analysis is needed.
Figure 4.4 C-His-λN and N-His-λN degradation in the presence of AMPPNP for Edman sequencing.

C-His-λN and N-His-λN protein (65 μM) was digested with 6 μM ELon and quenched at 60 minutes. Arrows point to degradation products. The red box indicates the band that was cut out for Edman sequencing.
4.3.3  Steady-state kinetic comparison of NsFR\(\lambda N\) versus CsFR\(\lambda N\)

The fact that both his-tagged C- and N-terminal were detected in western blot indicates that no order of peptide bond cleavage occurred in the degradation reactions. In order to further confirm the possibility that all sites are cleaved without preference, two constructs were developed based on the theory behind the small peptide substrate. Figure 4.2A shows how two \(\lambda N\) constructs were generated, one with Lon cleavage sites flanked by \(\text{NO}_2\text{Tyr}\) and \(\text{Abz}\) at the amino end, and one with Lon cleavage sites flanked by the same set of chromophores in the carboxyl end. These were generated by chemical synthesis without any His-tag. Since Trp also internally quenches the fluorescence of Abz, the intrinsic Trp residues in \(\lambda N\) were replaced with Phe to ensure the observed changes in the Abz signal were attributable to peptide bond cleavage. The resulting N-versus C-labeled \(\lambda N\) substrates are referred to as NsFR\(\lambda N\) and CsFR\(\lambda N\), respectively, and their sequences are found in Figure 4.2B.

One technical issue that has been seen with fluorogenic assays is inner filter effect at high concentrations of substrate. In order to overcome this, a substrate cocktail that contains 10 % of the fluorescently labeled \(\lambda N\) was mixed with 90 % of the unlabeled \(\lambda N\) to make up the desirable substrate concentration for the kinetics of peptide bond cleavage in synthetic peptides. Using the fluorogenic assay, the \(k_{\text{cat}}\) and \(K_m\) values for the cleavage of the N-terminal in NsFR\(\lambda N\) and C-terminal in CsFR\(\lambda N\) at 1 mM [ATP] were determined by fitting the data in order to obtain the rate constant of peptide bond cleavage (\(k_{\text{obs}}\)) with the indicated [substrate]. Figure 4.5 shows the fit with equation 4.1 for both NsFR\(\lambda N\)
NsFR has a $k_{\text{cat}}$ value of 0.22 ± 0.001 and a $K_m$ of 5.55 ± 0.50 μM while CsFRλN has a $k_{\text{cat}}$ value of 0.15 ± 0.001 and a $K_m$ of 6.12 ± 0.53 μM (Table 4.1). The $k_{\text{cat}}$ and $K_m$ values of both substrates are comparable. The detection of comparable $k_{\text{cat}}$ and $K_m$ values for the cleavage of both sites indicates the same rate-limiting step. This further suggests that multiple cleavage sites in λN are cleaved at once, thereby supporting model 1.
The steady-state rate constants \( k_{\text{obs,m,ATP}} \) of protein hydrolysis with varying concentrations of \( \text{NsFR} \) (●), and \( \text{CsFR} \) (■) were determined using the continuous fluorescence based proteinase assay. The \( k_{\text{obs,m,ATP}} \) values, determined by dividing reaction rates with enzyme monomer concentration, were plotted as a function of peptide concentration. The data were best fit with equation 4.1 to yield the kinetic parameters summarized in Table 4.1. The fit of the data is illustrated by the solid lines. Error bars show the experimental standard deviations of at least three independent trials from the averaged values.

<table>
<thead>
<tr>
<th></th>
<th>( k_{\text{cat}} ) (sec(^{-1}))</th>
<th>( K_{m} ) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{NsFR} )</td>
<td>0.22 ± 0.01</td>
<td>6.12 ± 0.53</td>
</tr>
<tr>
<td>( \text{CsFR} )</td>
<td>0.15 ± 0.01</td>
<td>5.55 ± 0.50</td>
</tr>
</tbody>
</table>
4.3.4 Pre-steady-state stopped flow time courses of peptide bond cleavage in λN under pseudo first order conditions

In the presence of ATP, lag phase at saturating concentrations of NsFRλN and CsFRλN can be detected. The lag phase suggests a buildup of an intermediate prior to the steady-state reaction, the formation of the rate-limiting step. The lag phase that was observed in NsFRλN and CsFRλN cleavage can be contributed to two different possibilities. One possibility is that it can be attributed to a slow step prior to peptide bond cleavage. Another possibility is that it can be attributed to the slow dissociation of the donor from the quencher. Figure 4.6 examines if the slow dissociation of peptide products caused the lag. 1 μM and 5 μM ELon in the presence of 8 μM CsFRλN was monitored over a time course using the discontinuous acid-quench assay. An aliquot of the reaction was quenched with HCl at the times indicated in Figure 4.6. The fluorescence intensity at each time point was measured to yield the time course for CsFRλN cleavage. The lag phase still remains in the fluorescence time course of CsFRλN cleavage, despite the acid denaturation that released hydrolyzed peptides. This indicates that the separation of the hydrolyzed peptide from Lon does not contribute to the observed lag phase of the reaction, which is similar to the result found for substrate FR89-98. Since the separation of hydrolyzed peptide from Lon does not contribute to the observed lag phase, this indicates that substrate release is not rate-limiting as well.
Figure 4.6 Detection of ATP-dependent CsFRλN cleavage by discontinuous acid-quench assay.

Lon (A. 1 μM, B. 5 μM) was incubated with 8 μM CsFRλN, and reaction aliquots were quenched at the indicated time points. The fluorescence signals associated with peptide cleavage were measured and plotted against their corresponding reaction time points. The experiment was performed at least 3 times, and the average data was plotted. The data was fitted with equation 4.2 to yield the lag rate constant of 0.039 ± 0.030 sec⁻¹ for 1 μM CsFRλN and 0.12 ± 0.044 sec⁻¹ for 5 μM CsFRλN.
In order to examine the rate-limiting step in the ATP dependent degradation of CsFRλN and NsFRλN, the cleavage of the NO\textsubscript{2}Tyr/Abz-labeled site in CsFRλN and NsFRλN was monitored under excess [substrate] over [Lon] (~2-fold $K_{m,\lambda N}$, λN; > 10-fold $K_{d,\lambda N}$, λN) in the presence of 1 mM ATP (Figure 4.7) by a stopped-flow apparatus. Lag phases were detected in both reactions. For each time course trace was fitted with equation 4.2 to yield the kinetic parameters found in Table 4.2. The steady-state $k_{obs}$ value corresponds to the cleavage at 8 μM of NsFRλN or CsFRλN. The $k_{lag}$ value corresponds to the rate constant for the transition of the slow to the fast phase of the time course. Looking at the reciprocal of $k_{lag}$ provides an estimate for the duration of the lag phase, which is found to be 1.3 sec for NsFRλN and 1.15 sec for CsFRλN under ATP conditions. When comparing the $k_{lag}$ and $k_{obs}$ of the ATP-dependent cleavage, the values for CsFRλN are comparable to those for NsFRλN; indicating the same rate-limiting step was detected in the cleavage of both sites. The detection of a lag phase indicates that both peptide bond cleavage reactions need a buildup of the same enzyme intermediate before peptide bond cleavage occurs. The lag kinetic profiles detected in the cleavage of CsFRλNA and NsFRλNA are reminiscent of the rate-limiting substrate translocation step previously detected in the ATP-dependent cleavage of a fluorogenic peptide constituting residues 89-98 of λN.\textsuperscript{42}
Figure 4.7 Stopped-flow analysis of ATP-dependent FR\(\lambda\)N cleavage by WT ELon under excess FR\(\lambda\)N conditions.

5 μM WT ELon was incubated with 8 μM NsFR\(\lambda\)N with no nucleotide (○), 1 mM ADP (□) or 1 mM ATP (■), or 8 μM CsFR\(\lambda\)N with no nucleotide (x), 1 mM ADP (▲), or 1 mM ATP (●). The fluorescent changes associated with peptide cleavage were converted to product concentrations. Each time course shown is an average of 4 traces. The traces with ATP were set to equation 4.2 to determine the kinetic parameters listed in Table 4.2. No change in fluorescence was observed without nucleotide or with ADP.

<table>
<thead>
<tr>
<th></th>
<th>(k_{\text{lag}} , (\text{sec}^{-1}))</th>
<th>(k_{\text{obs}} , (\text{sec}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsFR</td>
<td>0.77 ± 0.08</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>NsFR</td>
<td>0.87 ± 0.12</td>
<td>0.08 ± 0.01</td>
</tr>
</tbody>
</table>
In order to observe the lag rate further, we wanted to see if varying enzyme and substrate concentrations would yield the same lag rate constant. Figures 4.8 A and B were done at 1 mM ATP with varying concentrations of NsFR\N (A) and CsFR\N (B), while Figures 4.8 C and D were done at 8 μM NsFR\N (C) and 8 μM CsFR\N (D) with varying concentrations of ATP. As shown in Figure 4.8, a lag in peptide hydrolysis was observed in all the time courses. The lag rate constants ($k_{\text{lag}}$) and the observed steady-state rate constants for peptide cleavage ($k_{\text{ss,CsFR}}$ and $k_{\text{ss,NsFR}}$) were obtained by fitting each time course with a linear function. The plots of steady state rate constants as a function of peptide substrate and ATP concentrations are shown in Figure 4.9 A-D. The data were fitted with equations 4.5 and 4.6, and all kinetic parameters are summarized in Table 4.3 and 4.4. The Hill coefficients from the steady-state rate constants at varied sFR\N and ATP concentrations show comparable positive cooperativity. Positive cooperatively indicates that when one ligand molecule is bound to the enzyme, the enzyme's affinity for other ligand molecules increases. The steady state kinetic parameters determined in this study are comparable to the steady state parameters that were determined at saturating conditions (Table 4.2), indicating that these kinetic parameters are independent of the enzyme concentration under the conditions examined. Since pre-steady-state lag kinetics could be connected to the binding of substrate at low concentrations being rate-limiting, the $k_{\text{lag}}$ of substrate cleavage at increasing sFR\N (Figure 4.10A) or ATP (Figure 4.10B) concentration was examined. Figure 4.10 A-B shows that the dependence of $k_{\text{lag}}$ towards sFR\N and ATP
concentrations reaches saturation with maximum $k_{lag}$ value. The data is best-fit with equation 4.5 to yield the kinetic parameters $k_{lag}$, $K_{NsFR\lambda N}$ or $CsFR\lambda N$, and $K_{ATP}$, $NsFR\lambda N$ or $ATP,CsFR\lambda N$ as summarized in Table 4.5 and 4.6. The rate constants for both $NsFR\lambda N$ and $CsFR\lambda N$ at varying substrate and ATP concentrations are similar. The Hill coefficient for both $NsFR\lambda N$ and $CsFR\lambda N$ at varying substrate and ATP concentrations showed positive cooperativity. At high protein substrate and ATP concentrations, substrate binding no longer limits the rate of the first protease turnover. Therefore, the maximum $k_{lag}$, $CsFR$ and $k_{lag}$, $NsFR$ values reflect the rate constant for the build-up of a reaction intermediate that leads to protein hydrolysis at the active site.
Figure 4.8 Stopped flow analysis of ATP-dependent NsFRλN and CsFRλN cleavage by E. coli Lon.

ATP (1 mM) was incubated with 5 μM ELon in the presence of 0.1 μM (pink), 0.3 μM (blue), 0.5 μM (green), 0.8 μM (black), 1 μM (red), 3 μM (gray), 5 μM (purple), 8 μM (maroon) NsFRλN (A) and CsFRλN (B). The values on the left y axis represent the concentration of peptide hydrolyzed, whereas the values on the right y axis represent the mole equivalent of peptide digested by each Lon monomer. NsFRλN (C) and CsFRλN (D) at 500 μM were digested by 5 μM Lon monomer in the presence of 10 μM (red), 20 μM (blue), 30 μM (green), 50 μM (black), 100 μM (pink), 250 μM (gray), 500 μM (orange) ATP. Each time course is an average of at least 4 traces.
Figure 4.9 Steady-state kinetics of ATP-dependent NsFR\(\lambda\)N and CsFR\(\lambda\)N cleavage by WT ELon.

The \(k_{\text{ss,NsFR}}\) and \(k_{\text{ss,CsFR}}\) values were obtained by dividing the steady-state rates of the reaction by [Lon]. The steady-state rates of CsFR\(\lambda\)N (A) and NsFR\(\lambda\)N (B) cleavage were obtained by fitting the stopped-flow time courses of peptide cleavage at varying [\(\lambda\)N] with equation 4.4. The data presented was fitted with equation 4.5 and the kinetic parameters obtained were \(k_{\text{cat},\text{NsFR}\lambda\lambda\lambda\lambda} = 0.14 \text{ sec}^{-1}\), \(K_{\text{NsFRN}} = 4.07 \mu\text{M}\) and \(n = 1.22 \pm 0.46\), \(k_{\text{cat},\text{CsFR}\lambda\lambda\lambda\lambda} = 0.10 \text{ sec}^{-1}\), \(K_{\text{CsFRN}} = 4.40 \mu\text{M}\) and \(n = 1.7 \pm 0.79\). Each data point was obtained from the average of at least three independent trials. The error bars represent the experimental deviation among the different trials. Steady-state rate constants for NsFR\(\lambda\)N (C) and CsFR\(\lambda\)N (D) at varying ATP concentrations were obtained by fitting the stopped-flow traces with equation 4.6 to yield \(k_{\text{cat},\text{NsFRN,ATP}} = 0.13 \text{ sec}^{-1}\), \(K_{\text{ATP,NsFRN}} = 265.74 \mu\text{M}\), and \(n = 0.98 \pm 0.35\), \(k_{\text{cat},\text{CsFRN,ATP}} = 0.07 \text{ sec}^{-1}\), and \(K_{\text{ATP,CsFRN}} = 209.76 \mu\text{M}\) and \(n = 0.73 \pm 0.16\).
Table 4.3 Steady-state parameters obtained from pre-steady-state Kinetic characterization of ATP-dependent FR\(\lambda\)N at varying FR\(\lambda\)N concentrations.

<table>
<thead>
<tr>
<th></th>
<th>(k_{\text{cat}, \lambda \text{N}}) (sec(^{-1}))</th>
<th>(K_{\lambda \text{N}}) ((\mu)M)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsFR(\lambda)N</td>
<td>0.10 ± 0.01</td>
<td>4.40 ± 0.77</td>
<td>1.70 ± 0.79</td>
</tr>
<tr>
<td>NsFR(\lambda)N</td>
<td>0.14 ± 0.05</td>
<td>4.07 ± 2.46</td>
<td>1.22 ± 0.46</td>
</tr>
</tbody>
</table>

Table 4.4 Steady-state parameters obtained from pre-steady-state Kinetic characterization of ATP-dependent FR\(\lambda\)N at varying ATP concentrations.

<table>
<thead>
<tr>
<th></th>
<th>(k_{\text{cat,ATP}}) (sec(^{-1}))</th>
<th>(K_{\text{ATP}, \lambda \text{N}}) ((\mu)M)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsFR(\lambda)N</td>
<td>0.07 ± 0.01</td>
<td>209.76 ± 70.91</td>
<td>0.98 ± 0.35</td>
</tr>
<tr>
<td>NsFR(\lambda)N</td>
<td>0.13 ± 0.02</td>
<td>265.74 ± 64.73</td>
<td>0.73 ± 0.16</td>
</tr>
</tbody>
</table>
Figure 4.10 Substrate dependencies of the lag rate constants of NsFR\(\lambda\)N and CsFR\(\lambda\)N cleavage.

(A) Lag rate constant for CsFR\(\lambda\)N and NsFR\(\lambda\)N peptide degradation by 5\,\mu M ELon at varying [ATP] was determined by fitting the stopped-flow time courses of peptide cleavage as shown in Figure 4.8 with equation 4.4. The data were collectively fit with equation 7.4 to yield a maximal \(k_{\text{lag. NsFR\(\lambda\)N}} = 0.89\,\text{sec}^{-1}\), \(K_{\text{NsFR\(\lambda\)N}} = 39.04\,\mu M\) and \(n = 1.51 \pm 0.50\), \(k_{\text{lag. CsFR\(\lambda\)N}} = 0.90\,\text{sec}^{-1}\), \(K_{\text{CsFR\(\lambda\)N}} = 33.38\,\mu M\) and \(n = 2.43 \pm 0.03\). The error bars represent the experimental deviations among the different trials. Each endpoint was obtained from the average of at least three independent experiments, with each experiment containing at least 4 stopped-flow traces. (B) Lag rate constant for CsFR\(\lambda\)N and NsFR\(\lambda\)N peptide degradation by 5\,\mu M ELon at varying \(\lambda\)N was determined by fitting the stopped-flow time courses of peptide cleavage with equation 4.4. The data were collectively fit with equation 4.5 to yield a maximal \(k_{\text{lag. NsFR\(\lambda\)N}} = 0.74\,\text{sec}^{-1}\), \(K_{\text{ATP,NsFR\(\lambda\)N}} = 0.52\,\mu M\) and \(n = 2.38 \pm 0.26\), \(k_{\text{lag. CsFR\(\lambda\)N}} = 0.78\,\text{sec}^{-1}\), \(K_{\text{ATP,CsFR\(\lambda\)N}} = 0.62\,\mu M\) and \(n = 2.14 \pm 0.4\). The error bars represent the experimental deviations among the different trials. Each endpoint was obtained from the average of at least three independent experiments, with each experiment containing at least 4 stopped-flow traces.
Table 4.5 Lag rate constant for pre-steady-state Kinetic characterization of ATP-dependent FR\(\lambda\)N at varying ATP concentrations.

<table>
<thead>
<tr>
<th></th>
<th>(k_{\text{lag}}) (sec(^{-1}))</th>
<th>(K_{S_{\text{FR(\lambda)N,ATP}}}) ((\mu)M)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsFR(\lambda)N varied ATP</td>
<td>0.90 ± 0.04</td>
<td>33.38 ± 1.35</td>
<td>2.43 ± 0.03</td>
</tr>
<tr>
<td>NsFR(\lambda)N varied ATP</td>
<td>0.89 ± 0.17</td>
<td>39.04 ± 8.68</td>
<td>1.51 ± 0.50</td>
</tr>
</tbody>
</table>

Table 4.6 Lag rate constant for pre-steady-state Kinetic characterization of ATP-dependent FR\(\lambda\)N at varying \(\lambda\)N concentrations.

<table>
<thead>
<tr>
<th></th>
<th>(k_{\text{lag}}) (sec(^{-1}))</th>
<th>(K_{S_{\text{FR(\lambda)N,(\lambda)N}}}) ((\mu)M)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsFR(\lambda)N varied (\lambda)N</td>
<td>0.78 ± 0.03</td>
<td>0.62 ± 0.05</td>
<td>2.13 ± 0.40</td>
</tr>
<tr>
<td>NsFR(\lambda)N varied (\lambda)N</td>
<td>0.74 ± 0.02</td>
<td>0.52 ± 0.02</td>
<td>2.38 ± 0.26</td>
</tr>
</tbody>
</table>
4.4 Conclusion

Processive protein degradation is a common feature found in ATP-dependent proteases. Proteins substrates are degraded into peptides ranging from 5 to 20 amino acids long without generation of partially digested substrate. In order to identify whether there is a preference for cleavage, Edman sequencing was done. The first 5 amino acid residues of the C-his-λN were KPVNR, cleavage at residues A30-K31, at the N-terminal of WT λN. Attempts to identify the cleavage site in the N-terminally His-tagged λN by the same approach were unsuccessful. Further supporting model one, Lon may recognize a sequence on the C-terminal; however, cleavage does not necessarily occur at the C-terminal first.

Using a similar approach as FR89-98, two synthetic λN variants, each lacking intrinsic Trp residues, were labeled with the internally fluorescent quenching group, NO₂Tyr and Lys(Abz) such that the kinetics of peptide bond cleavage of two different scissile sites could be quantified. These two scissile sites are separated by 77 residues; placing the spectroscopic probe at these sites allows for the detection of the difference in substrate interaction and the existence of directionality in the substrate translocation event if this process is initiated at one terminal of the substrate. The steady-state kinetics studies of the two different synthetic λN proteins indicate that the $K_m$ and $k_{cat}$ are comparable. The detection of comparable $k_{lag}$ and $K_m$ values for the cleavage of both sites indicated the same rate-limiting step. To further investigate the mechanism of ATPase-dependent protease activity in ELon, varying concentrations of substrate and ATP under pre-steady-state conditions were also studied. The steady-state and
pre-steady-state showed similar rates, suggesting that peptide bond cleavage occurs all at once. The data obtained from peptide bond cleavage further supports model 1. The next step is to look at the translocation substrate to the proteolytic site.
Chapter 5

Pre-steady State Dansyl\(\text{H}\)N Translocation into the Proteolytic Site
5.1 Introduction

The kinetic rates of peptide bond cleavage in both steady state and pre-steady-state reactions were found to be similar as shown in chapter 4. A lag phase was also observed. Our lab has hypothesized that the lag phase corresponds the time needed for substrate translocation to occur. Based on the generally accepted mechanism, proposed by Kenniston, J. A. et al., the unfolding and delivery of the substrate to the active site is considered to be the rate-limiting step and is dependent by ATPase activity.\textsuperscript{71} When the substrate is bound and ATP hydrolysis takes place, a conformational change happens in the enzyme which leads to the unfolding of the substrate so that it can be translocated to the active site.\textsuperscript{3, 35} The unfolded and translocated substrate is sequestered within the central cavity of the proteolytic chamber, where peptide bond cleavage occurs.\textsuperscript{71}

Our current understanding of processivity in ATP-dependent proteolysis is primarily from studies on another ATP-dependent protease called ClpXP.\textsuperscript{38, 72, 73} Clp protease complexes recognizes a peptide sequence tag, such as SsrA tag, it has been shown that the translocation of protein or polypeptide substrates by the ATPase unit subunit in Clp X originates from the SsrA tag and exhibits processibility.\textsuperscript{74} Although these studies show the contribution of the ATPase activity in rendering processivity in substrate interaction, it does not explain how the ATPase and peptidase activities are coordinated to generate only completely digested products. This chapter compares how translocation is coordinated with peptide bond cleavage in order to completely digest substrates during the reaction time course.
There are two possible processive degradation pathways (Model 1 and Model 2) that peptide bond cleavage in Lon can take: Peptide bond cleavage can either occur after complete translocation of the substrate, or the peptide bond cleavage happens in a cycle of translocation/peptide bond cleavage. These two possible pathways are shown in Figure 4.1. We studied the mechanism of ELon using λN protein as a substrate. Previously Jessica Patterson-Ward studied the delivery of a synthetic dansylated peptide substrate containing residues 89-98 of λN to the proteolytic site of ELon was detected by using FRET. In S679W ELon, the Ser located in the proteolytic site was mutated to Trp such that the mutant possesses WT-like ATPase activity but is proteolytically inactive. Using this approach, the reaction was excited at the Trp donor wavelength of 295 nm, and the fluorescence was monitored at the dansyl acceptor wavelength of 520 nm. An increase in fluorescence indicates the delivery of the substrate as it approaches the Trp active site, which occurs prior to peptide bond cleavage. Using this approach, three dansylated λN with N-terminal His-tag protein were constructed. Dansyl C42 was provided by Natalie Mikita. These constructs are shown in Figure 5.1A.
Figure 5.1 λN model and sequence of DansylλN.

