DEVELOP SPECTROSCOPIC APPROACHES TO STUDY NON-PROTEOSOMAL ATP-DEPENDENT PROTEOLYSIS

By

NATALIE MIKITA

Submitted in partial fulfillment of the requirements

For the degree of Doctor of Philosophy

Dissertation Advisor: Irene Lee

Department of Chemistry

CASE WESTERN RESERVE UNIVERSITY

August, 2014
CASE WESTERN RESERVE UNIVERSITY

SCHOOL OF GRADUATE STUDIES

We hereby approve the thesis/dissertation of

NATALIE MIKITA

Candidate for the degree of Doctor of Philosophy*

Committee Chair: MARY BARKLEY

Committee Member: IRENE LEE

Committee Member: BLANTON TOLBERT

Committee Member: YANMING WANG

Committee Member: PATRICK WINTRODE

Date of Defense

May 30, 2014

*We also certify that written approval has been obtained for any proprietary material contained therein.
TABLE OF CONTENTS

Title page..................................................................................................................1
Committee Sign-off sheet..........................................................................................2
Table of Contents......................................................................................................3
List of Tables............................................................................................................8
List of Figures..........................................................................................................9
Acknowledgements.................................................................................................12
List of Abbreviations...............................................................................................13
Abstract..................................................................................................................16
Chapter 1: Introduction to Lon protease.................................................................17
  1.1 Structure of Lon..............................................................................................18
  1.2 Function of Lon............................................................................................21
  1.3 Substrates of Lon..........................................................................................24
  1.4 Role of Lon in protecting the cell..............................................................25
  1.5 Monitoring peptide bond hydrolysis.........................................................27

Chapter 2: Evaluating the contributions of the N- vs C- terminal of λN towards nucleotide-dependent degradation by E. Coli Lon protease.............31
  2.1 Introduction.................................................................................................32
  2.2 Materials and Methods...............................................................................36
      2.2.1 Materials............................................................................................36
      2.2.2 General Methods...............................................................................36
      2.2.3 λN001, λN002, and λN006 plasmid construction.............................36
      2.2.4 Purification of his-tagged λN...............................................................37
2.2.5 λN001, λN002, and λN006 dansyl labeling..................................................37
2.2.6 Western blot analysis of λN degradation.................................................37
2.2.7 Characterization of the AMPPNP-dependent degradation of
dansyl λN..............................................................................................................38
2.2.8 λNΔ99-107 plasmid generation.................................................................38
2.2.9 Purification of non-histagged λN proteins................................................39
2.2.10 λN degradation assay...............................................................................40
2.2.11 Radiolabeled ATPase assay......................................................................40
2.3 Results and Discussion................................................................................41
2.3.1 AMPPNP-dependent degradation of his-tagged λN.................................41
2.3.2 Generation of fluorescent λN constructs to evaluate nucleotide
dependent proteolysis......................................................................................43
2.3.3 Evaluation of the AMPPNP-dependent degradation of
dansylated λN....................................................................................................46
2.3.4 Degradation profiles of WT and truncated λN.......................................47
2.3.5 Evaluate the effect of a his-tag on degradation of λN.........................49
2.3.6 Evaluate the contribution of residues 99-107 in the
binding of λN..................................................................................................50
2.4 Conclusion......................................................................................................51

Chapter 3: Evaluating the coordination between substrate translocation and peptide bond
Hydrolysis........................................................................................................53
3.1 Introduction..................................................................................................54
3.2 Materials and Methods...............................................................................58
3.2.1 Materials..........................................................................................58
3.2.2 General Methods..............................................................................58
3.2.3 Monitoring the ATP-dependent degradation of fluorescently labeled
\(\lambda N\) (FR\(\lambda N\)) by steady state kinetics...........................................58
3.2.4 Pseudo-first order time course of FR\(\lambda N\) cleavage by fluorescent
stopped flow.............................................................................................59
3.2.5 Fluorescence Emission scans of dansyl\(\lambda N\) interaction with S679W
ELon............................................................................................................59
3.2.6 Pseudo first order translocation of dansyl\(\lambda N\) by fluorescent stopped
Flow...........................................................................................................60
3.3 Results and Discussion.........................................................................61
3.3.1 Fluorescently labeled \(\lambda N\) as a substrate of ELon.........................61
3.3.2 Pre-steady-state stopped flow time courses of peptide bond cleavage
in \(\lambda N\) under pseudo first order conditions.............................................64
3.3.3 Dansylated \(\lambda N\) as reporters to monitor translocation of N- versus
C-terminal of \(\lambda N\) and the utilization of S679W ELon.........................67
3.3.4 Pre-steady-state FRET time course for the delivery of a specific
dansylated site in \(\lambda N\) under pseudo first order conditions.................74
3.3.5 AMPPNP-dependent Delivery of \(\lambda N\) to 679W..............................79
3.4 Conclusion.............................................................................................82