(A) Design of fluorescent λN protein used to monitor degradation and translocation by ELon. (B) Amino acid sequences of the λN protein used to monitor substrate translocation by WT ELon. FRET was detected when the donor from the Trp in S679W Lon interacted with the dansylated substrate, which acts as an acceptor. Each λN substrate sequence shows the dansyl location; dansylλNC26 in the 26th position, dansylλNC42 in the 42nd position, and dansylλNC99 in the 99th position. The FRET signal was detected at excitation 295 nm (Trp) and emission of 520 nm (Dansyl).
The three constructs differ by the location of the dansylated Cys inserted upstream and downstream of Lon cleavage sites. dansyl\(\lambda\)NC26 contains a dansyl label at position C26 and is four residues downstream of cleavage site A30-K31; this substrate has the dansyl label furthest away from C-terminal. dansyl\(\lambda\)NC42 contains a dansyl label at position C42 and is 1 residue upstream from the cleavage site of L40-N41. Dansyl\(\lambda\)NC99 is the closest to the C-terminal, where the fluorescent label is located at position 99 and is near cleavage site L93-S94 (Figure 5.1B).\(^7\) We were able to consider the translocation step of Lon protease due to the lack of structure for all three of the dansylated labeled substrates. All three dansyl\(\lambda\)N had their tryptophans mutated to a phenylalanine in order to prevent false reading from the FRET interaction between dansyl and typtophan. All other cysteines in the amino acid sequence were mutated to a leucine in order to prevent dansyl from reacting at multiple places on the \(\lambda\)N. A correlation between translocation and peptide bond cleavage was determined using this approach.
5.2 Methods and Material

5.2.1 Materials

Solvents, buffers, chromatography resin, antibiotics, culture media and PEI cellulose TLC plates were purchased from Fisher Biotechnology and Sigma/Aldrich. Plasmids used for protein expression and competent cells were purchased from Invitrogen and Novagen. \( \alpha^{32} \text{P} \)ATP was purchased from Perkin-Elmer Life Science.

5.2.2 General Methods

ELon (WT, S679W and S679A; plasmids for S679W and S679A were generated by Jessica Patterson-Ward) and \( \lambda \)N substrate purification procedures were performed as described in chapter 2. Experiments were performed in triplicate at minimum. In all cases, comparable results were obtained. Either representative data or averaged data is shown.

5.2.3 Preparation of dansylated \( \lambda \)N variants

The purified \( \lambda \)NC26, \( \lambda \)NC42 and \( \lambda \)NC99 were labeled with dansyl aziridine (Invitrogen). Dansyl aziridine was prepared in dimethylsulfoxide and was added to the \( \lambda \)N protein in 5-fold excess. The reaction mixture was gently agitated at room temperature overnight and then quenched with \( \beta \)-mercaptoethanol. Unreacted dye was removed by dialyzing \( \lambda \)N into storage buffer containing 20 mM Tris, 50 mM NaCl, 1 mM \( \beta \)-mercaptoethanol and 20 % glycerol. The labeled protein was then quantified using the Bradford assay. The extent of dansyl labeling in each protein was determined by the absorbance of dansyl at \( A_{340} \) and compared to the concentration of protein.
5.2.4 *Degradation of DansylλN versus λN*

Reactions contained 50 mM Tris (pH 8), 15 mM Mg(OAc)\(_2\), 5 mM DTT, 10 μM λNC26, dansylλNC26, λNC42, dansylλNC42, λNC99, dansylλNC99, N-his-λN, C-his-λN, or Δ99-107λN, and 1 μM WT Lon and were initiated with 5 mM ATP at 37 °C. Aliquots were quenched at 0, 1, 3, 5, 7, and 10 min in 5 x SDS-PAGE loading buffer. All quenched reactions were resolved on a 12.5 % SDS-PAGE gel and stained with Coomassie Brilliant Blue to detect the undigested substrates. Lon and λN bands were quantified with the program ImageJ.¹ The amount of λN remaining at each time point was normalized relative to the amount of Lon at each time point. The amount of λN present at the initial time point was then set to 1 and each remaining time point value was normalized relative to the initial value. Time point values were plotted out and fitted to a single exponential decay. Each reaction and quantification was done at least in triplicate.

5.2.5 *Delivery of dansylλN to the protoelytic active site in S679W ELon by fluorescent stopped flow*

Experiments to monitor the translocation of DansylλN with ELon (S679W and S679A) were performed on a KinTek Stopped Flow instrument controlled by the data collection software Stop Flow version 7.50 β with a 0.5 cm path length. The sample syringes were maintained at 37 °C by a circulating water bath. Syringe A contained 10 μM S679A or S679W ELon monomer with 10 μM dansylλN (dansylλNC26, dansylλNC42, or dansylλNC99), and reaction buffer (5 mM HEPES pH 8.0, 75 mM KOAc, 75 mM KPi, 5 mM Mg(OAc)\(_2\) and 5 mM DTT). Syringe B contained 1 mM ATP, 10 μM dansylλN, and reaction buffer. DansylλN
translocating to S679A or S679W ELon was monitored by an increase in fluorescence (excitation 295 nm emission 450 nm long-pass filter) upon rapid mixing of the syringe contents over 20 sec. In addition to monitoring with excitation 295 nm and emission with 450 nm long-pass filter, experiments were performed with excitation 295 nm emission with a 340 nm band-pass filter monitor to view the changes in Trp fluorescence. The data shown are a result of averaging at least four traces. Each reaction was performed in triplicate. PMT (photomultiplier tube) sensitivity was automatically adjusted by the instrument to optimize signal-to-noise. As a result, the relative amplitudes of the time courses do not reflect the stoichiometries of the enzyme intermediates monitored by the signals. The first-order rate constants of the reactions do not change because the dansyl absorbance in each reaction remains constant. The first 0.5 sec of the average time courses of S679W ELon with ATP and dansylλN were fitted with equation 5.1 describing a single exponential

\[
F = A_1 \exp^{-k_{1,FRET,ATP}t} + C \tag{5.1}
\]

Where \(F\) is relative fluorescence, \(A_1\) is the amplitude in relative fluorescence units, \(t\) is time in seconds, \(C\) is the endpoint and \(k_{1,FRET,ATP}\) is the first order rate constant associated with protein initiation.

Varied concentrations of ATP (10-500 \(\mu\)M in syringe B) with 10 \(\mu\)M dansylλN and varied concentration of dansylλN with 500 \(\mu\)M ATP were performed as well.
5.2.6 Chemical-Quench ATPase Activity Assays

The pre-steady-state time courses for ATP hydrolysis were measured using a rapid chemical-quench-flow instrument from KinTek Corporation. The instrument was maintained at 37 °C by a circulating water bath. Syringe A contained 5 μM Lon (WT), with 0.1-8 μM λN substrate (λNC26 or λNC99), 5 mM Mg(OAc)$_2$, 50 mM HEPES (pH 8.1), 5 mM DTT, 75 mM KOAc, and 75 mM KPi. Syringe B contained 0.1-8 μM λN substrate (λN001 or λN006), 5 mM Mg(OAc)$_2$, 50 mM HEPES (pH 8.1), 5 mM DTT, 75 mM KOAc, 75 mM KPi, and 200 μM ATP containing 0.01 % of [α-32P]ATP at times (0-1.8 sec) before quenching with 0.5 N formic acid and then extracting with 200 μL of phenol/chloroform/isoamyl alcohol at pH 6.7 (25:24:1). A 3 μL aliquot of the aqueous solution was spotted directly onto a PEI-cellulose TLC plate (10 X 20 cm), and the plate was developed in 0.75 M potassium phosphate buffer (pH 3.4) to separate ADP from ATP. The relative amount of radiolabeled ADP and ATP at each time point was quantified by a Cyclone Phosphor imager (Perkin-Elmer Life Science). To compensate for the slight variations in spotting volume, the concentration of the ADP product obtained at each time point was corrected for by using an internal reference as shown in equation 5.3. Where [ADP] is the concentration of ADP, ADP$_{dlu}$ is the ADP density light unit produced, ATP$_{dlu}$ is the ATP density light unit, and [ATP] is the concentration of ATP. All assays were performed at least in triplicate, and the average of those traces was used for data analysis. The burst amplitude and the burst rates were determined by fitting the $k_{obs}$ data from 0 to 400 msec with equation 5.4.
where $t$ is time in seconds, $Y$ is [ADP] in micromolar, $A$ is the burst amplitude in micromolar, $k_{\text{burst}}$ is the burst rate constant in per sec, and $C$ is the end point. The observed steady-state rate constants ($k_{\text{obs,ATP}}$) were determined by fitting the data from 600 ms to 1.8 sec with equation 5.5,

$$ Y = k_{\text{obs,ATP}} X + k_{\text{obs,ATP}} C $$  \hspace{1cm} (5.4)

where $X$ is time in sec, $Y$ is [ADP]/[E], $k_{\text{obs,ATP}}$ is the observed steady-state rate constant in per sec, and $C$ is the y intercept. Data fitting was accomplished using the nonlinear regression program Kaleida-Graph (Synergy).

Varied concentrations of ATP with 8 μM $\lambda$N were also performed.

**Results and discussion**

**5.3.1 Degradation of Dansyl$\lambda$N versus $\lambda$N**

Dansyl dye was attached to the thiol side chain of cysteine in $\lambda$N through reaction with dansyl aziridine in each of the $\lambda$N constructs. The fluorescence of the dansylated protein can be visualized and quantified by UV transillumination on the SDS-PAGE gel. To ensure that the dansyl label did not interfere with substrate degradation, control experiments were conducted to verify that the rates of labeled and unlabeled $\lambda$N were similar. Figure 5.2 shows the gel.
representation of labeled versus unlabeled λN substrate. The intensity of λN over time was quantified by ImageJ, and the amount remaining was plotted versus time.
Figure 5.2 Comparing rates of cleavage for different λN adducts.\textsuperscript{20} Representative gels for the degradation of (A) dansylλNC26, (B) λNC26, (C) dansylλNC42, (D) λNC42, (E) dansylλNC99, (F) λNC99, (G) C-His-λN, (H) N-His-λN, (I) Graphical representation of amount of dansylλNC26 (red), λNC26 (blue), dansylλNC42 (gray), λNC42 (pink), dansylλNC99 (green), λNC99 (black), C-His-λN (orange), and N-His-λN (teal) at each time point.
5.3.2 Dansylated λN as reporters to monitor the translocation of N- versus C-terminal of λN and the utilization of S679W Lon

S679A Lon was tested with dansylλNC26 substrate, in order to determine that the FRET signal was from dansyl interacting with the Trp located in S679W and not from other Trps present in the enzyme. Using S679A we can determine if the signal being observed is from the dansylated protein interacting with the Trp located in the active site or dansylated protein interacting with other Trps present in the protein. The stopped flow fluorescence time courses of dansylλNC26 interacting with S679W versus S679A in the presence of 0.5 mM MgATP were monitored. Also, for comparison, 0.5 mM MgADP and no nucleotide were also studied. I wanted to make sure that the FRET signal was only generated when ATP and dansylated substrate were present; that way, translocation of protein substrate to the central cavity can be clearly examined. The time courses show an increase in dansyl fluorescence and a decrease in Trp fluorescence, as shown on Figure 5.3 A and B, in the presence of ATP. Figure 5.3 also shows that there is a small amount of dansyl fluorescence when the substrate interacts with S679A; however, the most noticeable changes occur when dansyl interacts with the Trp in S679W. These results suggest that the FRET signal is generated by 679W interacting with the dansyl located on the λN substrate in the presence of ATP.
Figure 5.3 Dansyl\(\lambda\)N substrate deliver to the proteolytic site of S679W versus S679A.\(^7\)

(A) Dansyl signal of 5\(\mu\)M S679W ELon was incubated with 10 \(\mu\)M dansyl\(\lambda\)NC26 with no nucleotide (●), 0.5 mM ADP (■), or 0.5 mM ATP (♦). 5 \(\mu\)M S679A ELon was incubated with 10 \(\mu\)M dansyl\(\lambda\)NC26 with 0.5 mM ATP (▲). (B) Tryptophan signal of 5 \(\mu\)M S679W ELon was incubated with 10 \(\mu\)M dansyl\(\lambda\)NC26 with no nucleotide (●), 0.5 mM ADP (■), or 0.5 mM ATP (♦). 5 \(\mu\)M S679A ELon was incubated with 10 \(\mu\)M dansyl\(\lambda\)NC26 with 0.5 mM ATP (▲).
Since Lon is an ATP-dependent protease, I wanted to examine how Lon translocates in the presence of ATP. Figure 5.4 shows the dansyl emission of dansyl\(\lambda\)NC26, dansyl\(\lambda\)NC42, and dansyl\(\lambda\)NC99 after incubation with S679W in the presence of ATP. The time courses show three-, two- and one-stepwise increases in dansyl fluorescence before attaining a slow steady-state phase. The dansyl reactions were excited at the Trp wavelength (295 nm) and changes in fluorescence intensity were recorded at the dansyl excitation wavelength. The FRET signal generated from the ATP-dependent cleavage of CsFR\(\lambda\)N by WT ELon was included for comparison. The duration of the lag phase in peptide bond cleavage amounts for NsFR\(\lambda\)N and CsFR\(\lambda\)N were 1.15- to 1.3 sec, respectively. The lag phase of peptide bond cleavage denotes the buildup of an enzyme intermediate needed to initiate peptide bond cleavage (translocation of substrate prior to cleavage). The end of the lag phase sets the end point for assigning FRET signals attributed to substrate translocation occurring before peptide bond cleavage. Comparing the FRET signals associated with S679W interacting with the dansyl from any of the three substrates within the first 1.15-1.3 sec of the time course reveals a maximum increase in FRET signal for two of the dansyl\(\lambda\)N substrates (dansyl\(\lambda\)NC42 and dansyl\(\lambda\)NC99), which is shown in Figure 5.4. The lag phase of peptide bond cleavage in NsFR\(\lambda\)N or CsFR\(\lambda\)N spans approximately the duration needed to complete the translation of dansyl\(\lambda\)NC26. Based on the kinetic data, the idea that peptide bond cleavage occurs after almost complete translocation of the scissile sites in the \(\lambda\)N substrate was proposed. The observed FRET signal could also be from the changes in orientation of the
dansyl label with respect to S679W once it is translocated into the proteolytic chamber. The one or more steps detected in the reaction within dansylλNC26 and dansylλNC42 could be caused by conformation changes in the proteolytic site that lead to changes in the FRET signal intensity. The FRET time course in this experiment allowed us to compare the kinetics by the different regions where dansyl is labeled on λN as it approaches the proteolytic site prior to their cleavages. The existence of directionality in λN interacting with S679W can be implied based on the data shown in Figure 5.4. The C-terminal is preferred since dansylλNC99 interacts with S679W in one step, followed by the successive delivery of the dansylated site in dansylλNC42 (2-steps) and then the dansylated site in dansylλNC26 (3-steps).

All three dansylλN show difference in relative fluorescence intensity, this could be due to the fact that trp and dansyl are environmentally sensitive dyes. The FRET efficiency between the dansyl group and Trp will vary due to the different peptide sequences surround each dansyl probe in the three different dansylλNs. Once dansylλN is bound to the active site of Lon, conformational changes in the enzyme may occur to cause additional changes in the orientation of dansyl and Trp, thereby producing further changes in the FRET kinetics. Each kinetic step could be attributed to the translational movement or orientation of the fluorescent probes due to conformational changes in the enzyme. Given these consideration, the amplitudes of the FRET time courses could not be directly compared. Only kinetic parameters were extracted from the FRET time courses to deduce the order of substrate translocation to the active site.
To further investigate the interaction between S679W and dansylλN varied ATP and dansylated λN protein concentrations were examined. Figure 5.5 (B, D, F) shows selected time courses reflecting the changes in the dansyl fluorescence of dansylλN following the excitation of the Trp residue in S679W at varying concentrations of ATP (0.01-0.5 mM).
Figure 5.4. Protein translocation to S679W ELon can be monitored using the dansyl\(\lambda N\) protein and ATP.\(^7\)

5 \(\mu\)M S679W ELon was incubated with 10 \(\mu\)M dansyl\(\lambda N\)C26 (▲), dansyl\(\lambda N\)C42 (■), or dansyl\(\lambda\)C99 (●) and 1 mM ATP (A). The left y-axis shows relative FRET signal between S679W ELon and dansylated \(\lambda N\). Cleavage of CsFR\(\lambda N\)A (▼) by WT ELon with ATP is also shown for comparison. The right y-axis shows relative fluorescence due to peptide bond cleavage. The end of lag phase is indicated in a dotted black line at 1.3 seconds. Dansyl\(\lambda N\) reactions were excited at wavelength 295 nm and emission 450 nm long-pass filter. CsFR\(\lambda N\) reaction was excited at wavelength 320 nm and emission at 420 nm.
Figure 5.5 Representative time courses for S679W interacting with dansyl\(\lambda\)N.

All the reactions were excited at 295 nm, and the emission signals from the dansyl\(\lambda\)N were detected using a 450 nm long-pass filter. (A, C, E) 5 μM S679W preincubated with 500 μM ATP was rapidly mixed with 0.3(●), 0.5(■), 0.8 (♦), 1(▲), 3(▼), 5 (+), 8 (X), 10 (▲) μM dansyl\(\lambda\)NC26 (A), dansyl\(\lambda\)NC42 (C), or dansyl\(\lambda\)NC99 (E) in the stopped flow apparatus. (B, D, F) 5 μM S679W was preincubated with 10 μM dansyl\(\lambda\)NC26 (B), dansyl\(\lambda\)NC42 (D), or dansyl\(\lambda\)NC99 (F) was rapidly mixed with 10 (●), 20 (■), 30 (♦), 50 (▲), 100(▼), 500 (+) μM ATP in the stopped flow apparatus.
Varied concentrations of dansyl\(\lambda N\) were also performed (Figure 5.5 A,C,E). As both \(\lambda N\) and ATP concentrations increased, the relative intensity increased as well. Also, as the concentrations increased, a more pronounced curve was observed. Dansyl\(\lambda N\)C99 shows a one-step interaction with S679W, followed by the 2-step interaction of dansyl\(\lambda N\)C42, then finally the dansyl\(\lambda N\)C26 (3-step).

5.3.3 Chemical Quench flow of \(\lambda NC99\) and \(\lambda NC26\)

Attempts to fit all 4 sec of the ATP-dependent delivery time courses using the traditional one to three exponential functions were unsuccessful. However, the first 0.5 sec of the ATP-dependent delivery time courses was fit with equation 5.1 (Table 5.1). The time course of each reaction shows resemblance to the pre-steady-state time course of ATPase activity that was obtained by the synthetic dansyl peptide containing residues 89-98 of \(\lambda N\) (data obtained by Diana Vineyard).\(^6^6\) It is reasonable to suggest that the step-like phase in the time course is coordinated with an ATPase cycle. The time course of ATP hydrolysis catalyzed by WT ELon stimulated by 8 \(\mu\)M of unlabeled \(\lambda NC26\) (Cys inserted at position 26), and \(\lambda NC99\) (Cys inserted at position C99) was determined in order to evaluate the ATPase activity. To examine if Dansyl interferes with the ATPase activity, Figure 5.6A-B shows both unlabeled \(\lambda N\) and Dansyl labeled \(\lambda N\) at 25 \(\mu\)M ATP and 200 \(\mu\)M ATP. The results suggest the Dansyl does not interfere with the ATPase activity. Figure 5.6C shows the time courses of the ATPase reaction that were obtained up to 1.8 sec for both unlabeled \(\lambda NC26\) and \(\lambda NC99\). Both \(\lambda N\) substrates show a rapid burst followed by a transient plateau and then the steady-state increase in ATP hydrolysis. These observations match the
previously published results by Diana Vineyard, that were obtained by using a
decapeptide that contains resides 89-98 of λN. Therefore, the substrate-
stimulating ATPase activity if ELon appears insensitive to the size or number of
the Lon cleavage sites present in a substrate. Figure 5.6D compares the time
course of ATP hydrolysis up to 0.4 sec, which is the burst phase of the reaction.
This phase was fitted with a single exponential function in order to yield the
kinetic parameters that are found in Table 5.2. The burst rate constant, $k_{burst}$, for
ATPase hydrolysis is $11.54 \pm 1.79$ sec$^{-1}$ for λNC26 and $6.49 \pm 1.08$ sec$^{-1}$ for
λNC99.

As shown in Figure 5.6D, both λNC26 and λNC99 are within error of each
other, although the burst rate constants appear to be different from each other.
When the burst rates constants of λNC26 was used to fit the curve of λNC99 the
curve was fit relatively well. Results are the same when the burst rate constant of
λNC99 was used to fit λNC26, suggesting the burst rate constants for both
substrates are comparable. The burst amplitude, which reflects the stoichiometry
of ADP generated per enzyme monomer during the first enzyme turnover, is $2.61$
$\pm 0.16 \mu M$ for λNC26 and $2.81 \pm 0.18 \mu M$ for λNC99. 5 μM of ELon was used in
the ATPase reactions, and only ~50 % burst was observed. This suggests that in
pre-steady-state burst Lon has half-site reactivity. This agrees with previous
results that were obtained by the peptide substrate. Burst kinetics displayed by
the two reactions are comparable within experimental error. The $k_{burst}$ substrate
stimulated ATPase is between 6.49-11.54 sec$^{-1}$, which is similar to the $k_{1,FRET,ATP}$
values (Table 5.1) obtained from fitting the first 0.5 sec of the ATP-dependent
dansylated translocation time course. This suggests that the initial burst of ATPase in ELon is coordinated with the first phase of substrate delivery in an ATP-dependent reaction.
Figure 5.6 Pre-steady time courses of ATP hydrolysis by ELon.

(A) [α-32P] ATP (200 µM) was incubated with 5 µM monomeric ELon in the present of 8 µM λNC26 (■), λNC99 (●), DansylλNC26 (♦), and DansylλNC99 (▲), and reactions were quenched with acid at the indicated times. (B) [α-32P] ATP (25 µM) was incubated with 5 µM monomeric ELon in the present of 8 µM λNC26 (■), DansylλNC26 (♦), and DansylλNC99 (▲), and reactions were quenched with acid at the indicated times. (C) [α-32P] ATP (200 µM) was incubated with 5 µM monomeric ELon in the present of 8 µM λNC26 (■) or λNC99 (●), and reactions were quenched with acid at the indicated times. The concentrations of [α-32P] ADP generated in the reactions were determined by TLC followed by PhosphorImaging. The values on the y axis were obtained by dividing the [ADP] produced by 5 µM monomeric ELon, which reflects the mole equivalent of ADP produced per Lon monomer.  

(D) Time points from 0 to 400 msec of λNC26 (■) or λNC99 (●) were fit with equation 5.3 to yield the kinetic parameters summarized in Table 5.2.
Table 5.1 Stopped Flow Translocation of Kinetic Constants for DansylλN interacting with S679W in the presence of ATP.  

<table>
<thead>
<tr>
<th></th>
<th>$k_{1\text{FRET, ATP}}$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DansylλNC26</td>
<td>16.63 ± 0.46</td>
</tr>
<tr>
<td>DansylλNC42</td>
<td>8.91 ± 0.47</td>
</tr>
<tr>
<td>DansylλNC99</td>
<td>5.82 ± 0.13</td>
</tr>
</tbody>
</table>

Table 5.2 Pre-steady-state ATPase activity of WT ELon with λNC26 and λNC99.  

<table>
<thead>
<tr>
<th></th>
<th>$k_{\text{burst}}$ (sec$^{-1}$)</th>
<th>Burst amplitude, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>λNC99</td>
<td>11.54 ± 1.79</td>
<td>2.81 ± 0.18</td>
</tr>
<tr>
<td>λNC26</td>
<td>6.49 ± 1.08</td>
<td>2.61 ± 0.16</td>
</tr>
</tbody>
</table>
ATP hydrolysis at saturating conditions was examined to compare with the saturating conditions of ATP-dependent translocation. To further assess ATP hydrolysis, varied λN substrate and ATP concentrations were examined. Figure 5.7 shows varied λN substrate (A) and ATP (B); both plots show an initial burst followed by the steady state curve. The burst phase is indicative of the rate-limiting step occurring after ATP hydrolysis. The first 400 msec of the data in Figure 5.7 (A-B) were fitted with equation 5.3; data is summarized in Table 5.3 and 5.4. The linear phase of the time course was fit with a linear function to yield the steady-state turnover numbers, also summarized in Table 5.3 and 5.4.
Figure 5.7 Pre-steady-state time courses of varying $[\lambda N]$ and [ATP].

(A) 5 μM ELon was incubated with 0 (●), 0.5(■), 1 (♦), 2(▲), 3(▼), 5 (+), 6 (X), and 8 (○) μM λNC26. (B) 5 μM ELon was incubated with 5 (●), 10(■), 25 (♦), 50 (▲), 100 (▼), and 200 (+) μM $[\alpha-^{32}P]$ ATP. The reactions were rapidly quenched with acid at the indicated times, and the amount of $[\alpha-^{32}P]$ ADP formed was determined by PhosphorImaging. Each time course was repeated at least 3 times, and the average data are reported.

Table 5.3 Kinetic parameters on varying λN from Chemical-Quench Flow.

<table>
<thead>
<tr>
<th>Burst amplitude (μM)</th>
<th>$K_{burst;amp,\lambda N}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.20 ± 0.12</td>
<td>3.97 ± 0.99</td>
</tr>
<tr>
<td>$k_{ss,\lambda N}$ (sec$^{-1}$)</td>
<td>$K_{ss,\lambda N}$ (μM)</td>
</tr>
<tr>
<td>0.90 ± 0.20</td>
<td>3.53 ± 0.34</td>
</tr>
</tbody>
</table>

Table 5.4 Kinetic parameters on varying ATP from Chemical-Quench Flow.

<table>
<thead>
<tr>
<th>Burst amplitude (μM)</th>
<th>$K_{burst;amp,ATP}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.00 ± 1.13</td>
<td>115.87 ± 6.45</td>
</tr>
<tr>
<td>$k_{ss,ATP}$ (sec$^{-1}$)</td>
<td>$K_{ss,ATP}$ (μM)</td>
</tr>
<tr>
<td>0.94 ± 0.01</td>
<td>83.15 ± 19.03</td>
</tr>
</tbody>
</table>
Figure 5.8 Fitting of pre-steady-state kinetic parameters of ATP hydrolysis by ELon.

Burst amplitudes for varying λN concentrations (A) were determined by fitting the data from 0 to 400 msec. The maximum burst amplitude obtained from the fit was 2.20 μM, which corresponds to ~30% of the enzyme present in the reaction; a $K_{\text{burst amp, } \lambda N} = 3.97$ μM, was determined. (B) Data from 600 msec to 1.8 sec were fit with a linear line in order to provide steady-state rates of varied λN concentration. The $k_{\text{ss, } \lambda N} = 0.90$ sec$^{-1}$ and a $K_{\text{ss, } \lambda N} = 3.97$ μM. (C) Burst amplitudes for varying ATP concentration were determined by fitting the data from 0 to 400 msec. The maximum burst amplitude obtained from the fit was 3.00 μM, which corresponds to ~60% of the enzyme present in the reaction; a $K_{\text{burst amp, ATP}} = 115.87$ μM was determined. (D) Data from 600 msec to 1.8 sec were fit with a linear line in order to provide steady-state rates of varied ATP concentration. The $k_{\text{ss, ATP}} = 0.73$ sec$^{-1}$ and a $K_{\text{ss, ATP}} = 48.15$ μM. All time points reported are an average of at least three different trials. The error bars represent the standard error of the fit.
In order to determine how ATP hydrolysis and peptide bond cleavage of full length λN are correlated, the time courses of peptide cleavage and the protein-stimulated ATP hydrolysis were examined. Despite the usage of 5 μM ELon, only ~2 μM of ADP formation was detected in the burst phase of the ATPase time course. Since acid-quench experiments measure the ADP formation at the active site of the enzyme, the detection of a substoichiometric burst could be attributed to ~50 % of the enzyme saturated with ATP due to low ATP concentration. Therefore, when I varied ATP concentration, an increase in burst amplitude was observed. Varying λN concentrations were also performed for comparison. The burst amplitude and steady state were fit and plotted (Figure 5.8 A-F). As the concentration of ATP or λN protein was increased, there was also an increase in burst amplitude and steady state rate. Using these parameters, Kintek Global Explorer, an enzyme fitting program, can be used to globally fit the data in order to determine a working mechanism for ELon.

5.4 Conclusion

Comparing the degradation of dansyl labeled λN versus non-labeled λN shows that the addition of dansyl does not interfere with degradation and ATPase efficiency. Currently, there is no recognition tag known in λN substrate; however, based on the rate constants of translocation the results show that ATP-dependent dansylated translocation show a preference for C-terminal translocation. Results from chemical-quench flow show a rapid burst followed by the steady-state. The burst rate was compared to the first 0.5 sec of the ATP-dependent dansylated translocation. The rate constants from the burst phase
and the translocation phase show comparable kinetic values, suggesting that the first phase in translocation is correlated with the burst rate of ATPase.

Comparing the lag phase with the translocation experiments indicates that almost complete translocation occurs before degradation of the substrate can take place. Since peptide cleavage occurs after complete translocation of λN, any partially translocated λN would have been detected as undigested λN in SDS-PAGE. The delivery of the scissile site beginning at the C-terminal was implicated by comparing the kinetic time course of translocation on the different regions of λN, represented by dansylλNC26, dansylλNC42 and dansylλNC99 by S679W in the presence of ATP.

In order to look further into the mechanism of Lon, varying concentrations of dansylλN and ATP in ATP-dependent translocation were examined. Attempts to fit each trace using the traditional method of double or triple exponential equation have failed. However, it was observed that dansylλNC99 displays a one-step curve, while dansylλNC42 displays a two-step curve, and finally dansylλNC26 displays a three-step curve. The observed change in FRET signal could be due to the change in conformation as dansyl translocates closer to the proteolytic chamber. Based on this, it can be implied that there is a preference for which terminal of λN is translocated first. It was found that the C-terminal end of the substrate is preferred since dansylλNC99 shows a one-step signal compared to dansylλNC42 and dansylλNC26.

Varying λN and ATP concentrations were also monitored using chemical quench flow. The burst in ATP hydrolysis indicates that the rate-limiting step of
the reaction occurs after ATP hydrolysis. Therefore, it could be ADP product release. The detection of the rate-limiting step following ATP hydrolysis is consistent with the fact that ADP release limits ATPase turnover in ELon.\textsuperscript{3, 40} Taken collectively, the results support a reaction model where peptide cleavage occurs after ATP hydrolysis and the first turnover of peptide cleavage is coupled with the rate-limiting step in ATP hydrolysis. The steady state rates and burst amplitudes were comparable with the rates that were obtained at saturating conditions. Taking all the kinetic data together, can help determine a working mechanism for ELon in the future.
Chapter 7

Summary Findings and Future Directions
7.1 Summary Finding

In chapter 2, the function of the N-terminal in ELon was examined. Three different truncated ELon mutants were generated to determine the importance of the N-terminal in ELon. Based on the ATPase results, WT and Δ1-239 show comparable $k_{\text{obs}}$ values, while Δ232-252 and Δ1-252 were ~4-fold lower. This suggests that ATP hydrolysis of ELon is compromised when residues 240-252 are missing from the N-terminal of ELon.

It is known that ELon degrades unstructured λN proteins. WT and Δ1-239 show degradation of λN within 10 min. Δ232-252 and Δ1-252 show a reduction in protein degradation. The reduction of λN degradation from Δ232-252 and Δ1-252 suggests that despite the removal of 30% of the N-terminal Lon still remains functional as an ATP-dependent protease in processing unstructured substrate. The degradation profile was also examined using a structured protein substrate, *Caulobacter crescentus* CcrM. Under equal stoichiometric amounts of enzyme and substrate concentrations, WT ELon shows a slight degradation of the structured substrate, while the three mutants show no degradation. Since only WT possess these residues, it is clear that when degrading *Caulobacter crescentus* CcrM, Lon needs residues 232-239 (amino acid sequence: EQMKAIQK). This suggests that residues 232-239 may be responsible for unfolding the protein substrate or conformational changes of ELon prior to substrate translocation and cleavage. Based on the degradation and ATPase results, this suggests that the region between residues 232-252 is responsible for the reduction of the ATPase activity in Lon. Furthermore, λN degradation was
observed at high concentrations for Δ232-252 and Δ1-252. This is because more enzyme:substrate complex is formed, and therefore substrate degradation becomes more apparent. The observed $K_{MN}$ in WT and Δ1-239 are ~3 times lower than the other two mutants. This proves that the region containing residues 240-252 contributes to substrate interaction.
Figure 7.1 Proposed Mechanism for ATP-Dependent Proteolysis.\textsuperscript{38}
General mechanism of ATP-dependent proteolysis, red box indicates the step where substrate cleavage was being studied in chapter 3.
In chapter 3, a quantitative approach was taken in order to determine the rate of substrate cleavage (step 4 in Figure 7.1). A previously engineered, fluorogenic substrate, was used in order to determine the peptidase results for the truncated mutants. In the steady-state peptidase study, it was found that Δ1-239 and Δ232-252 ELon mutants catalyze cleavage of FR 89-98 with a comparable steady-state rate as WT Lon. The $k_{cat}$ and $n$ values of the truncated mutants Δ1-239 and Δ232-252 were similar to WT Lon. However, Δ1-252 has a $k_{cat}$ that is ~1.8-fold lower, which shows that all the mutants possess intact ATP-dependent peptidase activity, despite the reduced ATPase activity observed in Δ232-252 and Δ1-252 Lon. Although a majority of the N-terminal was removed, peptidase activity was still observed.

After determining that ATPase and peptidase activity in ELon was still functional despite the removal of most of the terminal, chapter 4 examined the directionality and preference of substrate cleavage. In order to identify whether there is a preference for cleavage, Edman sequencing was done on the partially digested product of C-his-λN. The results showed that the first 5 amino acid residues of the C-his-λN were KPVNR (residues 31-35), indicating that the cleavage occurred between residues A30-K-31 of the N-terminal. Attempts to identify the cleavage site in the N-terminally his-tagged λN by the same approach were unsuccessful. To further examine the rate of cleavage, FRET of NO$_2$Y and Abz was used. Two synthetic λN (CsFRλN and NsFRλN) were used to study the kinetics of peptide bond cleavage at each terminal. The steady-state kinetic results of CsFRλN and NsFRλN are comparable. The detection of the
comparable \( k_{\text{lag}} \) and \( K_m \) values suggest the same rate-limiting step may occur. At saturating conditions, pre-steady-state results are comparable. The presence of a lag phase was observed in the cleavage of full length protein substrate with ATP. The similarity in the lag rates confirms that Lon has no preference for directionality of cleavage.

In chapter 5, the translocation of substrate into the proteolytic site was examined. To monitor this step, dansyl labeled \( \lambda N \) was used with FRET with a Trp in a proteolytically inactive S679W ELon mutant. During the excitation of the Trp, the fluorescence of the dansyl signal increases as it approaches the Trp located in the active site. In order to determine that the dansyl does not interfere with the efficiency of degradation, the degradation of labeled substrate versus non-labeled substrate was examined. It was determined that both labeled \( \lambda N \) and non-labeled \( \lambda N \) show similar degradation profiles, suggesting that dansyl does not interfere with the efficiency of substrate degradation. After determining that dansyl does not interfere with protein degradation, translocation of dansyl\( \lambda N \) into the active site by using a proteolytically inactive Lon was examined. In the presences of ATP it was found that dansyl\( \lambda NC99 \) shows a one-step curve, while dansyl\( \lambda NC42 \) shows a two-step curve, and dansyl\( \lambda NC26 \) shows a three-step curve. Comparing the lag phase with the translocation experiments indicates that almost complete translocation occurs before degradation takes place. The translocation data obtained in the presence of ATP was too complicated to fit with the traditional double or triple exponential equation. Therefore, AMPPNP was used by Natalie Mikita to determine the translocation rate.

The rate
indicates that the C-terminal enters the active site first.\textsuperscript{70} Comparing the rate of
the lag reaction to the rate of translocation in the presences of ATP indicate that
\( \lambda N \) is translocated before cleavage. This indicates that protein degradation in
ELon occurs through model 1 in Figure 4.1, where the \( \lambda N \) is fully translocated
before cleavage occurs.
Figure 7.2 Model 1 of $\lambda N$ degradation by Lon.\textsuperscript{70}

In model 1, the protein substrate is completely translocated before peptide bond cleavage occurs, resulting in fully digested products. Lon N-terminal domain is indicated in blue, ATPase domain in red and proteolytic domain in green.
7.2 Future directions

Using different types of kinetics, I have learned a lot about the substrate translocation and cleavage of ELon. I have confirmed that ELon degrades NsFR\(\lambda\)N and CsFR\(\lambda\)N with comparable rates, suggesting that all \(\lambda\)N cleavage sites are cleaved at the same time. Based on the translocation data, full substrate translocation appears to take place prior to substrate degradation. Finally, ATPase results indicate that there is an initial burst of ATP hydrolysis prior to substrate cleavage. Now we have kinetic information on the individual steps from ATP hydrolysis to substrate degradation, but the full detailed mechanism is still unknown. The next step is to take the data from translocation, pre-steady-state peptide bond cleavage, and chemical quench flow and import them into Kintek Global Explorer. This program will be able to globally fit the collected data and determine a working mechanism for Lon protease. Once a working model is determined for ELon, the same method can be applied to hLon.

ADP inhibition towards hLon needs to be explored in the same fashion as ELon. The differences can be significant in determining specific inhibitors that can inhibit bacterial Lon while keeping the function of hLon. Based on the chemical quench flow, approximately half site reactivity was observed in the stimulated ATPase reaction of ELon, however hLon is unknown. Half-site reactivity in ELon was originally indicated by three different methods. The first was a filter-binding study, where all the enzyme subunits bind to ADP.\(^{66}\) The second method was pulse chase, where a full burst of ADP production was observed when the reaction is chased with unlabeled ATP. Even though the
theory of half-site reactivity is supported by the kinetic evidence; another theory that can explain the observed 50% burst amplitude of ATP hydrolysis is the idea that ATP hydrolysis is reversible. In order to address this issue in hLon and compare it with ELon, positional isotope exchange experiments can be done in order to show how many $^{18}$O is incorporated; if one $^{18}$O is incorporated it would indicate that ATPase is irreversible. To provide a better understanding of $^{18}$O incorporation reactions done at various time points can provide a more accurate understanding and ensure that over 1 hour the $^{18}$O incorporation is still one. Aside from looking into the $^{18}$O experiment, a filter binding assays can be performed to further confirm half-site reactivity.

In order to study further into the difference in ATP hydrolysis between hLon and ELon, ATP binding was studied using MANT-ATP. MANT-ATP is a fluorescent nucleotide analogue, 2’-(or 3’-) O-(N-methylanthraniloyl) adenosine triphosphate used to monitor ATP binding. MANT-ATP is known to have an increase in fluorescence once it is bound to protein. Based on a method that we previously published, I can use MANT-ATP, MANT-ADP or MANT-AMPPNP to observe the on/off-rate of ATP binding using stopped-flow fluorescence spectroscopy. Using varying amounts of MANT-ATP, the time courses can be fitted with a double exponential equation. Fitting the data to a double exponential equation will yield two observed rate constants. Plotting the rate constants to the corresponding nucleotide will reveal a dependency of the observed rate constant against the nucleotide. The on-rate and off-rate of nucleotide binding and release can be determined by this type of experiment. It is possible that the binding
mechanism of nucleotides may be different compared to ELon, therefore a
different kinetic model may be needed to fit the time courses in order to
determine the differences in ATP hydrolysis between the two.

Another approach is to create a hLon mutant where the serine in the
active site is mutated to a tryptophan. Using a previously published technique\textsuperscript{67}, I
can determine if hLon is proteolytically inactive but still have comparable ATPase
activity of wild-type hLon. Using dansyl fluorescence, it will allow us to determine
if the translocation step in hLon is the same as ELon. In our previously published
results, it was indicated that the first 0.5 ms of \( \lambda N \) translocation in ELon is
correlated with the ATPase step\textsuperscript{70}. Creating the proteolytically inactive hLon we
can monitor to see if the initial milliseconds of translocation are the same as the
ATPase step. The amplitude of the burst in ADP production, which generally
reflects the concentration of active enzyme used in the reaction. Based on the
results from the chemical quench flow, it has indicated that only 50\% of ELon is
active. According to the pulse-chase experiment it was determine that the burst
amplitude was close to the amount of enzyme used, indicating that the low burst
amplitude is not due to inactive enzyme. To further confirm this, filter binding
assay can be used to determine if the low burst rate is from inactive enzyme. The
difference obtained from the hLon when compared to ELon can provide
information in the development of therapeutic procedures to cure certain bacterial
diseases in humans.

In summary, the experiments performed have indicated differences
between hLon and Elon. The next step is to take the data that was collected from
ELon and map out a working mechanism using Kintek Global Explorer. After a
detailed mechanism is determined, this information can be used to determine the
mechanism of hLon. By doing so, one can determine similarities and differences
in bacterial Lon and hLon. Knowing the differences in functionality is essential for
therapeutic purposes.
Identification of a Region in the N-Terminus of *Escherichia coli* Lon That Affects ATPase, Substrate Translocation and Proteolytic Activity

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**Keywords:** Lon protease; allosteric modulation of ATPase; coiled-coil structure; substrate translocation; hydrogen–deuterium exchange

Lon, also known as protease La, is an AAA+ protease machine that contains the ATPase and proteolytic domain within each enzyme subunit. Three truncated *Escherichia coli* Lon (ELon) mutants were generated based on a previous limited tryptic digestion result and hydrogen–deuterium exchange mass spectrometry analyses performed in this study. Using methods developed for characterizing wild-type (WT) Lon, we compared the ATPase, ATP-dependent protein degradation and ATP-dependent peptidase activities. With the exception of not degrading a putative structured substrate known as CcrM (cell-cycle-regulated DNA methyltransferase), the mutant lacking the first 239 residues behaved like WT ELon. Comparing the activity data of WT and ELon mutants reveals that the first 239 residues are not needed for minimal enzyme catalysis. The mutants lacking the first 252 residues or residues 232–252 displayed compromised ATPase, protein degradation and ATP-dependent peptide translocation abilities but retained WT-like steady-state peptidase activity. The binding affinities of WT and Lon mutants were evaluated by determining the concentration of λ N (\(K_{\lambda N}\)) needed to achieve 50% maximal ATPase stimulation. Comparing the \(K_{\lambda N}\) values reveals that the region encompassing 232–252 of ELon could contribute to λ N binding, but the effect is modest. Taken together, results generated from this study reveal that the region constituting residues 240–252 of ELon is important for ATPase activity, substrate translocation and protein degradation.

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**Introduction**

Lon, also known as protease La, is one of the simplest ATP-dependent proteases found in a variety of organisms including plants, bacteria and mammals.\(^1^–^4\) Based on the amino acid sequence of the ATPase domain exhibiting significant homology with AAA+ (AAA, ATPase associated with various cellular activities) proteins, Lon is assigned to this super protein family.\(^5^–^6\) As such, it is anticipated that the ATPase domain of Lon acts as a molecular motor, which couples the binding and hydrolysis of ATP to activate proteolysis.\(^7\) Since its discovery, Lon...
has been found to display diverse biochemical properties including binding to nucleic acids,\textsuperscript{8–11} unfolding and degrading certain improperly folded or unstructured proteins.\textsuperscript{12,13} As an oligomeric protease machine, Lon contains an amino-terminus, an ATPase and a proteolytic domain within each enzyme subunit.\textsuperscript{14} While the ATPase domain and proteolytic sites of most lon homologs share high sequence homology, the amino acid sequences of the amino-termini are diverse.\textsuperscript{6} Recently, the crystal structures containing residues 7–245 of ELon,\textsuperscript{15} TonLon [(Protein Data Bank ID 3K1J) Thermococcus onnurineus NA1 structure] lacking residues 134–170\textsuperscript{16} and BsLon lacking the first 245 residues [BsLon-AP\textsuperscript{17} [(Protein Data Bank ID 3M6A) truncated Bacillus subtilis Lon structure]] were solved, with ADP bound to the latter two structures. The amino acid sequence of TonLon aligns with residues 305–778 of Escherichia coli Lon and is proposed to be a general structural model for studying the proteolytic mechanism of lon homologs.\textsuperscript{16} BsLon-AP constitutes residues 246–770 of the full-length protein and bears >70% sequence homology with residues 248–772 of ELon, which also contains the ATPase and proteolytic domains. Comparing these structures suggests that the N-terminal domain of Lon connects to the rest of the ATPase/proteolytic subunit via a long helix that has a high propensity to form a coiled-coil structure.\textsuperscript{15,17,18} Within this proposed coiled-coil structure lies one or multiple nonspecific proteolytic sites that are found in BsLon,\textsuperscript{17} ELon\textsuperscript{9,20} and Mycobacterium smegmatis Lon (MsLon\textsuperscript{21}). Limited proteolytic digestion of these lon homologs typically generates Lon fragments lacking the N-terminal domain but possessing an intact ATPase and proteolytic domain located within the same polypeptide.