Chapter 4: Probing the structural dynamics and conformational changes of E. Coli Lon
protease using HXMS..............................................................................84
4.1 Introduction............................................................................................85
5.3.1 Cleavage of 8998Ac versus 8998Bz..................................................120
5.3.2 Purification of recombinant human Lon (hLon) and mutants……..123
5.3.3 ATP-dependent peptidase activity of hLon and mutants..........124
5.4 Conclusions.........................................................................................125

Chapter 6: Conclusion and future directions........................................128

BIBLIOGRAPHY.........................................................................................136
LIST OF TABLES

Chapter 2

2.1 Kinetic parameters for λN cleavage by ELon………………………………………50

Chapter 3

3.1 Kinetic parameters for protein degradation of FR λN by WT ELon………………64

3.2 Kinetic constants for AMPPNP-dependent dansyl λN interaction with S679W ELon……………………………………………………………………80

Chapter 4

4.1 Percent of the folded state envelope at each time point for peptide 233-244, QMKAIQKELGEM…………………………………………………………....105
LIST OF FIGURES

Chapter 1

1.1 Domain layout of Lon protease monomer.................................................................19
1.2 Hydrolysis of ATP to ADP and P, by Lon.................................................................22
1.3 Proposed mechanism for peptide bond cleavage in Lon...........................................22
1.4 Structure of Lon and simplified mechanism of proteolysis.......................................23
1.5 Degradation profile of λN protein...............................................................................24
1.6 Normal and Lon-deficient mitochondria....................................................................26
1.7 Explanation of the continuous fluorescent peptidase assay.....................................29

Chapter 2

2.1 Proposed mechanism for ATP-dependent proteolysis..............................................32
2.2 Structure of adenylyl imidodiphosphate, AMPPNP.................................................34
2.3 Degradation of λN by ELon in the presence of AMPPNP or ATP............................35
2.4 Western blot analysis of AMPPNP-dependent C-his-λN and N-his-λN Degradation.........................................................................................................................43
2.5 Amino acid sequences of the λN proteins used to evaluate the contribution of the C-terminal versus the N-terminal.................................................................45
2.6 Graphical representation of amount of λN left over time........................................45
2.7 Degradation of dansyl λN001, dansyl λN002 and dansyl λN006 in the presence of AMPPNP.....................................................................................................................47
2.8 Compare the time courses of λN deletion mutant degradations by WT ELon..........48
2.9 Graphical representation comparing the effect of a his-tag on the amount of λN left over time......................................................................................................................49
2.10 Graphical representation of the stimulatory effect of $\lambda N$ on ATPase activity of $\text{ELon}$…………………………………………………………………………………………………51

Chapter 3
3.1 Cartoon illustration for two possible models of protein degradation………………..56
3.2 Design of fluorescent $\lambda N$ protein used to monitor peptide bond cleavage by Lon…..62
3.3 Steady-state kinetics of ATP-dependent hydrolysis of FR$\lambda N001$ versus FR$\lambda N006$ cleavage by WT ELon……………………………………………………………………………63
3.4 Stopped-flow analysis of nucleotide-dependent FR$\lambda N$ cleavage by WT ELon under excess FR$\lambda N$ conditions……………………………………………………………………..67
3.5 Fluorescence emission scan of S679W ELon with unlabeled $\lambda N$…………………..69
3.6 Fluorescent emission scans of S679W ELon interacting with dansyl$\lambda N$…………70
3.7 Fluorescent emission scans of S679W interacting with dansyl$\lambda N001$ at increasing concentrations………………………………………………………………………………..71
3.8 Representative time courses for S679W interacting with dansyl$\lambda N$ through fluorescence stopped-flow………………………………………………………………………………..73
3.9 Substrate delivery to S679W ELon active site can be monitored using fluorescence stopped-flow with dansyl $\lambda N$ and ATP……………………………………………………77
3.10 Representative time courses for dansylated substrate delivery to S679W with AMPPNP using fluorescent stopped-flow…………………………………………………80

Chapter 4
4.1 Domain compositions for ELon and BsLon…………………………………………85
4.2 View of the crystal structure of ELon-N………………………………………………86
4.3 Crystal structure of the $\alpha$ domain of the ATPase domain of WT ELon……………87
4.4 Crystal structure of the protease domain of WT ELon

4.5 Three groups of hydrogens in proteins

4.6 The MS spectra resulting from two exchange kinetics, EX2 and EX1

4.7 Overview of the experimental procedure for monitoring deuterium uptake

4.8 MS spectra of the double-charged peptide 253-260 illustrating time-dependent deuterium uptake

4.9 Percent exchange of peptides from WT ELon

4.10 Mass spectra 233-244 (QMKAIQKELGEM) exhibiting EX1 kinetics

Chapter 5

5.1 Structure of nonfluorescent 8998Bz and 8998Ac substrates

5.2 HPLC traces of 8998Bz and 8998 Ac degradations by hLon viewed at 220 nm

5.3 Degradations of 8998Ac versus 8998Bz by WT hLon

5.4 ATP-dependent degradation of fluorgenic peptide S3 by WT hLon and CODAS mutants

Chapter 6

6.2 Structure of nonfluorescent 8998Cbz

11
Acknowledgements

I would first like to thank my advisor, Dr. Irene Lee, for her willingness to work with me and push me in the directions I needed to go. She has been a great teacher and always there to help me when I needed it.