Comparing the sequences of BsLon and ELon reveals that the only structurally undefined region in the former consists of residues 210–245, which corresponds to residues 212–247 in ELon. The trypsin-sensitive lysine residues are found in the structurally unknown region (K235 and K239 in ELon\textsuperscript{20} and K233 and K237 in BsLon). In a publication by Patterson et al.,\textsuperscript{20} K235 and K239 were labeled as K234 and K238. In the most recent accession number of ELon (AAC36871), these two residues are K235 and K239, respectively. In the rest of the study, the latter numberings are used. Given the conserved nature of residues surrounding positions 200–240 of the two bacterial Lon and the lack of protection from limited trypsin digestion from nucleotide binding, this stretch of peptide may be important for enzyme function. To narrow the region to be evaluated for functionality, we employed hydrogen–deuterium exchange coupled with liquid chromatography/mass spectrometry (LC-MS) [hydrogen–deuterium exchange by mass spectrometry (HXMS\textsuperscript{22})] technique to probe the solvent accessibility and structural dynamic of the region flanking residues 232–252 of ELon in nucleotide-free and MgATP-bound enzymes. As K239 of ELon was accessible to limited tryptic digestion,\textsuperscript{20} the peptide backbone flanking this residue should be susceptible to deuterium exchange and be detected by mass spectrometry. Because the structure dynamics of residues 232–252 were altered by ATP binding, the truncated Lon mutants Δ1–239, Δ232–252 and Δ1–252 were generated and examined to elucidate the function of these residues. To better understand the role played by the deleted residues in mediating ELon catalysis, we quantitatively compared the ATPase, the peptidase and the binding of λ N by mutants versus wild-type (WT) enzymes using kinetic techniques. This study differs from previous structure–function evaluations of the amino-terminal domain of Lon by employing HXMS to define the boundaries of the proposed coiled-coil region in ELon to be examined and a quantitative approach to measure the effects of losing sections of this region on the enzymatic activities.\textsuperscript{18,21} We discovered that residues within position 239–252 of ELon played an important role in the maintenance of optimal ATPase turnover. Mutants lacking this region also showed deficiencies in substrate translocation and protein degradation. Additionally, the amino-terminus of ELon might function to aid substrate unfolding, as the Δ1–239 mutant failed to degrade the putative structured protein substrate CcrM (cell-cycle-regulated DNA methyltransferase) despite its ability to translocate substrate and degrade unstructured protein such as λ N.

Results

Peptide mapping and coverage

ELon was digested with pepsin to yield ~160 peptide fragments whose sequences were identified by LC-MS/MS analysis using the Sequest program. Compiling the mass spectrometry data reveals 78% peptide coverage of the ELon sequence. Previously, we showed that nucleotide-free ELon and MgATP-bound ELon were cleaved at K239 by trypsin, suggesting that this region was solvent accessible.\textsuperscript{20} The detection of residues 232–252 as one of the covered peptide sequences suggests that HXMS could be used to assess the solvent accessibility or structural dynamics of the region surrounding K239. To this end, nucleotide-free ELon and ELon:MgATP were independently incubated with deuterated buffer for various times and then digested with pepsin under acidic conditions for subsequent LC-MS analysis. It was anticipated that the amide hydrogens in the backbone of ELon would exchange with deuterium in the buffer, with the more solvent-
exposed hydrogens exchanging faster than the structured ones. Monitoring the deuteration of ELon as a function of time by LC-MS would provide insights into the solvent accessibility of the amide backbone of the peptide containing residues 232–252 of ELon.

To add confidence to the interpretation that region 232–252 is partially protected from solvent in the presence of MgATP, we included the peptide sequence flanking residues 42–52, which showed very little changes in its m/z distribution in the presence versus absence of MgATP, as a negative control. As seen in Fig. 1a, the mass spectra at each time point are only shifted at most 0.3 m/z units if at all (determined using eq 2). This equates to at most 1.5 more amide hydrogens being exchanged into the peptide backbone of residues 42–52 of ELon. To further validate our findings, we also probed the peptide region containing the proteolytic S679 of ELon by HXMS (Fig. 1b). Previous kinetic experiments revealed that the environment surrounding S679 was altered when ELon was bound to MgATP, suggesting the presence of at least one conformational change. Therefore, the MgATP-induced conformational change at the vicinity of S679 in ELon would be detectable by HXMS and served as a positive control. For comparison, the m/z data of fully deuterated and undeuterated ELon at residues 232–252, from residues 659 to 681 and from residues 42 to 52, were obtained as described in Materials and Methods.

In proteins, hydrogen–deuterium exchange may occur via two different mechanisms: EX1 and EX2. In an EX2 mechanism, the exchange reaction occurs slower than the unfolding and refolding of the peptide region of interest, whereas in an EX1 mechanism, the exchange reaction occurs at a much faster rate. Experimentally, EX2 kinetics are revealed by a gradual shift of a single isotopic peak of the data to a higher m/z value in the time course of the reaction. EX1 kinetics, however, are characterized by the detection of two isotopic peaks in the mass spectrum time courses. The two peaks with low and high m/z values correspond to two states of the peptide: a folded, more protected and less solvent exposed state (with a lower m/z value) and an unfolded, less protected and more solvent exposed state (with a higher m/z value). These two peaks can be set to a double Gaussian distribution, while EX2 kinetics can only be set to a single Gaussian distribution.

Figure 1b shows the HXMS result of residues 659–681 in ELon versus ELon:MgATP complex. The top panel reveals the mass shifts for the peptide region encompassing residues 659–681 of ELon upon complete exchange with deuterium. The bottom panel shows the extent to which structural changes associated with ELon binding to ATP could be discerned in the bottom panels. The 659–681 peptide shows EX2 kinetics, due to the appearance of just one peak shifting to a higher m/z value over time, and a single Gaussian can be fit to each peak (Fig. 2). Comparing the mass spectra of ELon versus ELon: MgATP complex reveals that the latter is more resistant to deuterium exchange, due to the detection of a lower m/z isotopic peak in all the time points. At each time point, the centroid is about 0.7 m/z unit higher in the nucleotide-free enzyme; using Eq. (2), we deduced that about four more amide hydrogens were exchanged into the non-nucleotide-bound enzyme form at each time point. Therefore, MgATP indeed caused a localized structural change in the peptide region encompassing residues 659–681 of ELon. Given that S679, which is located in the 659–681 peptide region, is important for enzymatic activity, it is possible that any structural difference observed in the peptide sequence containing 232–252 due to MgATP binding will be associated with enzyme activity. While previous limited tryptic digestion studies could not identify structural differences at K239 in the absence versus the presence of ATP bound to ELon, HXMS results (Fig. 3, bottom panels) show that the peptide region containing residues 232–252 exhibits different exchangeability of D2O as a function of time between the two enzyme forms. Figure 3 further shows that residues 232–252 in the ELon-bound MgATP form adopts an EX1 mechanism in deuterium exchange, which is illustrated by the presence of two Gaussian peaks shifting to a single higher m/z peak at increasing incubation time with deuterated buffer. These Gaussian peaks can be used to determine the amount of each peptide form present. Fitting the area of each Gaussian into Eq. (3) provided a percentage of the folded, or protected, form of the peptide. These percentages were then used to determine if a region was more or less folded in the presence of MgATP due to the change in percentages of each form. Using Eq. (3), we calculated the extent of peptide protected from H/D exchange at each time point. In the nucleotide-free ELon exchange reactions (Fig. 3, left panels), the 5- and 10-s time points detected a 70% protection from deuterium exchange. By contrast, the 500-s time point detected only fully exchanged peptide, indicating the presence of only unprotected state of the same region (a fact determined because the 500-s mass spectrum was unable to be fit to a double Gaussian). In the reactions performed with ELon:MgATP (Fig. 3, right panels), the 5-s and 10-s time points detected 66% and 63% of the unexchanged peptide, respectively. By 500 s into the reaction, 28% of the peptide was still protected from H/D exchange, a result that was not seen in the 500-s nucleotide-free Lon H/D exchange reaction. Taken together, the HXMS

Fig. 1 (legend on next page)
data show that when bound to MgATP, the peptide region consisting residues 232–252 of ELon was more protected from deuterium exchange, thereby suggesting the formation of a more folded or conformationally rigid local structure in this region. Additionally, ELon might exist in two different conformations that were in equilibrium. Binding of MgATP to ELon favored the formation of one of the more “folded” enzyme forms.

**Overexpression and purification of ELon mutants**

To evaluate the function(s) of the region containing residues 232–252 in ELon, we generated the
Fig. 3. Mass spectra of peptide 232–252 (EQMKAIQKELGEMDDAPDENE) after various incubation times in ELon buffer with D$_2$O. Undeuterated and fully deuterated spectra are shown in the top panel. UD, undeuterated protein; TD, totally deuterated protein. The mass spectra comparing ELon preincubated with and without ATP are shown in the bottom panels. In the HXMS time points, double Gaussian distribution of isotopic peaks is detected, indicating the existence of an EX1 H/D exchange mechanism. Upon incubation with ATP, there is a shift in the isotopic distribution of the peptide, indicating that ATP binding to ELon hinders deuterium from exchanging with hydrogen in the NH of this region.
Surveying the ATPase activity of ELon mutants

Lon possesses intrinsic ATPase activity that is stimulated by protein or certain peptide substrates.\textsuperscript{14,26,27} To evaluate the effects of enzyme truncation, we used the physiological substrate λ N to stimulate the ATPase activity of WT and Lon mutants.\textsuperscript{28} The ATPase activity of Lon was measured by the radiometric ATPase assay that was used to characterize the mechanism of WT enzyme.\textsuperscript{20} Figure 4 shows the plot correlating the $k_{\text{obs}}$ of intrinsic ATPase of the three Lon mutants, determined by dividing the steady-state rates with enzyme monomer concentration, with corresponding [ATP] to provide the $k_{\text{cat}}$ and $K_{m}$ values of the respective protein as summarized in Table 1. For comparison, the kinetic parameters of WT Lon were included. According to Table 1, neither the $K_{m}$ of ATP nor the ability of λ N to stimulate ATP hydrolysis in each protein is altered by any of the truncations, but the $k_{\text{cat}}$ of ATPase is reduced by 3.5- to 10-fold in Δ232–252 and Δ1–252 compared to WT and Δ1–239 ELon. Since the peptide region containing residues 232–252 is not part of the AAA+ domain, one possibility to account for the observed reduction in $k_{\text{cat}}$ is that residues within the 232–252 region play a role in modulating the ATPase activity. Alternatively, excision of this region in Lon may compromise the folding/structural integrity of the hexameric enzyme that adversely affects the ATPase function. While the data presented here cannot distinguish the two possibilities, it does highlight the importance of region 232–252 in rendering Lon function despite its distal location from the ATPase site.

A mechanistic feature of most ATP-dependent proteases is the stimulation of ATPase by protein or peptide substrates.\textsuperscript{14,29} In ELon, the ATPase stimulation can be accomplished independent of proteolytic or peptidase activity, as the proteolytically inactive Lon mutant S679A or S679W exhibits WT-like ATPase activity upon interaction with peptide substrate.\textsuperscript{23} Through monitoring of the concentration of substrate needed to attain 50% maximal ATPase stimulation, the affinity of ELon for a model peptide substrate known as S3 [a mixed peptide substrate containing 10% of the fluorescent peptide

![Graphical comparison of $k_{\text{obs}}$ values of the intrinsic ATPase in Δ1–239 (▲), Δ1–252 (○) and Δ232–252 (□) Lon. The $k_{\text{obs}}$ values for ATP hydrolysis were determined at 25, 50, 100, 250 and 500 μM ATP containing [32P]ATP using the ATPase assay described in Materials and Methods. The time courses of ATP hydrolysis were quantified by the increase in the amount of [32P]ADP over time. The data presented are the average of at least three independent assays. Error bars reflect values obtained from experimental deviations.](image)

Table 1. Steady-state kinetic parameters for the ATP hydrolysis activity and λ N binding of WT and mutant ELon

<table>
<thead>
<tr>
<th></th>
<th>$k_{\text{cat}}$</th>
<th>$K_{m}$</th>
<th>$k_{\text{cat}}$ (λ N)</th>
<th>$K_{N}$</th>
<th>ATPase enhancement\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.43 ± 0.12 (0.26 ± 0.02)\textsuperscript{b}</td>
<td>47 ± 10\textsuperscript{b}</td>
<td>1.32 ± 0.15</td>
<td>1.4 ± 0.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Δ232–252</td>
<td>0.12 ± 0.01</td>
<td>72 ± 15</td>
<td>0.18 ± 0.02</td>
<td>5.3 ± 2.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Δ1–252</td>
<td>0.063 ± 0.006</td>
<td>53 ± 12</td>
<td>0.12 ± 0.01</td>
<td>4.8 ± 1.7</td>
<td>1.9</td>
</tr>
<tr>
<td>Δ1–239</td>
<td>0.65 ± 0.16</td>
<td>50 ± 12</td>
<td>1.25 ± 0.20</td>
<td>1.6 ± 0.9</td>
<td>1.9</td>
</tr>
</tbody>
</table>

\textsuperscript{a} These values were calculated by $k_{\text{cat}}$(+λ N)/$k_{\text{cat}}$.

\textsuperscript{b} These values were obtained from Ref. 20.

Fig. 5. Graphical representation of the stimulatory effect of λ N on the $k_{\text{cat}}$ of the ATPase activity of WT (X), Δ1–239 (▲), Δ1–252 (○) and Δ232–252 (▲) Lon. The $k_{\text{cat}}$ values of ATP hydrolysis were determined at 500 μM ATP containing α$[^{32}\text{P}]$ATP in the presence of 0, 1.5, 3, 7.5, 10 and 15 μM λ N using the ATPase assay described in Materials and Methods. The data presented are the average of at least three independent assays. Error bars reflect values obtained from experimental deviations.

Eq. (6) provides the apparent affinity of various Lon constructs for λ N ($K_{\text{N}}$) that are summarized in Table 1. As in the case of intrinsic ATPase, the $k_{\text{cat}}$ values of λ-N-stimulated ATPase are lowered in the mutants lacking residues 232–252. A modest variation (~3-fold) in the $K_{\text{N}}$ values is detected among the enzymes, but the standard errors associated with data fitting suggest that the $K_{\text{N}}$ values are probably comparable. Therefore, despite the fact that 232–252 constitutes the putative coiled-coil region in Lon that may be involved in substrate recognition, the contribution of this region toward λ N binding is modest or minimal. Taken together, the loss in catalytic turnover of ATPase activity is the most pronounced effect associated with the removal of 232–252 in ELon. Since Δ1–239 displays WT-like ATPase activity, we conclude that the region within 240–252 most likely contributes to the observed ATPase effect. As such, additional scanning mutagenesis studies should be able to further identify specific residues with enzyme functions. As residues 240–252 are not part of the ATPase domain, the effect of this region on the ATPase activity of ELon could be allosteric. Alternatively, the removal of residues 240–252 in ELon may lead to structural changes that interfere with the AAA+ domain function. Given that a 78% of peptide coverage was obtained in the HXMS experiment, additional analysis comparing the structural dynamics with alteration in enzyme function could potentially provide insights to further distinguish these two possibilities.

### ATP-dependent proteolytic activity of Lon

Lon is a processive protease that unfolds and translocates multiple Lon cleavage sites during protein degradation.28,31 As the proteolytic activity of Lon is dependent on its ATPase activity,74 reduction in the efficiency of ATPase in the Δ1–252 and Δ232–252 ELon may affect proteolytic activity. The endogenous unstructured ELon substrate λ N and the putative substrate caulobacter CcrM32 were used to assess the importance of each truncation in the enzyme. The time courses of WT and Lon mutants degrading λ N and the putative structured protein substrate caulobacter CcrM were obtained and compared by SDS-PAGE (Figs. 6 and 7). The concentration of λ N used was 20 μM, which was 2-fold $K_m$ based on the value extracted from the Maurizi study.28,29 At 1 μM protease, only WT and Δ1–239 exhibited λ N degradation. Judging from the 30-min time point, WT Lon was more efficient than Δ1–239 in degrading λ N (Fig. 6). When enzyme concentration was increased to 3 μM, λ N degradation was also detected in the Δ232–235 and the Δ1–252 mutants, albeit with reduced efficiency compared to WT and Δ1–239 Lon. Taken together, these data reveal that, despite the removal of the...
Fig. 6. ATP-dependent degradation of λ N protein by WT and Lon mutants. Reaction aliquots containing ELon or mutant [1 μM, (a–d); 3 μM (e–h)], λ N (20 μM) and 5 mM ATP in reaction buffers were quenched at the indicated times as stated in Materials and Methods. The quenched time points were resolved by 17.5% SDS-PAGE and visualized by Coomassie brilliant blue. Lanes 1–6 (in order) contain the time points when the reaction was quenched at 0, 1, 2, 5, 10 and 30 min. WT (a and e) and Δ1–239 (b and f) degrade λ N protein more efficiently than do Δ232–252 (c and g) and Δ1–252 (d and h).
N-terminal domain, which constitutes \( \sim 30\% \) of the protein size, Lon remains functional as an ATP-dependent protease in processing unstructured substrate such as \( \lambda \) N. By contrast, CcrM, which is an adenine DNA methyltransferase and is anticipated to adopt a globular structure like those found in certain DNA adenine methyltransferase,\(^{33,34}\) is a poor substrate of WT ELon (Fig. 7). Under equal stoichiometric amount of enzyme and substrate, CcrM degradation was only observed in the reaction containing WT protease. Compared with the results shown in Fig. 6, the CcrM degradation results suggest that residues 1–239 contribute to the processing of a putative structured substrate such as CcrM. Because \( \Delta232–252 \) also failed to degrade CcrM, residues flanking 232–239 are likely involved in mediating CcrM degradation.

ATP-dependent peptidase activity of Lon

Lon needs to unfold and deliver/translocate multiple scissile bonds within the unfolded substrate to the proteolytic site for peptide bond cleavage to occur.\(^{35}\) As such, deficiency in protein degradation does not necessarily reflect the competency of the proteolytic site in catalyzing peptide bond cleavage. For quantitative assessment of the functionality of the proteolytic site, a peptide substrate whose degradation does not require unfolding should be used. A fluorogenic peptide substrate cocktail known as S3, which contains residues 89–98 of \( \lambda \) N and a Lon cleavage site, has been routinely used to quantify the ATP-structured peptidase activity of Lon.\(^{29,36}\) This substrate contains an anthraniloyl group whose fluorescence is quenched by nitrotyrosine in the peptide substrate. Proteolytic digestion of S3 liberates the anthraniloyl fluorescence that was detected at 420 nm when excited at 320 nm. The amount of S3 hydrolyzed was calculated from the fluorescence signal generated using the method reported by Vineyard \textit{et al.}\(^{36}\) and is described briefly in Materials and Methods. Through prior mechanistic characterization studies, N-terminal domain, which constitutes \( \sim 30\% \) of the protein size, Lon remains functional as an ATP-dependent protease in processing unstructured substrate such as \( \lambda \) N. By contrast, CcrM, which is an adenine DNA methyltransferase and is anticipated to adopt a globular structure like those found in certain DNA adenine methyltransferase,\(^{33,34}\) is a poor substrate of WT ELon (Fig. 7). Under equal stoichiometric amount of enzyme and substrate, CcrM degradation was only observed in the reaction containing WT protease. Compared with the results shown in Fig. 6, the CcrM degradation results suggest that residues 1–239 contribute to the processing of a putative structured substrate such as CcrM. Because \( \Delta232–252 \) also failed to degrade CcrM, residues flanking 232–239 are likely involved in mediating CcrM degradation.

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the steady-state kinetic parameters of WT ELon catalyzing the degradation of S3 as well as intrinsic versus peptide-stimulated ATPase activity have been determined. Employing the same methodologies to evaluate the activities of the Lon mutants should allow direct quantitative comparison of the extent to which each mutation affects ATP-dependent peptide bond cleavage. The ATP-dependent peptidase of Lon mutants is shown in Fig. 8. Fitting the data with Eq. (7) from Materials and Methods generates the steady-state kinetic parameters summarized in Table 2. Overall, the peptidase results reveal that the Δ1–239 and Δ232–252 Lon mutants catalyzed cleavage of S3 with $k_{cat}$ and $n$ values matching with the ones determined for WT Lon. With regard to Δ1–252, the $n$ value matches well with that of WT Lon, but the $k_{cat}$ value is ∼1.8-fold lower. In all mutants, the $K_m$ for S3 is ∼2.5-fold higher than that for WT Lon. Taken together, all the mutants possess intact ATP-dependent peptidase activity despite the reduced ATPase activity observed in Δ232–252 and Δ1–252 Lon.

Previous studies showed that the translocation and cleavage of the peptide bond in the S3 substrate exhibited ATP dependency. Using fluorescent stopped-flow kinetic techniques, we identified an ATP-dependent peptide translocation step occurring in the pre-steady-state phase of the peptidase reaction catalyzed by WT ELon. The substrate translocation step was detected as a lag phase in the peptidase time course. To evaluate if the compromised ATPase in ELon mutants affects the peptide translocation step, we compared the time courses of S3 cleavage catalyzed by WT and Lon mutants in the presence of 1 mM ATP (∼10-fold $K_m$, see Table 1) and S3 (>3.5-fold $K_m$, see Table 2). Under this pseudo-first-order reaction condition, enzyme was predominantly bound with ATP and S3; therefore, substrate binding should not be partially rate limiting and be detected in the lag phase. Consequently, the lag kinetic time courses shown in Fig. 9 reveal the conversion of the enzyme:ATP:S3 complex to enzyme:ADP:Pi:hydrolyzed peptides. Fitting each time course with Eq. (10) and the steady-state rates of S3 hydrolysis obtained from the kinetic experiments described above yielded the respective lag rate constants ($k_{lag}$) that are summarized in Table 2. Based on previous kinetic studies on WT

### Table 2. Kinetic parameters of the ATP-dependent peptidase activity of WT and ELon mutants

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$k_{lag}$ (s$^{-1}$)</th>
<th>Hill coefficient (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>9.0 ± 0.5$^a$</td>
<td>102 ± 30$^a$</td>
<td>0.158 ± 0.009$^a$</td>
<td>1.60$^a$</td>
</tr>
<tr>
<td>Δ232–252</td>
<td>8.65 ± 0.52</td>
<td>261 ± 27</td>
<td>0.0287 ± 0.0001</td>
<td>1.67 ± 0.16</td>
</tr>
<tr>
<td>Δ1–252</td>
<td>5.97 ± 0.35</td>
<td>254 ± 24</td>
<td>0.0263 ± 0.0052</td>
<td>1.95 ± 0.21</td>
</tr>
<tr>
<td>Δ1–239</td>
<td>9.40 ± 0.68</td>
<td>280 ± 20</td>
<td>0.262 ± 0.042</td>
<td>1.20 ± 0.15</td>
</tr>
</tbody>
</table>

* Data obtained from Ref. 20.

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ELon, $^{23,30} k_{lag}$ is assigned to the ATP-dependent substrate translocation step occurring prior to peptide bond cleavage in the substrate. The fact that $k_{cat}$ values of S3 hydrolysis among WT and Lon mutants are comparable but their $k_{lag}$ values differ suggests that the efficiencies of ATP-dependent substrate translocation vary in these enzymes. Because $\Delta 232$–252 and $\Delta 1$–252 exhibit ~10-fold reduction in the $k_{lag}$ of ATP-dependent S3 cleavage and are deficient in ATPase turnover but not ATP binding, we conclude that substrate translocation is impaired in the mutants because the $k_{cat}$ of ATPase is significantly reduced. In conjunction with the observation that $\Delta 1$–239 and WT ELon produce comparable peptidase time courses, we conclude that residues 240–252 in ELon facilitate substrate translocation by maintaining optimal ATPase turnover.

**Discussion**

Aside from an apparent lack of CcrM degradation, $\Delta 1$–239 possesses WT-like ATPase, ATP-dependent peptidase and $\lambda$ N degradation activities (Figs. 4–9 and Tables 1 and 2). Comparing the activity profiles of WT with all three mutants reveals that only the degradation of CcrM is affected by residues 232–239, as only WT ELon, which contains these residues, degrades this protein substrate; $\Delta 1$–239, $\Delta 1$–252 nor $\Delta 232$–252 degrades CcrM. Therefore, residues within position 232–239 are needed to process CcrM. Furthermore, $\lambda$ N degradation was observed in the reaction containing 3 but not 1 $\mu$M $\Delta 232$–252 or $\Delta 1$–252 (Fig. 6). A lack of protein degradation in the reactions containing 1 $\mu$M mutants could be attributed to subsaturation of protease with substrate, due to reduced affinity. At 3 $\mu$M enzyme, more enzyme: substrate complex was formed, and thus, substrate degradation became apparent. This proposal is supported by the observation that the $K_{AN}$ values of WT and $\Delta 1$–239 are ~3-fold lower than those of the other two mutants. Taken together, the results support the idea that the region containing residues 240–252 contributes to substrate interaction. As has been proposed by others, ELon binds protein substrates at multiple sites, $^{12,35}$ and residues 232–239 and residues 240–252 likely constitute two of these sites. Another possibility to account for the apparent lack of CcrM degradation is that residues found in positions 232–239 are needed to unfold substrates such as CcrM. As $\lambda$ N lacks a defined structure, $^{36}$ no unfolding will be needed to accomplish protein translocation and subsequent degradation. The data presented in this study cannot distinguish these possibilities; therefore, additional studies will be required to evaluate the two possibilities.

In a study performed by Ebel et al., mutation of E240 to K abolished the degradation of RscA but not SulA in cells presumably because the two substrates interact with Lon at different sites. $^{18}$ Independently, Roudiak and Shrader demonstrated that MsLon lacking the first 91 or 226 residues had difficulties degrading casein but not peptide substrates and the ATPase activity in all mutants is significantly lower than that in WT enzyme. $^{21}$ While these studies reveal that the N-terminus of ELon is important for protein substrate degradation, they do not provide information on the location of the substrate binding site(s) and the extent to which substrate binding is affected by the mutation. When compared to the N-terminal deletion mutants of MsLon, the ELon mutants examined in this study are more active in degrading protein substrates. The differences between the two studies could be attributed to different substrates used. The sites needed to interact with the MsLon substrates were removed. In this study, we demonstrated that residues within 240–252 participate in substrate translocation and degradation of $\lambda$ N. This observation supports the proposal that region 240–252 constitutes a coiled-coil region designed for protein interaction. $^{15,17,18}$ As a 3-fold increase in $K_{AN}$ was observed in the $\Delta 232$–252 and $\Delta 1$–252 mutants (Table 1), additional sites in the mutants must be present to interact with $\lambda$ N to provide the observed $K_{AN}$ values.

An intriguing result generated from this study is the detection of EX1 H/D exchange behavior in residues 232–252 but not residues 659–681 of ELon (Figs. 2 and 3). Conceivably, Lon exists in two conformations in equilibrium with each another: one that is more susceptible to H/D exchange at residues 232–252 than the other. The binding of ATP shifts the equilibrium to the more protected or folded enzyme form (the lower $m/z$ peak in Fig. 3). This postulate is consistent with the cryo-electron microscopy study showing that the binding of ATP to yeast Lon causes a change in the symmetry of the heptameric oligomer. $^{37}$ Steady-state enzyme inhibition studies of ELon by a hydrolyzed peptide product also implicated the existence of two enzyme forms along the ATP-dependent peptidase pathway. $^{38}$ It should be noted that the H/D exchange mechanism of NH in amide backbone of residues 659–681 is EX2 (Fig. 2). In this case, the binding of ATP to ELon stabilizes the folded state of that region. Taken together, these data indicate that ATP binding to ELon allosterically induces structural changes in residues 232–252 of the N-terminal domain and in residues 659–681 of the proteolytic domain. Additionally, ELon may exist in equilibrium with different conformations that are perturbed by ATP binding. Alternatively, ELon may contain structural heterogeneity among its subunits such that the observed EX1 behavior is a manifestation of structural changes within the subunits induced by ATPase. Despite existing as a homohexamer, a set of high- and low-affinity ATPase sites has been present in the ELon subunits.
identified in WT ELon. Therefore, the EX1 behavior could be attributed to structural changes associated with the interconversion of the two kinds of ATPase among the subunits. The data presented in this study lack the resolution needed to distinguish the two possibilities. Additional experiments to further investigate the molecular basis of the EX1 behavior detected in this study are in progress.

Despite the presence of the majority of amino-terminus, the Δ232–252 mutant behaves like Δ1–252 ELon, which shows deficiency in catalyzing ATP hydrolysis in the absence and presence of protein substrate. ELon lacking the first 239 residues displays WT-like ATPase activity, indicating that residues located within positions 240–252 of ELon contribute to the efficiency of ATPase turnover. In heterosubunit ATP-dependent proteases such as ClpA, the D2 loop functions to unfold and translocate protein substrates. Given that the region flanking residues 240–252 interacts weakly with λ N and affects the catalytic efficiency of ATPase and substrate translocation, it is plausible that this region, which constitutes part of the proposed coiled-coil motif in Lon, adopts a similar function as the D2 loop in catalyzing substrate translocation, thereby affecting λ N degradation. This hypothesis is supported by the pre-steady-state kinetic experiments showing that the $k_{\text{lag}}$ values of Δ232–252 and Δ1–252 are ~10-fold lower than those found in WT and Δ1–239 ELon. As the lag phase of the ATP-dependent cleavage of S3 measures the kinetics of the first pass of substrate translocation, the mutants (Δ1–252 and Δ232–252), which lack residues 232–252, have low $k_{\text{lag}}$ values because of impaired substrate translocation capability. As the degradation of λ N also requires substrate translocation, the reduced λ N degradation activity detected in Δ1–252 and Δ232–252 is consistent with the above proposal.

At present, the structure of the region in ELon containing residues 239–252 has not been solved in full-length enzyme. Therefore, the mechanism by which this region modulates enzyme activity remains to be experimentally determined. Residues 210–245 of BsLon have been proposed to constitute a coiled-coil region in the N-terminal domain, and the N-terminal region of BsLon-AP is very flexible. Therefore, it is plausible that residues 232–252 of ELon, which bear high sequence homology with residues 230–250 of BsLon, function to coordinate the movement of N-terminal domain with the rest of the protease to maintain optimal ATPase function, substrate translocation and consequently protein degradation efficiencies. The result observed in this study is consistent with the idea that the movement of the N-terminal fragment of ELon is used to interact with unfolded proteins or exert forces to disrupt local structures of bound proteins. In related AAA+ proteins such as the ClpA or ClpX chaperones, flexible movement of the N-terminal domain is crucial to chaperone activity. Since Lon belongs to the same protein family, similar N-terminal domain movement may be required to attain optimal protein degradation activity.

Aside from mechanistic characterization of Lon, this work also demonstrates that HXMS can be used as general approach to complement existing structural characterization of ATP-dependent proteases such as Lon. Using HXMS, we were able to map the local structural changes within residues 232–252 in ELon and detected that this region is “rigidified” upon binding to MgATP. LC/MS mapping of pepsin digestion of Lon yields 78% peptide coverage. Based on the HXMS discovery reported here, we anticipate that comparative studies of free Lon with nucleotide-bound Lon in the presence and absence of substrates should provide unique insights into structural dynamic and functions of this protease and related systems.

In summary, this article reports the identification and characterization of a potential allosteric site in ELon that modulates the ATPase functionality. Since Δ1–239 and Δ1–252 ELon share significant sequence homology with the amino acid sequence of BsLon-AP, whose three-dimensional structure is known, the structure of the truncated Bs Lon should provide the framework needed to conduct structure–function relationship studies. As truncated Lon mutants display WT-like peptidase activity and they tend to be more amenable to crystallization studies than full-length enzyme, we anticipate that truncated Lon will be a useful surrogate for generating crystal structures of enzyme:inhibitor complexes to advance the design of more potent or selective inhibitors in the future.

### Materials and Methods

#### Materials

Restriction endonucleases were purchased from Promega or New England Biolabs. Oligonucleotides were custom-synthesized by IDT, Inc. Solvents, buffers, chromatography resin, antibiotics, culture media and PEI cellulose TLC plates were purchased from Fisher Biotechnology or Sigma/Aldrich. Plasmids used for protein expression and competent cells were purchased from Invitrogen and Novagen. [γ-32P]ATP was purchased from Perkin-Elmer Life Science.

#### General methods

Fluorogenic peptide substrates were synthesized as previously described. WT and Lon mutants were purified by a previously established procedure. Unless specified, all proteins and reagents were reported as final concentrations. The HXMS experiments were performed in duplicate. The rest of the experiments were performed...
at least in triplicate. In all cases, comparable results were obtained. Either representative data or averaged data were shown.

**Plasmid construction**

The genes encoding Lon mutants were amplified from WT Lon (pHF004) using oligonucleotides 5’-GGAATTC-CATATGGAACCGCTGAATGCACG-3’ and 5’-GGCGGATCCACTCTGACAGTCGTC3’ as forward and reverse primers to create Δ1–239, oligonucleotides 5’-GGAATTCCTATGGCCCTGAACCGCAAAAT-3’ and 5’-GGCGGATCCACCTACGACGGACGC-3’ as forward and reverse primers to create Δ1–252 and oligonucleotides 5’-GAGTACTATCTGACAGTC-3’ and 5’-GTCGATTTTGCGCTTCAGTTGCTCGTTCAGTAGT-3’ as forward and reverse primers to create Δ232–252. The resultant PCR products were cloned into the NdeI and BamHI sites of pET24c(+) (Novagen). The sequences were verified by DNA sequencing. All three plasmids were transformed into BL21 (DE3); the mutants were expressed and purified to homogeneity using the protocols described previously for WT enzyme.

**Protein quantification**

Enzyme concentrations were reported as monomer concentrations. The concentrated Lon mutants were quantified using Bradford assay. Purified ELon of known concentrations were used as calibration standards. The concentration of each mutant was calculated using their MWs, Δ232–252 (85,000 g/mol), Δ1–252 (58,764 g/mol) and Δ1–239 (60,338 g/mol). To confirm accuracy, we also determined the concentrations by using molar extinction coefficients of each mutant. The concentration of protein can be determined by using absorbance spectroscopy at 280 nm. The molar absorption coefficient of each mutant at 280 nm was calculated based on Eq. (1).

\[ \varepsilon(280) = \frac{(\text{Tyr})(1490) + (\text{Trp})(5500) + (\text{cystine})(125)}{280} \]

The molar absorption coefficients were determined for Δ232–252 (46,300 M⁻¹ cm⁻¹), Δ1–252 (37,360 M⁻¹ cm⁻¹) and Δ1–239 (46,300 M⁻¹ cm⁻¹). The concentration can be calculated using the Beer–Lambert law.

**Peptide mapping by tandem mass spectrometry**

A total of 5 μg (0.57 μM) of ELon in 100 μL of ELon buffer [50 mM Tris (pH 8), 10 mM Mg(OAc)₂ and 2.5 mM tris(2-carboxyethyl)phosphine] was mixed with quench buffer [200 mM sodium phosphate (pH 2.3), 2.5 mM tris(2-carboxyethyl)phosphine and 10% 1M HCl] followed by addition of 5 μg porcine pepsin in 0.05% trifluoroacetic acid in H₂O for 5 min on ice. This was injected into a micropeptide trap (Michrom Bioresearches) connected to a C18 HPLC column (50 mm × 1 mm, Grace) coupled to a Finnigan LTQ quadrupole ion trap mass spectrometer (ThermoElectron). Peptide fragments were eluted using a gradient of acetonitrile at a flow rate of 50 μL/min. Fragments were identified using the search algorithm Sequest.

**Hydrogen–deuterium exchange by mass spectrometry**

ELon (5 μg, 5.7 μM) was preincubated in ELon buffer for 5 min at room temperature. Protein was then diluted 10-fold into ELon buffer with D₂O (pD 7.6) and incubated for various times at room temperature. For the experiments containing ATP, ELon was preincubated with 10 mM ATP prior to dilution into D₂O buffer containing 10 mM ATP. Exchange was quenched by 5-fold dilution into quench buffer at 4 °C. An undeuterated sample was prepared by preincubating ELon in ELon buffer and then diluting 10-fold into ELon buffer with H₂O. For a fully deuterated sample, ELon was preincubated in ELon buffer and then diluted 10-fold into ELon buffer with D₂O containing 6 M guanidine deuterochloride for 3 h before quenching.

Deuterium-labeled protein was digested on ice with 5 μg of porcine pepsin in 0.05% trifluoroacetic acid in H₂O for 5 min and immediately analyzed by injecting into a micropeptide trap connected to a C18 HPLC column coupled to a Finnigan LTQ quadrupole ion trap mass spectrometer (ThermoElectron). Peptides were eluted for 12 min using a gradient of 12–25% acetonitrile at a flow rate of 50 μL/min. The micropeptide trap and column were put on ice to minimize back-exchange. The raw mass spectrometry data were obtained using Xcalibur (Thermo Fisher Scientific, Inc.) and imported into Origin (OriginLab Corporation).

Deuterium levels for the 659 and 425 peptide were corrected for back-exchange using Eq. (2).

\[ D = \frac{m - m_0}{m_{100} - m_0} \times N \]  

where \( D \) is the number of amide hydrogens exchanged, \( m \) is the centroid mass of the peptide at a given time, \( m_0 \) is the mass of the undeuterated peptide, \( m_{100} \) is the mass of the fully deuterated peptide and \( N \) is the number of amide hydrogens present in the peptide. Centroid masses of peptides were calculated using HX Express and confirmed with the center of the Gaussian curve. Gaussian distributions of the MS data were determined from the preloaded equation in Origin. To determine the percent of folded peptides in EX1 kinetics, we used Eq. (3):

\[ \%\text{folded} = \frac{A_{\text{folded}}}{A_{\text{folded}} + A_{\text{unfolded}}} \times 100 \]

where \( A_{\text{folded}} \) and \( A_{\text{unfolded}} \) are the areas of the folded peak and unfolded peak, respectively, as determined from the Gaussian equation from Origin.

**Radiolabeled ATPase assays**

Steady-state velocity data for ATP hydrolysis were measured as described elsewhere, and all reactions were performed at least in triplicate. Briefly, each reaction mixture (see below) was initiated and incubated at 37 °C. Subsequently, 4- or 5-μL aliquots were quenched at various time points from 0 to 7 min in 10 μL 0.5 N formic acid. A 3-μL aliquot of the reaction was spotted directly
onto a PEI cellulose TLC plate (10 cm × 20 cm) and developed in 0.3 M potassium phosphate buffer (pH 3.4). Radiolabeled ATP nucleotide was then quantified using the Packard Cyclone storage phosphor screen Phosphor imager purchased from Perkin-Elmer Life Science. To compensate for slight variations in spotting volume, we corrected the concentration of ADP product obtained at each time point using an internal reference as shown in Eq. (4)

\[
[\text{ADP}] = \left( \frac{\text{ATP}_{\text{dil}}}{\text{ATP}_{\text{dil}} + \text{ADP}_{\text{dil}}} \right) [\text{ADP}]
\]  

The rates of the reactions for ADP production were converted to \( k_{\text{obs}} \) values by dividing the steady-state rates with enzyme concentration. The kinetic parameters for intrinsic ATPase were determined by fitting the \( k_{\text{obs}} \) data with Eq. (5), where \( k_{\text{obs}} \) is the observed rate constant, \( k_{\text{obs, max}} \) is the maximal rate, \( B \) is the ATP concentration and \( \kappa_m \) is the Michaelis–Menten constant.

\[
k_{\text{obs}} = \frac{k_{\text{obs, max}}[B]}{\kappa_m + [B]}
\]

For the Michaelis–Menten plots, each reaction mixture contained 50 mM Tris–HCl (pH 8.1), 5 mM Mg(OAc)\(_2\), 2 mM DTT and varied ATP concentrations from 25 to 500 mM. Reactions were initiated by 600 nM \( \lambda \) and \( \Delta \) S1 or S3 peptide concentration (excitation at 320 nm and emission at 420 nm) and 150 nM Lon; reaction was incubated for 1 min at 37 °C before being initiated with 1 mM ATP. S1 is 100% fluorescently labeled peptide Y(NO\(_2\))RGITCSGRQK(Azb), and S3 is 10% fluorescently labeled peptide with 90% nonfluorescent analog of S1. S3 is a substrate mixture used to correct for the inner filter effect otherwise observed at high peptide substrate concentration. All assays were performed at least in triplicate, and the averaged value of the rates determined for each set of mutants was fit to Eq. (7) as described previously.

\[
k_{\text{obs}} = \frac{k_{\text{cat}}[S]^n}{(\kappa_m + [S])^n}
\]

where \( k_{\text{obs}} \) is the observed rate, \( k_{\text{cat}} \) is the max rate constant of product formation at saturating substrate, \( \kappa_m \) is the Michaelis–Menten constant, [S] is the substrate concentration, and \( n \) is the hill coefficient.

### C99 \( \lambda \) N degradation assay

\( \lambda \) N degradation assays contained 50 mM Hepes (pH 8.0), 15 mM magnesium acetate, 5 mM DTT, 1 or 3 mM WT or \( \lambda \) N protein, and reaction was initiated with 5 mM ATP at 37 °C. At various time points, reaction aliquots (10 μL) were quenched with 5× SDS-PAGE loading dye (2 μL). The aliquots were loaded and run on 17.5% SDS-PAGE gel and stained with Coomassie stain to detect the protein. The gel was viewed using the Biorad Gel Doc 2000 and Quantity One quantitation software.

### His-CcrM degradation assay

His-tagged CcrM was a gift from the Benkovic laboratory. The degradation assays contained 50 mM Hepes (pH 8.0), 15 mM Mg(OAc)\(_2\), 5 mM DTT, 1 μM WT or His-CRM mutants (\( \Delta \)232–252, \( \Delta \)1–252, \( \Delta \)1–239) and 20 μM WT \( \lambda \) N protein, and reaction was initiated with 5 mM ATP at 37 °C. At various time points, reaction aliquots (10 μL) were quenched with 5× SDS-PAGE loading dye (2 μL). The aliquots were loaded and run on 15% SDS-
PAGE gel and stained with Coomassie stain to detect the protein. The gel was viewed using the Biorad Gel Doc 2000 and Quantity One quantitation software.

Pseudo-first-order time course of S3 cleavage by fluorescent stopped flow

Pre-steady-state experiments were performed on a KinTek Stopped Flow controlled by the data collection software Stop Flow version 7.50. The sample syringes were maintained at 37 °C by a circulating water bath. Syringe A contained 1 μM Lon (mutants or WT), with 1 mM peptide substrate S3, 5 mM Mg(OAc)₂, 50 mM Tris-HCl (pH 8.1), 5 mM DTT, 30 mM KPi and 1 mM ATP. Peptide cleavage was detected by an increase in fluorescence (excitation of 320 nm and emission of 420 nm) following rapid mixing of the syringe contents in the sample cell over 120 s (600 points for the first 20 s and 400 points from 21 to 120 s). The baseline of the fluorescence was normalized to zero, and the data shown are the results of averaging at least four traces. The concentration of the hydrolyzed peptide was calibrated by determining the maximum fluorescence generated per micromolar peptide; this was done by complete digestion by trypsin under identical conditions on the stopped flow assembly of protein complexes.

\[
Y = A \exp^{-k_{\text{SS}}t} + v_{\text{lag}} t + C
\]

where \(t\) is the time in seconds, \(Y\) is the concentration of hydrolyzed peptide S3 in micromolar, \(A\) is the amplitude of the reaction, \(k_{\text{SS}}\) is the pre-steady-state rate constant in seconds, \(v_{\text{lag}}\) is the steady-state rate in units of micromolar product per second and \(C\) is the endpoint. The \(v_{\text{lag}}\) value can be converted to a first-order rate constant \((k_{\text{SS}})\) in the unit of per second) by division with the enzyme concentration. The \(v_{\text{lag}}\) values were obtained from those reported for the respective enzyme in Table 2. Since Eq. (8) is the general function that quantifies a biphasic time course, when \(Y = 0\) at \(t = 0\), \(C = A\) and the equation changes to

\[
0 = -A + A \exp^{-k_{\text{SS}}t} + v_{\text{lag}} t + C
\]

Such that

\[
Y = -A (\exp^{-k_{\text{SS}}t}) + v_{\text{lag}} t
\]

When \(A\) is defined as \(v_{\text{lag}} - v_{i}/k_{\text{SS}}\), Eq. (8) becomes equivalent to the equation defining hysteresis, where \(v_{i}\) is the rate corresponding to the initial phase of the time course. For WT and \( \Delta 1-239\), only the first 20 s were used to obtain the pre-steady-state rate constant; for \(\Delta 232-252\), only the first 60 s were used; and for \(\Delta 1-252\), all 120 s were used to obtain the pre-steady-state rate constant.

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References


Processive Degradation of Unstructured Protein by *Escherichia coli* Lon Occurs via the Slow, Sequential Delivery of Multiple Scissile Sites Followed by Rapid and Synchronized Peptide Bond Cleavage Events

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**Supporting Information**

**ABSTRACT:** Processive protein degradation is a common feature found in ATP-dependent proteases. This study utilized a physiological substrate of *Escherichia coli* Lon protease known as the lambda N protein (\(\lambda N\)) to initiate the first kinetic analysis of the proteolytic mechanism of this enzyme. To this end, experiments were designed to determine the timing of three selected scissile sites in \(\lambda N\) approaching the proteolytic site of ELon and their subsequent cleavages to gain insight into the mechanism by which ATP-dependent proteases attain processivity in protein degradation. The kinetic profile of peptide bond cleavage at different regions of \(\lambda N\) was first detected by the iTRAQ/mass spectrometry technique. Fluorogenic \(\lambda N\) constructs were then generated as reporter substrates for transient kinetic characterization of the ATP- versus AMPPNP-dependent peptide bond cleavage and the delivery of the scissile sites near the amino- versus carboxyl-terminal of the \(\lambda N\) protein to the proteolytic site of ELon. Collectively, our results support a mechanism by which the cleavage of multiple peptide bonds awaits the “almost complete” delivery of all the scissile sites in \(\lambda N\) to the proteolytic site in an ATP-dependent manner. Comparing the time courses of delivery to the active site of the selected scissile sites further implicates the existence of a preferred directionality in the final stage of substrate delivery, which begins at the carboxyl-terminal. The subsequent cleavage of the scissile sites in \(\lambda N\), however, appears to lack a specific directionality and occurs at a much faster rate than the substrate delivery step.