Thank you so much to my parents and siblings. You have helped me so much along the way. I would also like to thank my labmate Iteen Cheng, who went through this whole process with me, step for step. I would also like to thank previous lab members especially Jennifer Fishovitz, who was like a mom to me when I first started and introduced me to pretty much every technique I learned. I would also like to thank my lunch crew for providing me with laughs and encouragement, and many strange and random conversations.

A special thanks to Dr. Patrick Wintrode and Daniel Deredge for helping me with the H/D exchange. Collaborations with Dr. Carolyn Suzuki at Rutgers New Jersey Medical School, Dr. Robert Jinks at Franklin and Marshall, and Dr. Anthony Berdis at Cleveland State University have all been invaluable to me.

And finally, I want to thank all my other family and friends for their unwavering support and encouragement.
LIST OF ABBREVIATIONS

\( \lambda_{\text{emission}} \) emission wavelength
\( \lambda_{\text{excitation}} \) excitation wavelength
\( \lambda_{\text{max}} \) wavelength at which the maximal optical signal is observed
\( \lambda N \) Lambda N protein: a \( \lambda \) phage protein that allows \( E. \ coli \) RNA polymerase to transcribe through termination signals in the early operons of the phage
\( \lambda N001 \) \( \lambda N \) with a cysteine at the 26 position, all other cysteines mutated to leucine, and all tryptophans mutated to phenylalanine
\( \lambda N001A \) \( \lambda N \) cocktail consisting of 10% \( \lambda N001 \) and 90% \( \lambda N001 \)
\( \lambda N002 \) \( \lambda N \) with a cysteine at the 26 position, all other cysteines mutated to leucine, and all tryptophans mutated to phenylalanine
\( \lambda N006 \) \( \lambda N \) with a cysteine at the 26 position, all other cysteines mutated to leucine, and all tryptophans mutated to phenylalanine
\( \lambda N001A \) \( \lambda N \) cocktail consisting of 10% \( \lambda N006 \) and 90% \( \lambda N006 \)
8998AC YRGITCSGRQ-K(\text{Ac})
8998Bz YRGITCSGRQ-K(\text{Bz})
AAA+ ATPases Associated with a variety of cellular Activities
Abz Anthranilamide
ACN Acetonitrile
ADP Adenosine diphosphate
AMPPNP Adenyl 5-imidotriphosphate – a nonhydrolyzable ATP analogue
ATP Adenosine triphosphate
BCIP 5-bromo-4-chloro-3-indolyl phosphate
BME Beta-mercaptoethanol
BSA Bovine Serum Albumin
BsLon Bacillus Subtilus Lon
BsLon-N Bacillus Subtilus Lon N-terminal crystal structure containing residues 1-209
BsLon-AP Bacillus Subtilus Lon ATPase and protease crystal structure containing residues 246-770
Bz Benzoic acid amide
C-His-\( \lambda N \) C-terminal his-tagged \( \lambda N \)
Cam Chloramphenicol
Cbz Carboxybenzyl
Cleptide Y(NO2)-FAPHMALVPV-K(\text{Abz})
dansyl 5-(dimethylamino)naphthalene-1-sulfonyle
dlu Density light units
dansyl\( \lambda N001 \) \( \lambda N001 \) with a dansyl attached to the cysteine at the 26 position
dansyl\( \lambda N002 \) \( \lambda N002 \) with a dansyl attached to the cysteine at the 42 position
dansyl\( \lambda N006 \) \( \lambda N006 \) with a dansyl attached to the cysteine at the 99 position
DNA Deoxyribonucleic acid
DMSO Dimethyl sulfoxide
DTT Dithiothreitol
ESI Electrospray ionization
E. coli
Escherichia coli, a gram negative bacteria
EDTA
Ethylendiaminetetraacetic acid
ELon
E. coli Lon protease
ELon-N
ELon crystal structure of N-terminus consisting of residues 1-245
ELon-A
ELon crystal structure of ATPase domain consisting of residues 491-584
ELon-P
ELon crystal structure of Protease domain consisting of residues 585-784
EtOH
Ethanol
FRET
Fluorescence Resonance Energy Transfer
FRET N 89-98
Y(NO2)-RGITCSGRQ-K(Abz)
FRLN
λN with a FRET pair attached
FRLN001
λN with a