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Lon, also known as protease La, is an ATP-dependent protease functioning to degrade certain damaged proteins and short-lived regulatory proteins in the cell.1–7 This enzyme possesses intrinsic ATPase activity that is enhanced during protein or peptide degradation; however, the two processes are not stoichiometrically linked.8 As a homo-oligomer, Lon possesses intrinsic ATPase activity that is enhanced during protein or peptide degradation; however, the two processes are not stoichiometrically linked.8 As a homo-oligomer, Lon is composed of an ATPase and a protease domain in each enzyme subunit.9,10 Oligomerization requires Mg\(^{2+}\) but not ATP.9 Analytical ultracentrifugation and electron microscopy studies reveal that bacterial Lon proteases are hexameric ring-shaped structures containing a central cavity, where the proteolytic sites reside.9,10 Limited proteolytic footprinting studies of ELon reveals that adenine nucleotide protects the enzyme from nonspecific proteolysis, thereby indicating the existence of at least one conformational change generated by binding to ATP.11,12 The presence of a proteolytic dyad consisting of a conserved Ser and Lys has been implicated.13 Within the full-length Lon protein, mutation of either Ser or Lys to Ala in the catalytic dyad abolishes proteolytic but not ATPase activity.13–16 By contrast, mutation of the ATP-binding site abolishes both the ATPase and proteolytic activities of Lon.17 Therefore, despite the presence of a proteolytic dyad, the proteolytic activity of Lon is affected by the binding and hydrolysis of ATP.

Based upon the general mechanism of ATP-dependent proteolysis, it is proposed that Lon, like other proteases in the family, coordinates repeated cycles of ATPase and peptidase activity to completely degrade a protein substrate containing multiple sites, without generating partially digested substrate intermediates. This is referred to as processive degradation in the field.18–20 It is suggested that the unfolding and vectorial delivery of substrate to the proteolytic site constitutes the rate-limiting step, and is dependent on the ATPase activity.21 The binding and hydrolysis of ATP induces a series of conformational changes within the enzyme subunits that leads to unfolding of substrate, followed by internalization (also known as translocation) of the protein substrate into the central cavity.22,23 Both unfolding and translocation are hypothesized to be carried out by repetitive cycles of ATP hydrolysis, and delivery into the central cavity is thought to happen via a threading mechanism18,24 (i.e., the polypeptide substrate is transported through the narrow central pore of the enzyme in a roughly linear conformation). The unfolded and translocated polypeptide substrates are sequestered within the central cavity of the protease machinery (the proteolytic chamber), where peptide bond cleavage occurs.25 Since the degradation of protein substrates only generates completely digested peptides ranging from 5 to 20 amino acids without generating partially digested protein intermediates, it is concluded that ATP-dependent proteases degrade their substrates processively.25,26 However, it is not clear how the substrate translocation event is
coordinated with peptide bond cleavage to completely digest substrates during the reaction time course.

Our current understanding of processivity in ATP-dependent proteolysis stems primarily from studies on the heterosubunit ATP-dependent proteases such as ClpXP or ClpAP.\textsuperscript{18,20,27–31} These Clp protease complexes recognize unique peptide sequence tags, such as the SsrA tag, on a protein substrate to initiate substrate translocation.\textsuperscript{32} Using model substrates containing the SsrA tag, it has been shown that the translocation of protein or polypeptide substrates by the ATPase subunit in ClpX or ClpP originates from the SsrA tag and exhibits processivity.\textsuperscript{33} While these studies reveal the contribution of the ATPase activity in rendering processivity in substrate interaction, they do not explain how the ATPase and peptidase activities are coordinated to generate only completely digested peptides. As illustrated in Scheme 1, depending on the timing by which the different scissile sites in a protein substrate gain access to the proteolytic site and when peptide bond cleavage occurs, two different protein degradation mechanisms could be expected. In model 1, peptide bond cleavage occurs after complete substrate translocation to generate only fully digested peptide products. The delivery of different Lon cleavage sites in the substrate to the proteolytic chamber should occur before initiation of any peptide bond cleavage. In model 2, the cleavage of each scissile peptide bond is flanked by a substrate translocation event, while partially digested substrate peptides will reveal a concerted (model 1) versus sequential (model 2) peptide bond cleavage mechanism.

\begin{scheme}
\textbf{Scheme 1. Two Possible Models for $\lambda\text{N}$ Degradation by Lon$^a$}

\begin{figure}[h!]
\centering
\includegraphics[width=\textwidth]{scheme1}
\caption{Two Possible Models for $\lambda\text{N}$ Degradation by Lon$^a$}
\end{figure}

\textsuperscript{a}Comparing the timing of the translocation and cleavage of the N-terminal versus the C-terminal site using pre-steady-state techniques will reveal a concerted (model 1) versus sequential (model 2) peptide bond cleavage mechanism.
\end{scheme}

The goal of this study is to determine the timing by which \textit{Escherichia coli} Lon (ELon) catalyzes the ATP- and AMPPNP-dependent cleavage of $\lambda\text{N}$ near the amino versus carboxyl termini in order to obtain insights into how processive peptide bond cleavage occurs. The $\lambda\text{N}$ protein is a transcriptional regulatory protein functioning to enable RNA transcription beyond the transcription termination signal in \textit{E. coli} cells during $\lambda$ phage infection.\textsuperscript{34,35} The timing of $\lambda\text{N}$ expression and degradation dictates whether $\lambda$ phage adopts a lytic or a lysogenic life cycle in the infected host. When free in solution, $\lambda\text{N}$ does not adopt any defined structure.\textsuperscript{36–39} \textit{In vitro}, ELon degrades the purified $\lambda\text{N}$ protein in the presence of ATP or AMPPNP (a nonhydrolyzable analog of ATP).\textsuperscript{22} Despite the differences in the rate of $\lambda\text{N}$ degradation exhibited by the two nucleotides (faster in the presence of ATP), the same hydrolyzed peptide products were generated upon complete degradation.\textsuperscript{35} Due to a lack of defined structure and its physiological relevance as a Lon substrate, $\lambda\text{N}$ serves as an ideal substrate for using a systematic approach to study the processive protein degradation of ELon. Since the unfolding of this substrate is not necessary, we will only need to consider the delivery to the active site and cleavage of the multiple peptide bonds within the substrate during its degradation. Because $\lambda\text{N}$ contains only 107 amino acids and lacks a defined structure, chemically modified forms of this protein can be readily synthesized to produce a large quantity of the reagents for transient kinetic studies. Elucidating the kinetic mechanism of $\lambda\text{N}$ degradation will reveal the enzyme intermediates generated along the reaction pathway whose formations are facilitated by ATP binding and hydrolysis, which are also responsible for mediating processive peptide bond cleavage.

\section{Materials and Methods}

\textbf{Materials.} Restriction endonucleases were purchased from Promega or New England Biolabs. Oligonucleotides were custom-synthesized by IDT, Inc. Solvents, buffers, chromatography resin, antibiotics, culture media, and PEI cellulose TLC plates were purchased from Fisher Biotechnology or Sigma/Aldrich. Plasmids used for protein expression and competent cells were purchased from Invitrogen and Novagen. [$\alpha^{32}\text{P}]$ATP was purchased from Perkin-Elmer Life Science. FRZ$\lambda$N001 and $\lambda\text{N}1$–34 were synthesized by GenScript, and FRZ$\lambda$N006 was synthesized by LifeTein.

\textbf{General Methods.} ELon purification procedures were performed as described elsewhere.\textsuperscript{39} All enzyme concentrations were reported as ELon monomer concentrations. Experiments were performed at least in triplicate. In all cases, comparable results were obtained. Either representative data or averaged data were shown.

\textbf{Generation of $\lambda\text{N}$ Variants.} For the construction of $\lambda$N001, $\lambda$N002, and $\lambda$N006, the DNA encoding for the respective gene was synthesized by Genescrypt in the pUC57 plasmid, with a cysteine inserted at position 26 for $\lambda$N001, position 42 for $\lambda$N002, and position 99 for $\lambda$N006. The endogenous Cys in WT $\lambda$N was replaced with a Leu and all endogenous Trp were replaced with Phe. The respective gene cassette was subcloned into the pCOLA-Duet vector using the BamHI and HindIII restriction sites. The resulting $\lambda$N constructs were expressed with an N-terminal 6x His tag in BL21 (DE3) \textit{E. coli}, which lacks endogenous Lon protease. N-his-$\lambda$N (N-terminal his-tagged $\lambda$N), C-his-$\lambda$N (C-terminal his-tagged $\lambda$N), N-his-$\lambda$NAA99–107, $\lambda$N001, $\lambda$N002, and $\lambda$N006 were expressed in BL21 (DE3) and purified as described previously.\textsuperscript{30} The protein was quantified using the Bradford assay.

\textbf{Preparation of Dansylated $\lambda\text{N}$ Variants.} The purified $\lambda$N001, $\lambda$N002, and $\lambda$N006 were labeled with dansyl aziridine
(Invitrogen). Dansyl aziridine was prepared in dimethylsulfoxide and was added to the λN protein in 5-fold excess. The reaction was gently agitated at room temperature overnight and then quenched with β-mercaptoethanol. Unreacted dye was removed by dialyzing λN into storage buffer containing 20 mM Tris, 50 mM NaCl, 1 mM β-mercaptoethanol, and 20% glycerol. The labeled protein was then quantified using the Bradford assay. The extent of dansyl labeling in each protein was determined by the absorbance of dansyl at $\lambda_{350}$ and compared to the concentration of protein.

Characterization of the Degradation of Full Length versus Truncated λN by SDS-PAGE. To compare the ATP-dependent degradation profiles of WT versus truncated λN, 10 μM of protein substrate (N-his-λN, Δ1–34λN, or N-his-λNΔ99–107) was incubated with 5 mM ATP and 1 μM WT ELon at 37 °C in a reaction buffer containing 50 mM Tris-HCl (pH 8.1), 15 mM Mg(OAc)$_2$, and 5 mM DTT. At 0, 5, and 10 min, reaction aliquots were quenched with 5x SDS-PAGE loading dye. All quenched reaction time points were resolved on a 12.5% SDS-PAGE gel and stained with Coomassie Brilliant Blue to detect the undigested substrates.

Measuring N-his-λNΔ99–107 Binding Using Radio-labeled ATPase Assay. N-His-λNΔ99–107 protein binding to WT ELon was detected by ATP hydrolysis at 37 °C by radiolabeled ATPase assay as described previously and all reactions were performed at least in triplicate. Briefly, each reaction mixture contained 50 mM Tris-HCl (pH 8.1), 15 mM Mg(OAc)$_2$, 2 mM DTT, 500 μM ATP, and varied λNΔ99–107 concentrations from 1 μM to 25 μM. Reactions were initiated by 100 nM WT ELon monomer and incubated at 37 °C. Subsequently, 5 μL aliquots were quenched at various time points from 0 to 8 min in 10 μL of 0.5 N formic acid. Radiolabeled ATP nucleotide was removed by dialyzing (Invitrogen). Dansyl aziridine was prepared in dimethylsulfoxide and was added to the 12.5% SDS-PAGE gel and stained with Coomassie Brilliant Blue to detect the undigested substrates.

Western Blot Analysis of λN Degradation. λN degradation assays were run as above with either 65 μM N-his-λN or C-his-λN and 5 mM AMPPNP. At 0 and 30 min, reaction aliquots were quenched with 5x SDS-PAGE loading dye and incubated at 100 °C for 1 min. The aliquots were loaded and run on a 17% SDS-PAGE gel for Western blotting. The proteins were transferred to a nitrocellulose membrane and blocked with TBST (Tris-buffered saline [pH 7.6], 0.1% Tween-20) with 1% bovine serum albumin overnight at 4 °C. The nitrocellulose was incubated with anti-His (1:500; Invitrogen, Life Technologies, Camarillo, CA, USA) for 1 h. After extensive washing with TBST, the membrane was incubated for 1 h in goat antimes IgG alkaline phosphatase secondary antibody (1:3000, Sigma-Aldrich, St. Louis, MO, USA). Protein bands were visualized using BCIP/NBT.

Quantification of the Time Courses of ATP-Dependent Degradation of Unlabeled λN by ELon Using Isobaric Tag for Relative and Absolute Quantization (iTRAQ)/Mass Spectrometry Analysis. Ten micromolar of monomeric WT ELon (final concentration) was mixed with 50 μM N-his-λN and 1 mM ATP (final concentration) in a chemical quench flow instrument (KinTek) in a reaction buffer containing 50 mM HEPES, pH 8; 2 mM Mg(OAc)$_2$, 2 mM TCEP; 0.05% Tween 20. Reaction time points at 1, 3, 5, 10, 20, 30, and 60 s were obtained by quenching a reaction aliquot with 0.5 N formic acid. For the 0 time point, reaction aliquot omitting ELon was quenched with 0.5 N formic acid. The quenched reaction time points were frozen at ~80 °C and submitted to the Center for Advanced Proteomics Research at the University of Medicine and Dentistry at New Jersey, NJ, USA for 8-plex iTRAQ analysis. In brief, the amino groups of the ELon-digested λN peptide products (from eight time points) were labeled by the respective iTRAQ tag and then desalted. The desalted time points were subjected to 2D liquid chromatography (cation exchange and C18 reverse phase) and then analyzed on an orbitrap mass spectrometer. Duplicate assays were performed. Peptides were identified from the degradation profile of λN by ELon and some sequences were further confirmed by manual evaluation of the MS/MS spectra performed by Dr. Tong Liu at UMDNJ. The standardized time courses for the generation of different hydrolyzed peptide products were obtained by plotting the ratio of the m/z intensity of the respective peptide at the indicated time point over the 60 s time point, versus the corresponding time of digestion. The raw data of the 8-plex iTRAQ data are supplied in Supporting Information (S3).

Monitoring the ATP-Dependent Degradation of Fluorescently Labeled λN (FRN) by Steady State Kinetics. The cleavage of fluorescently labeled sites in λN were measured using a Fluoromax 4 spectrophuorimeter (Horiba Group) as described previously.99 Reactions contained 50 mM HEPES pH 8.0, 5 mM Mg(OAc)$_2$, 2 mM DTT, 0.5, 1, 5, 10, 20, and 30 μM FRN001 or FRN006 (excitation at 320 nm and emission at 420 nm), and 150 nM WT ELon; the reaction was incubated for 1 min at 37 °C before being initiated by 1 mM ATP. All assays were performed at least in triplicate, and the averaged value of the rates determined for each substrate was fit to eq 3.

\[
[ADP] = \frac{ADP_{flu}}{ATP_{flu} + ADP_{flu}} [ADP]
\]

(1)

The rates of the reactions for ADP production were converted to $k_{obs,\lambda NΔ99–107}$ values by dividing the steady-state rates with enzyme concentration. The $k_{obs,\lambda NΔ99–107}$ values were fit to eq 2 where $k_{obs,\lambda NΔ99–107}$ is the observed rate constant, $k_{obs,max}$ is the maximal rate, $B$ is the N-his-λNΔ99–107 concentration, and $K_{l,\lambda NΔ99–107}$ is the binding constant for N-his-λNΔ99–107 constant and $M$ is the start point.

\[
k_{obs,\lambda NΔ99–107} = \frac{k_{obs,max} [B]}{K_{l,\lambda NΔ99–107} + [B]} + M
\]

(2)

Characterization of the ATP- and AMPPNP-Dependent Degradation of N-his-λN. λN degradation assays contained 50 mM HEPES (pH 8), 75 mM KOAc, 15 mM Mg(OAc)$_2$, 5 mM DTT, 65 μM N-his-λN, and 5 mM nucleotide and the reaction was initiated with 6 μM WT ELon at 37 °C. At 0, 5, 10, and 20 min, reactions with N-his-λN and ATP or AMPPNP were quenched with 5x SDS-PAGE loading dye and incubated at 100 °C for 1 min. N-his-λN aliquots were then run on a 12.5% SDS-PAGE gel and then stained and destained with Coomassie Brilliant Blue to detect the undigested substrates.
where \( k_{\text{obs},N,\text{ATP}} \) is the observed rate, \( k_{\text{cat},N,\text{ATP}} \) is the max rate constant of product formation at saturating substrate, \( K_m \) is the Michaelis–Menten constant, and \([S] \) is the substrate concentration.

**Monitoring the Pre-Steady-State ATP and AMPPNP-Dependent Degradation of Fluorescently Labeled λN (FRλN) by Stopped Flow Kinetics.** Pre-steady-state experiments were performed on a KinTek Stopped Flow controlled by the data collection software Stop Flow version 7.50. The sample syringes were maintained at 37°C by a circulating water bath. AMPPNP-dependent degradations, syringe A contained 10 \( \mu \)M WT ELon, 10 \( \mu \)M λN001A (10% FRλN001 and 90% λN001), or 10 \( \mu \)M λN006A (10% FRλN006 and 90% λN006), and reaction buffer (5 mM Mg(OAc)\(_2\), 50 mM Tris-HCl (pH 8.1), 5 \( \mu \)M DTT, 30 mM KOAc, and 30 mM KPi). Syringe B contained 10 \( \mu \)M λN001 or 10 \( \mu \)M λN006, reaction buffer, and 2 mM AMPPNP. For all other reactions, syringe A contained 10 \( \mu \)M WT ELon, 8 \( \mu \)M λN001A, or 8 \( \mu \)M λN006A, and reaction buffer. Syringe B contained 8 \( \mu \)M λN001 or 8 \( \mu \)M λN006, reaction buffer, and 2 mM ATP, ADP, or no nucleotide. Protein cleavage was detected by an increase in fluorescence (excitation of 320 nm and emission with a 400 nm long-pass filter) following rapid mixing of the syringe contents in the sample cell over 200 s for AMPPNP, and 10 s for all others. The baseline of the fluorescence was normalized to zero, and the data shown are the results of averaging at least four traces. The concentration of the hydrolyzed peptide was calibrated by determining the maximum fluorescence generated per micro-molar peptide due to complete digestion by trypsin under identical conditions on the stopped-flow. The lag equation was fit to the averaged time courses:

\[
F = \frac{k_{\text{lag}}}{k_{\text{lag}} \{1 - \exp(-k_{\text{lag}} t)\}}
\]

where \( F \) is the relative fluorescence intensity, \( v_1 \) is the final velocity, \( t \) is the time in seconds, and \( k_{\text{lag}} \) is the apparent lag rate of the time courses.

**Pseudo First Order Delivery of DansylλN to the Protoelytic Active Site in S679W ELon by Fluorescent Stopped Flow.** Experiments to monitor the translocation of DansylλN with ELon (S679W and S679A) were performed on a KinTek Stopped Flow controlled by the data collection software Stop Flow version 7.50 with a 0.5 cm path length. The sample syringes were maintained at 37°C by a circulating water bath. Syringe A contained 10 \( \mu \)M S679A or S679W ELon monomer with 10 \( \mu \)M dansylλN (dansylλN001, dansylλN002, or dansylλN006) and reaction buffer (5 mM HEPES pH 8.0, 75 mM KOAc, 75 mM KPi, 5 mM Mg(OAc)\(_2\), and 5 mM DTT). Syringe B contained 1 mM ATP or AMPPNP, 10 \( \mu \)M dansylλN, and reaction buffer. DansylλN translocating to S679A or S679W ELon was monitored by an increase in fluorescence (excitation 295 nm, emission 450 nm long-pass filter) upon rapid mixing of the syringe contents over 80 s for AMPPNP, 20 s for all others. In addition to monitoring excitation with 295 nm and emission with 450 nm long-pass filter, experiments were performed with excitation 295 nm and emission with a 340 nm band-pass filter monitor to view the changes in Trp fluorescence. The data shown are a result of averaging at least four traces. Each reaction was performed in triplicate. It should be noted that the PMT (photomultiplier tube) sensitivity was automatically adjusted by the instrument to optimize signal-to-noise. As a result, the relative amplitudes of the time courses do not reflect the stoichiometries of the enzyme intermediates monitored by the signals. The first-order rate constants of the reactions do not change because the dansyl absorbance in each reaction remains constant. The first 0.5 s of the average time courses of S679W ELon with ATP and dansylλN were fitted with eq 5 describing a single exponential

\[
F = A_1 \exp(-k_{1,\text{FRET},\text{ATP}} t) + C
\]

where \( F \) is relative fluorescence, \( A_1 \) is the amplitude in relative fluorescence units, \( t \) is time in seconds, \( C \) is the end point, and \( k_{1,\text{FRET},\text{ATP}} \) is the first order rate constant associated with protein initiation. The averaged time courses of S679W ELon with AMPPNP and dansylλN were fitted with eq 6 describing a double exponential

\[
F = A_1 \exp(-k_{1,\text{FRET},\text{AMPPNP}} t) + A_2 \exp(-k_{2,\text{FRET},\text{AMPPNP}} t) + C
\]

where \( F \) is relative fluorescence, \( A_1 \) and \( A_2 \) are amplitudes in relative fluorescence units, \( t \) is time in seconds, \( C \) is the end point, \( k_1 \) is the first-order rate constant associated with the first phase of the reaction in per seconds, and \( k_2 \) is the first-order rate constant associated with the second phase of the reaction in per seconds.

**Chemical-Quench ATPase Activity Assays.** The pre-steady-state time courses for ATP hydrolysis were measured using a rapid chemical-quench-flow instrument from KinTek Corporation. The instrument was maintained at 37°C by a circulating water bath. Syringe A contained 5 \( \mu \)M Lon (WT), with 8 \( \mu \)M λN substrate (λN001 or λN006), 5 mM Mg(OAc)\(_2\), 50 mM HEPES (pH 8.1), 5 \( \mu \)M DTT, 75 mM KOAc, and 75 mM KPi. Syringe B contained 8 \( \mu \)M λN substrate (λN001 or λN006), 5 mM Mg(OAc)\(_2\), 50 mM HEPES (pH 8.1), 5 \( \mu \)M DTT, 75 mM KOAc, 75 mM KPi, and 200 \( \mu \)M ATP containing 0.01% of \([\alpha-^{32}\text{P}]\)ATP at times (0–1.8 s) before quenching with 0.5 N formic acid and then extracting with 200 \( \mu \)L of phenol/chloroform/isooamyl alcohol at pH 6.7 (25:24:1). A 3 \( \mu \)L aliquot of the aqueous solution was spotted directly onto a PEI-cellulose TLC plate (10 × 20 cm), and the plates were developed in 0.75 M potassium phosphate buffer (pH 3.4) to separate ADP from ATP. The relative amount of radiolabeled ADP and ATP at each time point was quantified by a Cyclone Phosphor imager (Perkin-Elmer Life Science). To compensate for the slight variations in spotting volume, the concentration of the ADP product obtained at each time point was corrected for using an internal reference as shown in eq 1. All assays were performed at least in triplicate, and the average of those traces was used for data analysis. The burst amplitude and the burst rates were determined by fitting the \( k_{\text{obs}} \) data from 0 to 400 ms with eq 7

\[
Y = A \exp(-k_{\text{burst}} t) + C
\]

where \( t \) is time in seconds, \( Y \) is \([\text{ADP}] \) in micromolar, \( A \) is the burst amplitude in micromolar, \( k_{\text{burst}} \) is the burst rate constant in per seconds, and \( C \) is the end point. The observed steady-state rate constants (\( k_{\text{obs,ATP}} \)) were determined by fitting the data from 600 ms to 1.8 s with eq 8

\[
Y = k_{\text{obs,ATP}} X + k_{\text{obs,ATP}} C
\]
where $X$ is time in seconds, $Y$ is $[ADP]/[E]$, $k_{\text{obs, ATPase}}$ is the observed steady-state rate constant in per seconds, and $C$ is the $y$ intercept. Data fitting was accomplished using the nonlinear regression program Kaleida-Graph (Synergy).

### RESULTS

**Degradation Profiles of N-his-$\lambda$N.** In an earlier study, truncated N-his-$\lambda$N lacking residues 99–107 was shown to be degraded by ELon less efficiently than full length N-his-$\lambda$N, suggesting that the C-terminal of $\lambda$N plays a role in substrate degradation. By contrast, the contribution of the N-terminal of $\lambda$N is not known. As such, the degradation profiles of ATP-dependent degradation of N-his-$\lambda$N, truncated N-his-$\lambda$N lacking the C-terminal (N-his-$\lambda$NΔ99–107), and truncated $\lambda$N lacking the N-terminal and any his-tag (ΔNΔ1–34) were compared in this study. Figure 1A shows the degradation profiles of ELon degrading full-length N-his-$\lambda$N, ΔNΔ1–34, and N-his-$\lambda$NΔ99–107 in the presence of saturating [ATP] (5 mM) under identical conditions. Within 5 min into the reaction, full-length N-his-$\lambda$N and ΔNΔ1–34 were mostly degraded, but N-his-$\lambda$NΔ99–107 was still detected. According to Supporting Information S1 and S2, the time courses for the ATP-dependent degradation of full-length N-his-$\lambda$N and C-his-$\lambda$N are comparable, and ΔNΔ99–107 lacking a his-tag is still degraded less efficiently than full-length $\lambda$N lacking a his-tag.

Therefore, the addition of a his-tag to $\lambda$N does not affect protein degradation. Furthermore, the C- but not the N-terminal of $\lambda$N contributes to the degradation efficiency of the substrate.

**Evaluate the Contribution of Residues 99–107 in the Degradation of $\lambda$N.** Since the C-terminal containing residues 99–107 contributes to the degradation efficiency of $\lambda$N, this region likely interacts with ELon. To investigate this possibility, N-his-$\lambda$NΔ99–107 was titrated against 100 nM ELon at saturating [ATP] to determine the concentration of N-his-$\lambda$NΔ99–107 needed to attain 50% maximal ATPase stimulation. This assay was previously used to determine the $K_d$ of N-his full length $\lambda$N in ELon. The ATPase activity was measured using a radioactive assay that quantified the amount of ADP generated over time using eq 1 shown in Methods and Materials. Dividing the observed rate of ATP hydrolysis by [monomeric Lon] yields the observed rate constant at the corresponding [N-his-$\lambda$NΔ99–107]. As shown in Figure 1B, the $k_{\text{obs, ATPase}}$ values show a hyperbolic dependency against [N-his-$\lambda$NΔ99–107], which upon fitting the data to eq 2 provides a $K_{d, \lambda NΔ99–107}$ of 5.2 ± 1.7 μM. This value is ~3.7-fold higher than the $K_d$ of full-length N-his-$\lambda$N (1.4 ± 0.6 μM), which was determined in an earlier publication. Since the affinity as well as the degradation efficiency of the truncated substrate was reduced but not abolished, we conclude that residues in the deleted C-terminal of $\lambda$N contribute to substrate degradation. However, additional recognition site(s) not found in either terminal of $\lambda$N must be present to allow substrate recognition and degradation, albeit with reduced efficiency.

**ATP- and AMPPNP-Dependent Degradation of His-Tagged $\lambda$N.** Previously, Maurizi showed that $\lambda$N was degraded by ELon in the presence of the nonhydrolyzable ATP analog, AMPPNP, and the same peptide cleavage profile was detected in the ATP- versus AMPPNP-dependent degradation reaction. Based on the peptide products generated, the possibility of ELon conducting nonprocessive as well as processive protein degradation was considered. Using a fluorogenic peptidase assay, we demonstrated that the $k_{\text{cat}}$ of peptide bond cleavage was significantly reduced when ATP was substituted with AMPPNP. Therefore, the ATP-versus AMPPNP-dependent degradation of $\lambda$N may differ by the kinetics of substrate degradation. To evaluate this possibility, N-his-$\lambda$N was degraded by ELon in the presence of 5 mM ATP versus AMPPNP, from which the reaction time courses were monitored by SDS-PAGE as shown in Figure 2A. Comparing the intensities of the intact substrate band of the ATP- versus AMPPNP-dependent reaction at the 5, 10, and 20 min time points reveals that the N-his-$\lambda$N was degraded faster in the presence of ATP. Furthermore, a small amount of partially digested N-his-$\lambda$N was detected at the 10- and 20-min time points in the AMPPNP-dependent but not ATP-dependent reaction, suggesting that in addition to reduced protein degradation kinetics, the lack of ATP hydrolysis allowed nonprocessive as well as processive protein degradation to occur.
Figure 2. Degradation of λN by ELon in the presence of AMPPNP or ATP. Purified N-his-λN (65 μM) was digested by 6 μM ELon in the presence of 5 mM ATP or AMPPNP, and quenched at the times indicated. The degradations were monitored by 12.5% SDS-PAGE as described in Materials and Methods. Arrows point to degradation products only seen with AMPPNP. (B) Western blot of N-his-λN degradation in the presence of AMPPNP with an antibody against a his-tag. N-his-λN protein (65 μM) was digested with 6 μM ELon and quenched at the time points indicated. Arrows point to degradation products.

To further determine if the partially digested N-his-λN was generated due to the presence of the his-tag, full-length λN containing a his-tag at the C-terminal (C-his-λN) was digested with ELon in the presence of 5 mM AMPPNP and subjected to Western blot analysis with anti-his-tag antibodies. Figure 2B reveals that partially digested λN was generated regardless of the location of a his-tag at the N- or C-terminal of λN, as an anti-his positive band corresponding to the mobility of a partially digested λN lacking an N-terminal but containing a C-terminal his-tag or vice versa is detectable. Compared to the degradation profiles of λN in the presence of ATP, the results presented in this section indicate that the catalytic efficiency as well as the extent of processivity of λN degradation is affected by the lack of ATP hydrolysis in the reaction, as AMPPNP is bound but not hydrolyzed by ELon. However, ATP hydrolysis is not required for the progressive degradation of unstructured protein substrate. The fact that both his-tagged C- and N-termini were detected under identical time points indicates that no obligatory order of peptide bond cleavage occurred in the degradation of reactions.

Detecting the Kinetic Time Courses of WT ELon-Mediated Degradation of λN by iTRAQ Mass Spectrometry. To monitor the kinetics of N-his-λN degradation by WT ELon, a discontinuous chemical quench-flow mass spectrometry assay (RQ/MS) was used. In the RQ/MS assay, 5-fold the \( K_m \) level of N-his-λN was incubated with WT ELon at a saturating level of ATP (1 mM) and quenched at 0, 1, 3, 5, 10, 20, 30, and 60 s (see Supporting Information S3). These time points were chosen because no hydrolyzed peptide product was detected by LC/MS in the time points that were taken earlier than 1 s. The above reaction time points were quenched with 0.5 N formic acid to denature Lon and release digested λN products that were bound to Lon. The quenched time points were then derivatized with isobaric tagged succimidyl isothiocyanate44 such that the digested λN peptides in each time point were labeled with a specific m/z tag prior to analysis by LC/MS/MS. Peptides with identical sequences but tethered with different isobaric tags would have the same retention times on the LC traces. In the MS/MS spectra, the ratios of peptide quantities among different time points were deduced from the signal intensity ratios of the reporter groups in the isobaric tags, with m/z values of 113, 114, 115, 116, 117, 118, 119, and 121, respectively. The sequence of the digested peptide products was deduced and matched up with the published peptide sequences generated from the degradation of λN as summarized in S3. No partially digested λN sequence was detected. Of the peptides that were identified, the signal intensity of the reporter groups from each peptide was used to determine the ratio of the individual peptide at each time point. Analysis of the data done in duplicate showed approximately 40% of the digested λN peptides were identified and quantified. The time courses for the generation of three degraded λN peptides was used to illustrate the timing of peptide bond cleavage in the indicated regions in λN (Figure 3). Plotting the relative m/z intensity of the respective isobaric labeled peptides against the corresponding reaction time points yielded the data shown in Figure 3. According to Figure 3, the time courses for the cleavage of the various scissile sites in λN are comparable. Furthermore, biphasic kinetic time courses were detected, with the first phase displaying slower reaction kinetics. As ELon was denatured prior to LC/MS analysis of the hydrolyzed peptide
products, the lack of product detected prior to the 1 s time point suggests the presence of lag kinetics in the reaction. However, the apparent lag phase in product detection could have also been attributed to the detection limit of the technique. Therefore, the stopped flow kinetic techniques described below were used to further characterize the first four seconds of the degradation reactions.

Fluorescently Labeled λN as a Substrate of Elon. To quantify the ATP-dependent peptidase activity of Lon, fluorogenic decapeptide substrates containing different regions of λN, each containing a Lon cleavage site, were previously generated. Each intact peptide substrate contained a nitrotyrosine (NO2Tyr) at the N-terminal and a lysine-conjugated anthranilamide (Abz) group at the epsilon amino side chain such that the fluorescence emission signal of Abz (λex = 320 nm, λem = 420 nm) was internally quenched by nitrotyrosine due to proximity of the two moieties. Upon cleavage by Lon, the two chromophores separate from one another, leading to an increase in the Abz fluorescence over time, thereby allowing for the determination the kinetic parameters of the peptidase reaction. In this study, we utilized the same strategy to monitor the cleavage of the N- versus the C-terminal cleavage of λN by Elon in the presence of ATP and independently AMPPNP. Two λN constructs were generated, one with two Lon cleavage sites flanked by NO2Tyr and Abz at the amino end, and one with one Lon cleavage site flanked by the same set of chromophores at the carboxyl end (Figure 4A). These were generated by chemical synthesis without any his-tag. Since Trp also internally quenches the fluorescence of Abz, the intrinsic Trp residues in λN were replaced with Phe to ensure the observed changes in Abz signal were attributed to peptide bond cleavage. The resulting N- versus C- labeled λN substrates are referred to as FRλN001 and FRλN006, respectively. Due to the placement of the Abz-NO2Tyr dye pair, the cleavage of A30-K31 or L40-N41 will be detected in FRλN001 and the cleavage of L93-S94 will be detected in FRλN006. In a previous study, the kcat and Km of each Lon cleavage site in λN were determined in fluorogenic decapeptides containing λN sequence flanking each site. The cleavage sites at A30-K31 and L40-N41 are closer to the N-terminal whereas L93-S94 is closer to the C-terminal of λN, we place the Abz-NO2Tyr dye pair in FRλN001 to detect peptide bond cleavage at the N-terminal through monitoring the cleavage of either

Figure 4. (A) Amino acid sequences of the λN protein used to monitor peptide bond cleavage by WT Elon. A FRET pair of abz/nitrotyrosine was used in both λN proteins. Both sequences contain the same internal fluorescence quenching pair: NO2Tyr which quenches the fluorescence of Abz in the intact substrate, though the length between the two are different, with two cleavage sites between the fluorescent pair in FRλN001. Elon cleavage sites between the fluorescent pair are underlined. Upon peptide bond cleavage, separation of NO2Tyr from Abz allows the detection of fluorescence emission generated by the latter at excitation 320 nm and emission 420 nm. (B) Steady-state kinetics of ATP-dependent hydrolysis of FRλN001 versus FRλN006 cleavage by WT Elon. The steady-state rate constants (kobsN,ATP) of protein hydrolysis with varying concentrations of FRλN001 (●) and FRλN006 (□) were determined using the continuous fluorescence based proteinase assay as described in Materials and Methods. The kobsN,ATP values, determined by dividing reaction rates with enzyme monomer concentration, were plotted as a function of peptide concentration. The data were best fit with eq 3 to yield the kinetic parameters summarized in Table 1. The fit of the data is illustrated by the solid lines. Error bars show the experimental standard deviations of at least three independent trials from the averaged values.

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Table 1. Kinetic Parameters for Protein Degradation of $\lambda N$ by WT ELon

<table>
<thead>
<tr>
<th></th>
<th>ATP steady-state</th>
<th>ATP pre-steady-state</th>
<th>AMPPNP pre-steady-state</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{\text{cat,NATP}}$ (s$^{-1}$)</td>
<td>$K_m$ (µM)</td>
<td>$k_{\text{cat,ATP}}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>FRJN01</td>
<td>0.22 ± 0.06</td>
<td>6.12 ± 0.53</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>FRJN006</td>
<td>0.15 ± 0.01</td>
<td>5.55 ± 0.50</td>
<td>0.08 ± 0.01</td>
</tr>
</tbody>
</table>

A30-K31 or L40-N41. The cleavage of the C-terminal of $\lambda N$ will be detected by the separation of the dye pair flanking the L93-S94 in FRJN006. When excited at 320 nm, both FRJN001 and FRJN006 emitted maximum fluorescence signal at ~415 nm. Upon incubation with ELon and ATP, the signal intensity of FRJN001 and FRJN006 increased by 38 170 counts and 108 930 counts, respectively (see Supporting Information, S4). The difference in the signal intensity was attributed to the difference in the separation distance between NO$_2$Tyr and Abz in the two substrates. In FRJN001, NO$_2$Tyr was 16 residues from Lys(Abz), whereas in FRJN006, NO$_2$Tyr was 9 residues apart from Lys(Abz). Therefore the Abz moiety in FRJN006 had a lower intrinsic Abz fluorescence and was more effectively quenched. Using the fluorogenic assay described above, the $k_{\text{cat,NATP}}$ and $K_m$ values for the cleavage of the N-terminal in FRJN001 and C-terminal of FRJN006 at 1 mM [ATP] were determined by fitting the data that related the rate constant of peptide bond cleavage ($k_{\text{obs,NATP}}$ with the indicated [substrate]. The plots shown in Figure 4B fit well with eq 3 to provide the values summarized in Table 1. Within experimental deviation, the $k_{\text{cat,NATP}}$ and $K_m$ values of both substrates are comparable. The detection of comparable $k_{\text{cat,NATP}}$ and $K_m$ values for the cleavage of both sites indicated the same rate-limiting step occurred in both degradation reactions.

One technical issue associated with the fluorogenic assay described here is the presence of an inner filter effect at high substrate concentrations.45 To overcome this, a substrate cocktail containing 10% of the fluoroscently labeled $\lambda N$ was mixed with 90% of unlabelled $\lambda N$ to make up the desirable substrate concentration for the assay. This approach was successfully used in the past to determine the kinetics of peptide bond cleavage in synthetic peptides.46 In this study, controls were performed to ensure that the rate of peptide bond cleavage in the 10% labeled substrate was the same as the 100% labeled substrate using a substrate concentration that did not suffer from inner filter effect (see Supporting Information, S5). The substrate cocktail is referred to as $\lambda N001A$ and $\lambda N006A$, respectively. They are used in the pre-steady-state stopped flow assays described below.

Pre-Steady-State Stopped Flow Time Courses of Peptide Bond Cleavage in $\lambda N$ under Pseudo First Order Conditions. To gain insights into the rate-limiting step in the ATP-dependent degradation of $\lambda N001A$ and $\lambda N006A$, the cleavage of the NO$_2$Tyr/Abz-labeled site in $\lambda N001A$ and $\lambda N006A$ were monitored under excess [substrate] over [Lon] (~2-fold $k_{\text{cat,N}}$, $\lambda N$; >10-fold $k_{\text{cat,N}}$, $\lambda N$) in the presence of 1 mM ATP (Figure 5A) or AMPPNP (Figure 5B) using a stopped-flow apparatus. Lag kinetics were detected in both reactions. Fitting the respective time courses with eq 4 yielded the kinetic parameters summarized in Table 1. The $k_{\text{obs,NATP}}$ and $k_{\text{obs,AMPPNP}}$ values correspond to the steady-state rate constant of the cleavage of the labeled site at 8 µM of $\lambda N001A$ or $\lambda N006A$. The $k_{\text{obs}}$ values reflect the rate constant for the transition of the slow to the fast phase of the time course.

The $k_{\text{lag,NATP}}$ values for the ATP-dependent cleavage reactions are between 20- and 35-fold higher than the $k_{\text{lag,AMPPNP}}$ obtained for the AMPPNP-dependent reactions. A ~10-fold lower ratio in the $k_{\text{lag,AMPPNP}}$ of AMPPNP- versus the $k_{\text{lag,NATP}}$ ATP-dependent cleavage of a decapeptide containing residues 89–98 of $\lambda N$ (the labeled site in FRJN006) was previously detected.43 Taken together, a more pronounced dependency on the ATPase activity in $k_{\text{lag}}$ for the same scissile site (flanking residues 89–98) in full-length $\lambda N$ versus the decapetide was observed, suggesting that the length and/or presence of multiple Lon cleavage sites decreases the magnitude of $k_{\text{lag}}$ in peptide bond cleavage. The detection of lag kinetics in both reactions could be attributed to the buildup of the same enzyme intermediate before peptide bond cleavage occurs, or that the separation of the fluorescent donor and acceptor in the hydrolyzed $\lambda N$ products awaits the complete degradation of the entire substrate, which constitutes the rate-limiting step. The latter possibility was deemed unlikely because no hydrolyzed peptide products or partially digested $\lambda N$ were detected within the same time frame when the degradation reactions were quenched with denaturants to release enzyme bound $\lambda N$ products (see Supporting Information S6). The lag kinetic profiles detected in the cleavage of $\lambda N001A$ and $\lambda N006A$ are consistent with the rate-limiting substrate delivery step previously detected in the ATP- and AMPPNP-dependent cleavage of a fluorogenic peptide constituting residues 89–98 of $\lambda N$.43 The reciprocal of $k_{\text{lag}}$ provides an estimate for the duration of the lag phase ($\tau$) which is 1.3 s for $\lambda N001A$ and 1.15 s for $\lambda N006A$ in the presence of ATP. In the presence of AMPPNP, the $\tau$ values are 40 and 24 s for $\lambda N001A$ and $\lambda N006A$, respectively. As further discerned in Table 1, the $k_{\text{lag}}$ and $k_{\text{obs}}$ of the ATP- and, independently, AMPPNP-dependent cleavage of $\lambda N001A$ are comparable to the values obtained for $\lambda N006A$, indicating that the same rate-limiting step dictates the cleavage of both sites.

Dansylated $\lambda N$ as Reporters to Monitor Translocation of N- versus C-Terminal of $\lambda N$ and the Utilization of S679W ELon. In a previous study, the delivery of the synthetic peptide substrate constituting residues 89–98 of $\lambda N$ to the proteolytic site of ELon was detected by FRET, where the Trp in S679W ELon served as the donor and the dansylated peptide substrate acted as the acceptor.47 In S679W ELon, the proteolytic site Ser was mutated to Trp such that the mutant possessed WT-like ATPase activity but was proteolytically inactive. It was discovered that the FRET signal generated from S679W interacting with the dansylated peptide substrate occurred prior to peptide bond cleavage. As such, the timing of their delivery to the proteolytic site could be monitored by the FRET signal generated from exciting the Trps in S679W ELon and detecting dansyl emission. Capitalizing on this technology, we generated the three dansylated $\lambda N$ constructs shown in Figure 6A. The three constructs differ by the location of a dansylated Cys inserted upstream and downstream of the Lon cleavage sites at residues A30-K31, L40-N41, and L93-S94. The location of the dansylated sites are illustrated in Figure 6A. Dansyl$\lambda N001$ contains a dansyl label at position 26, 4 residues
dansyl\textsubscript{N001} to the C-terminal. The fluorescent label of dansyl\textsubscript{N006} is located at position 99, 5 residues from the Lon cleavage site at L93-S94, and is the closest to the C-terminal. Each dansylated site was located at the vicinity of a different Lon cleavage site, which differs by their respective locations from the C-terminal containing residues 99–107. As such, the timing of their delivery to the proteolytic site could be monitored by the FRET signal generated from exciting the Trps in S679W Lon and detecting dansyl emission.

The Trp fluorescence of S679W ELon was nonspecifically quenched by dansyl glutamic acid, but greater decrease in Trp fluorescence was detected in reactions containing any one of the dansylated \( \lambda \text{N} \) proteins (see Supporting Information S7). No change in Trp fluorescence was detected when Lon was incubated with unlabeled \( \lambda \text{N} \), \( \lambda \text{N}002 \), or \( \lambda \text{N}006 \) (data not shown). A decrease in Trp fluorescence was accompanied by an increase in dansyl fluorescence in a reaction containing S679W ELon, dansyl\textsubscript{N}, and ATP, indicating the presence of FRET. Since Trps are only present in Lon, the decrease in Trp fluorescence accompanied by the increase in dansyl fluorescence upon excitation of Trp at 295 nm demonstrates the existence of FRET signal between S679W ELon and dansylated \( \lambda \text{N} \). Furthermore, both the decrease in Trp and increase in dansyl fluorescence exhibit dependency on the concentration of dansyl\textsubscript{N} incubated with S679W ELon. These results indicate that the binding interaction between S679W ELon and the respective dansylated \( \lambda \text{N} \) at the specific site can be monitored by FRET.

The S679W ELon mutant contains 4 Trp residues, with the 679W located at the proteolytic site.\(^{47}\) To determine the extent to which 679W contributed to the FRET signal, the stopped flow fluorescence time courses of dansyl\textsubscript{N001}, dansyl\textsubscript{N002}, and dansyl\textsubscript{N006} interacting with S679W versus S679A ELon in the presence of 0.5 mM ATP were monitored. For comparison, the time courses of dansyl\textsubscript{N001}, dansyl\textsubscript{N002}, and dansyl\textsubscript{N006} mixed with S679W ELon in the absence of any nucleotide and in the presence of 0.5 mM ADP were also obtained. The time courses for the increases in dansyl fluorescence are shown in Figure 6B and the concomitant decreases in Trp fluorescence are shown in Figures 6C, D, and E. According to the data, the Trp fluorescence in S679A was affected by the presence of the dansylated \( \lambda \text{N} \) constructs, but these changes did not significantly affect the dansyl signal. Although a small amount of change in dansyl fluorescence was detected in the S679A reaction, the most noticeable changes in dansyl as well as Trp detection in S679W ELon was the presence of FRET. Since Trps are only present in Lon, the decrease in Trp fluorescence accompanied by the increase in dansyl fluorescence upon excitation of Trp at 295 nm demonstrates the existence of FRET signal between S679W ELon and dansylated \( \lambda \text{N} \).

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Pre-Steady-State FRET Time Course for the Delivery of a Specific Dansylated Site in \( \lambda \text{N} \) under Pseudo First Order Conditions. A unidirectional substrate translocation process has been found to exist in ATP-dependent protease complexes ClpXP and ClpAP, but has yet to be shown to exist in Lon.\(^{18,24,36,48,49}\) To evaluate the existence of directionality in the delivery of substrate to the proteolytic site of ELon, we determined the order by which dansyl\textsubscript{N001}, dansyl\textsubscript{N002}, and dansyl\textsubscript{N006} interacted with S679W ELon through monitoring the duration needed for each construct to obtain maximum FRET signal between the dansyl label and 679W Trp. The time courses for S679W ELon interacting with dansyl\textsubscript{N001}, dansyl\textsubscript{N002} and dansyl\textsubscript{N006} in the presence of ATP or AMPPNP were obtained by exciting the reactions at

Figure 5.Stopped-flow analysis of ATP-dependent FR\textsubscript{N} cleavage by WT ELon under excess FR\textsubscript{N} conditions. (A) 5 \( \mu \text{M} \) WT ELon was incubated with 8 \( \mu \text{M} \) \( \lambda \text{N} \textsubscript{001A} \) with no nucleotide (○), 1 mM ADP (□), or 1 mM ATP (▲), or 8 \( \mu \text{M} \) \( \lambda \text{N} \textsubscript{006A} \) with no nucleotide (●), 1 mM ADP (●), or 1 mM ATP (●). (B) 5 \( \mu \text{M} \) WT ELon was incubated with 10 \( \mu \text{M} \) \( \lambda \text{N} \textsubscript{001A} \) (■) or 10 \( \mu \text{M} \) \( \lambda \text{N} \textsubscript{006A} \) (○) with 1 mM AMPPNP. The fluorescent changes associated with peptide cleavage were converted to product concentrations as described in Materials and Methods. Each time course shown is an average of 4 traces. The traces with ATP and AMPPNP were set to eq 4 to determine the kinetic parameters listed in Table 1. No change in fluorescence was observed without nucleotide or with ADP.
Figure 6. (A) Design of fluorescent λN protein used to monitor degradation and translocation by ELon. (B) Stopped-flow analysis of ATP-dependent dansylλN delivery by S679W and S679A ELon under excess dansylλN conditions. 5 μM S679W ELon was incubated with 0.5 mM ATP and 10 μM dansylλN001 (○), dansylλN002 (□), or dansylλN006 (●). 5 μM S679W ELon was incubated with 1 mM ADP and dansyl λN001 (■, dark gray), no nucleotide and dansylλN001 (●, dark gray), 0.5 mM ADP and dansylλN002 (■, light gray), no nucleotide and dansylλN002 (●, light gray), 0.5 mM ADP and dansylλN006 (■, black) or no nucleotide (●, black). 5 μM S679A ELon was incubated with 10 μM dansylλN001 (▲, dark
at the dansyl excitation wavelength, the stepwise increases in the dansyl fluorescence are assigned to the FRET signal generated from the delivery of the respective dansylated site to 679W via translational movement and/or spatial orientation of the two groups due to conformational changes at the enzyme active site. It is discerned from the FRET time courses that the fluorescence probe located at residue 99 attains steady-state signal before the probe at residue 42 and then the probe at residue 26. The subsequent slow changes in FRET signal are assigned to the steady-state ATPase-mediated conformational changes in the enzyme’s proteolytic site that affect the local environment of dansyl and 679W FRET interaction. This speculation is supported by a previous study showing that the proteolytic site of ELon interacted with a synthetic peptide containing residues 89–98 of λN differently in the presence of ATP versus ADP.46 During the steady-state ATPase cycle, S679W is anticipated to undergo repeated cycles of conformational changes.

A comparison of the first 0.4 s of the ATP-dependent FRET time courses for the three dansylated AN constructs is shown in Figure 6F. The FRET signal originating from dansyl approaching 679W seems to initiate at dansyl N001, followed by dansyl N002 and then dansyl N006. Fitting the data spanning the first 0.4 s with eq 5 yields a rate constants, as seen in Table 2, $k_{FRET, ATP}$ values for dansyl N001 (○), dansyl N002 (□), and dansyl N006 (■) as summarized in Table 2. For comparison, cleavage of λN interacting with WT ELon with ATP is also shown. The right y-axis shows relative fluorescence due to peptide bond cleavage.

### Table 2. Kinetic Constants for DansylAN Interacting with S679W

<table>
<thead>
<tr>
<th>DansylAN</th>
<th>$k_{FRET,AMPPNP}$ ($s^{-1}$)</th>
<th>$k_{FRET,ATP}$ ($s^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N001</td>
<td>0.337 ± 0.029</td>
<td>16.63 ± 0.46</td>
</tr>
<tr>
<td>N002</td>
<td>0.404 ± 0.059</td>
<td>8.91 ± 0.47</td>
</tr>
<tr>
<td>N006</td>
<td>0.633 ± 0.035</td>
<td>5.82 ± 0.13</td>
</tr>
</tbody>
</table>

± 0.47 $s^{-1}$ for dansyl N002, and 5.82 ± 0.13 $s^{-1}$ for dansyl N006, respectively. This data suggests that within the detection limit of the Förster distance of the dansyl/Trp FRET dye pair, the N-terminal [represented by dansyl N001] begins to approach 679W before the other two sites, but it takes three steps spanning ~1.5 s to obtain steady-state FRET, implying that this is the last site to reach 679W. By contrast, attainment of FRET steady-state is completed in dansyl N002 in two steps within 1 s and in dansyl N006 in one step within 0.5 s. As extrapolated from the $k_{lag,INATP}$ of λN006A and λN001A found in Table 1 and discussed earlier, the duration of the lag phase ($\tau$) in peptide bond cleavage amounts to 1.15 to 1.3 s. This lag phase is attributed to the buildup of an enzyme intermediate needed to initiate peptide bond cleavage. The termination of this lag phase therefore sets the end point for considering the FRET signals associated with substrate delivery prior to peptide bond cleavage. Comparing the FRET signals associated with S679W interacting with any of the dansylated Cys in part A were fit with eq 5 to yield $k_{FRET,ATP}$ values for dansyl N001 (○), dansyl N002 (□), and dansyl N006 (■) as summarized in Table 2. For comparison, cleavage of λN006A (■) by WT ELon with ATP is also shown. The right y-axis shows relative fluorescence due to peptide bond cleavage.
burst in ATP hydrolysis suggests half-site reactivity, which λ enzyme turnover, is 2.61 of ADP generated per enzyme monomer during the catalyzed by WT ELon stimulated by 8 μM of unlabeled λN001, which contains a Cys insertion at position 26, and λN006, which contains a Cys insertion at position 99, were determined. Figure 7A shows the time courses of the ATPase reactions obtained up to 2 s. Both time courses show a rapid increase followed by a transient plateau and then a steady-state increase in ATP hydrolysis. This observation matches with the previously published results obtained using a decapetide containing residues 89−98 of λN.52 Therefore, it is plausible that each “step-like” phase of the time course is coordinated with an ATPase cycle. To evaluate such a possibility, the time courses of ATP hydrolysis catalyzed by WT ELon stimulated by 8 μM of unlabeled λN001, which contains a Cys insertion at position 26, and λN006, which contains a Cys insertion at position 99, were determined. Figure 7A shows the time courses of the ATPase reactions obtained up to 2 s. Both time courses show a rapid increase followed by a transient plateau and then a steady-state increase in ATP hydrolysis. This observation matches with the previously published results obtained using a decapeptide containing residues 89−98 of λN.52 Therefore, the substrate-stimulated ATPase activity of ELon appears insensitive to the size or number of Lon cleavage sites present in a substrate. Figure 7B compares the time course of ATP hydrolysis obtained up to 0.4 s. Fitting the data to the single exponential function yields the kinetic parameters found in Table 3. The burst rate constant, kburst, for ATP hydrolysis is 11.54 ± 1.79 s⁻¹ in the presence of λN001, and 6.49 ± 1.08 s⁻¹ in the presence of λN006. The burst amplitude, which reflects the stoichiometry of ADP generated per enzyme monomer during the first enzyme turnover, is 2.61 ± 0.16 and 2.81 ± 0.18 for λN001 and λN006, respectively. Since 5 μM of ELon monomer was used in the ATPase reactions, the observed ~50% pre-steady-state burst in ATP hydrolysis suggests half-site reactivity, which agrees with our previous finding when a peptide rather than λN was used as substrate.52 Despite an apparent 2-fold difference in the two kburst values, the error bars shown in Figure 7B reveal

![Figure 7](image_url)
that the burst kinetics displayed by the two reactions are comparable. Therefore the \( k_{\text{burst}} \) of \( \lambda N \)-stimulated ATPase is between 6.49 and 11.54 s\(^{-1}\), which is similar to the \( k_{\text{FRET,ATP}} \) values obtained in the fitting of the first 0.4 s of the ATP-dependent FRET time courses shown in Figure 6F. This result suggests that the initial burst of ATPase of ELon is coordinated with the first phase of substrate delivery.

### AMPPNP-Dependent Delivery of \( \lambda N \) to 679W.

As ELon catalyzed the delivery of the synthetic decapeptide containing residues 89–98 of \( \lambda N \) to the active site of ELon in the presence of AMPPNP,\(^{47} \) the kinetics of ELon delivering dansylated residues 89–98 of ELon were adapted.\(^{47} \) In this study, two synthetic \( \lambda N \) variants, each lacking intrinsic Trp residues, were labeled with the internally fluorescent quenching group, NO₂Tyr and Lys(Azb), such that the kinetics of peptide bond cleavage occurring at each end of \( \lambda N \) could be quantified. These two sites were separated by at least 50 residues. Independently, “trpless” \( \lambda N \) variants containing a unique Cys at sites proximal to the target scissile sites were labeled with dansyl to generate a site-specific labeled substrate that allowed for the detection of substrate delivery with the proteolytically inactive ELon mutant S679W, which possessed WT-like ATPase activity. Comparing the time courses of specific site cleavage versus delivery revealed that the three

![Figure 8](image-url)

**Figure 8.** Substrate delivery to S679W ELon active site can be monitored using the dansylated protein and AMPPNP. (A) 5 \( \mu \)M S679W ELon was incubated with 10 \( \mu \)M dansyl\( \lambda N \)001, dansyl\( \lambda N \)002, and dansyl\( \lambda N \)006 in the presence of this nonhydrolyzable ATP analog were also examined by the FRET technique. Figure 8 shows the time courses of the respective dansylated \( \lambda N \) approaching 679W (representatives of at least three independent experiments). For comparison, the time course of the AMPPNP-dependent degradation of \( \lambda N \)001A was also included. The y-axis reports the FRET signal intensity generated from each reaction. Unlike the ATP-dependent time courses shown in Figure 6B, the AMPPNP-dependent time courses are best fit with the double exponential function (eq 6) to yield the rate constants shown in Table 2. Both the \( k_{\text{FRET,AMPPNP}} \) and \( k_{\text{FRET,ATP}} \) values follow the same descending order in which dansyl\( \lambda N \)006 > dansyl\( \lambda N \)002 > dansyl\( \lambda N \)001, suggesting that C-terminal of \( \lambda N \) approaches and reaches 679W first. This result differs from the ATP-dependent reactions (Figure 6B) by the terminal of \( \lambda N \) that initiates movements toward 679W. In both cases, however, the C-terminal of \( \lambda N \) is the first to reach 679W.

The detection of two observed rate constants for each AMPPNP-dependent time course indicates the presence of at least two steps in the delivery event. The observation of a two-step substrate delivery step is reminiscent of our earlier discovery that the ATP- or AMPPNP-dependent delivery of the dansylated peptide containing residues 89–98 of \( \lambda N \) to S679W ELon involved the formation of a S679W:nucleotide:dansylated substrate complex followed by a second conformational change in the complex attributed to the delivery of the dansylated substrate to the proteolytic site at 679W.\(^{47} \) As revealed by the standard errors of the fits, the \( k_{\text{FRET,AMPPNP}} \) values of dansyl\( \lambda N \)001 and dansyl\( \lambda N \)002 are comparable to the \( k_{\text{FRET,AMPPNP}} \) of peptide bond cleavage in FR\( \lambda \)N001 and FR\( \lambda \)N002 (see Table 1), suggesting that the step corresponding to \( k_{\text{FRET,AMPPNP}} \) in dansyl\( \lambda N \)006 occurs faster. As FRET signal is generated from the dansyl label in \( \lambda N \) approaching 679W in Lon mutant, we conclude that, in the presence of AMPPNP, the C-terminal of \( \lambda N \), which is represented by dansyl\( \lambda N \)006, approaches the proteolytic site of ELon before the N-terminal, which is represented by dansyl\( \lambda N \)001 and dansyl\( \lambda N \)002. The similarity in the \( k_{\text{FRET,AMPPNP}} \) of dansyl\( \lambda N \)001 and dansyl\( \lambda N \)002 could be attributed to the location of these two probes being not far apart enough to detect noticeable difference in the kinetic experiment. Additional kinetic studies which involve determining the entire kinetic mechanism of \( \lambda N \) translocation will be required to further define the molecular nature of the two kinetic steps detected in the AMPPNP-dependent reactions.

### DISCUSSION

Procesive protein degradation is a common feature found in ATP-dependent proteases. Protein substrates are degraded into peptides ranging from 5 to 20 amino acids long without the generation of partially digested substrates. The study of processivity has been primarily conducted in the hererosubunit ATP-dependent proteases using FRET techniques.\(^{24,30} \) In these studies, Cys mutations were introduced into model substrate and enzyme subunits for chemical attachment of fluorescence donor and acceptor dyes containing overlapping spectral properties. The model substrates typically contain an enzyme recognition tag such as an ssrA tag to direct initiation of substrate binding to the ATPase subunit of the protease complex. Using this approach, the kinetics of substrate unfolding, translocation, and peptide bond cleavage have been obtained.\(^{30,31} \) In Lon protease, alkylation of Cys residues leads to enzyme inactivation.\(^{23} \) Therefore, the FRET approach used for studying the procesive mechanism of the Clp complexes cannot be used on Lon. As an alternative, the methodologies previously used to study the kinetic mechanism of ATP-dependent delivery to the active site and cleavage of the decapeptide containing residues 89–98 of \( \lambda N \) were adapted.\(^{47} \) In this study, two synthetic \( \lambda N \) variants, each lacking intrinsic Trp residues, were labeled with the internally fluorescent quenching group, NO₂Tyr and Lys(Azb), such that the kinetics of peptide bond cleavage occurring at each end of \( \lambda N \) could be quantified. These two sites were separated by at least 50 residues. Independently, “trpless” \( \lambda N \) variants containing a unique Cys at sites proximal to the target scissile sites were labeled with dansyl to generate a site-specific labeled substrate that allowed for the detection of substrate delivery with the proteolytically inactive ELon mutant S679W, which possessed WT-like ATPase activity. Comparing the time courses of specific site cleavage versus delivery revealed that the three
Dansylated sites were delivered to the 679W site in ELon before any peptide bond cleavage occurs.

In Lon, a naturally occurring substrate recognition tag has not been identified in physiological substrates. Gur and Sauer have identified hydrophobic peptide sequences that directed the degradation of non-natural substrates by Lon, but the physiological relevance of these peptide recognition tags was not clear.53 A review on the degradation profiles of physiological substrates of bacterial Lon reveals that recognition elements other than hydrophobic patches in these substrates can target proteins for degradation, and thus far, no consensus recognition tag has been identified for the recognition and degradation of physiological substrates of Lon.44 Given these considerations, we chose the $\lambda N$ protein, which is the endogenous protein substrate of ELon,55 to study the processive protein degradation mechanism of Lon. Structural studies have shown that full length and truncated $\lambda N$ variants do not contain defined secondary structures.35,36 The kinetic coordination between the delivery to the active site and cleavage of different regions of $\lambda N$ can thus be directly monitored without interferences from substrate unfolding. Based on the rate constants summarized in Tables 1 and 2 and the results shown in Figures 6B and 8, the order of scissile site delivery occurs from the C- to N-terminal. Although residues 99–107 contribute to the binding affinity of $\lambda N$ by ELon, it is currently not clear the extent to which the directionality of substrate delivery is affected by these residues, as $\lambda N$ lacking these residues was still degraded by ELon, albeit with lower efficiency than WT $\lambda N$.

To evaluate the timing of peptide bond cleavage at different scissile sites in $\lambda N$, an iTRAQ mass spectrometry experiment that allowed for the quantification of the degradation of different regions of $\lambda N$ was performed. The detection of comparable time courses in the generation of different hydrolyzed peptide products suggests that the different scissile sites in $\lambda N$ were cleaved with comparable timing during the reaction. This implication was corroborated by the kinetic results obtained for the cleavage of the two scissile sites at the N-terminal in FR$\lambda N$001 and one scissile site at the C-terminal in FR$\lambda N$006, which exhibited comparable kinetic parameters in their respective cleavages (Table 1). In an earlier study, the $k_{cat}$ and $K_m$ values for the ATP-dependent cleavage of the individual fluorescently labeled scissile sites of $\lambda N$ have been determined in decapeptides. Overall, the $K_m$ as well as the $k_{cat}$ values of the peptide analog of the same scissile site are larger than in full-length $\lambda N$. The difference in $K_m$ could be attributed to the ability of ELon to interact with multiple regions of $\lambda N$, which results in an apparently >100-fold lower $K_m$ of the substrate. As for the difference in the $k_{cat}$ value, the synthetic peptide substrates are ~10-fold shorter in length than $\lambda N$. The observed reduction in the $k_{cat}$ of $\lambda N$ cleavage obtained in this study could be attributed to the extra time needed to translocate the entire protein before initiation of peptide bond cleavage compared to the small peptides. In congruence with this finding was the detection of an “almost complete” delivery of the three dansylated sites in $\lambda N$ to the active site coinciding with the lag phase of the cleavage of the cognate sites in $\lambda N$ in the ATP- as well as AMPPNP-dependent reactions. Collectively these results also account for the observation that only completely digested peptide products are detected in a typical ATP-dependent $\lambda N$ degradation time course quenched with denaturant. As peptide cleavage only occurs after complete delivery of $\lambda N$, any partially translocated $\lambda N$ would have been detected as undigested $\lambda N$ in SDS-PAGE. The observed concerted peptide bond cleavage occurring after almost complete translocation of the entire substrate has also been detected in other heterosubunit ATP-dependent proteases.30 This result, however, contradicts the mechanism proposed by Choi and Licht, who observed that the extrusion of polypeptide substrate into the heterosubunit ATP-dependent protease ClpAP alternated with peptide bond cleavage events55 and will require further investigation to reconcile the differences observed in these studies.

A unidirectional substrate translocation process has been found to exist in the Clp complexes, but has yet to be shown to exist in Lon.18,24,48,49 Through comparing the kinetic time courses of the delivery of different regions of $\lambda N$, represented by dansyl$\lambda N$001, dansyl$\lambda N$002, and dansyl$\lambda N$006, to the active site S679W in the presence of ATP and independently AMPPNP, the delivery of the scissile site beginning at the C-terminal was implicated. As no putative recognition peptide tag for Lon has been identified in $\lambda N$, it is currently not clear if the region constituting residues 99–107, which affects substrate binding affinity, contributes to the directionality of substrate translocation. Since $\lambda N$ lacking residues 99–107 was also degraded processively by ELon, it is likely that internal region(s) within $\lambda N$ also participate in directing substrate translocation. As such, a more in-depth structure–function study on the translocation profile of $\lambda N$ by ELon will be needed. The kinetic approach described in this study will provide the conceptual and technological framework for completing such analysis.

In summary, we utilized a kinetic approach to demonstrate that the processive degradation of $\lambda N$ by ELon occurs via model 1 shown in Scheme 1. The transient kinetic data generated in this study reveals that the cleavage of the different scissile sites in $\lambda N$ occurs after the delivery of the protein substrate to the enzyme active site. The different scissile sites in $\lambda N$ are delivered at different times, but their subsequent cleavage encounter the same rate-limiting step. Despite the lack of a defined recognition peptide tag, the C-terminal of $\lambda N$ is delivered to the proteolytic site before the N-terminal. The residues located within positions 99–107 of $\lambda N$ contribute to the binding affinity of $\lambda N$ by ELon, but other residues beyond this region are also needed for substrate binding and translocation. Comparing the kinetic time courses of ATP-versus AMPPNP-dependent degradation and delivery of $\lambda N$ reveals that processive protein degradation requires ATP binding but not hydrolysis. However, ATP hydrolysis facilitates the efficiency of both events.

**ASSOCIATED CONTENT**

### Supporting Information

Details of the $\lambda N$ degradations, iTRAQ, changes in fluorescence of FR$\lambda N$, degradation of 100% FR$\lambda N$ versus 10% FR$\lambda N$, detection of lag kinetics in a discontinuous assay, and emission scans of Trp/dansyl FRET. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS:
Abz, anthranilamide; ADP, adenosine diphosphate; AMPPNP, adenylyl 5-imidodiphosphate; ATP, adenosine triphosphate; BCIP, 5-bromo-4-chloro-3-indolyolphosphate p-toluidine salt; C-his-ΔN, 6x his tag at the carboxyl end of wild type λN sequence; Clp, caseinolytic protease complex; C-terminal, carboxyl terminal; DTT, dithiothreitol; FRET, fluorescence resonance energy transfer; FRN001, a fluorescent protein substrate of Lon with a nitrotyrosine and anthranilamide at the amino end of λN; FRN002, a fluorescent protein substrate of Lon with a nitrosotyrosine and anthranilamide at the carboxyl end of λN; HEPES, N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid; iTRAQ, isobaric tag for relative and absolute quantitation; KOAc, potassium acetate; KPi, potassium phosphate; λN, also known as λN protein, a λ phage protein; λN001A, a fluorescent protein substrate of Lon containing 10% FRN001 and 90% λN001; λN001, cysteine at the 26th position of wild type λN; λN002, cysteine at the 42nd position of wild type λN; λN006A, a fluorescent protein substrate of Lon containing 10% FRN006 and 90% λN006; λN006, cysteine at the 99th position of wild type λN; λNΔ1–34, truncated λN lacking the N-terminal; N-his-ΔN99–107, truncated λN lacking the C-terminal; LC/MS/MS, liquid chromatography/mass spectrometry/mass spectrometry; Mg(OAc)2, magnesium acetate; MW, molecular weight; NBT, nitro-blue tetrazolium chloride; NO2Tyr, nitrotyrosine; N-terminal, amino terminal; PAGE, polyacrylamide gel electrophoresis; PEI-cellulose, polyethyleneimine-cellulose; PMT, terminal, amino terminal; PAGE, polyacrylamide gel electrophoresis; protease La from Escherichia-Coli. J. Biol. Chem. 268, 22502–22507.

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REFERENCE


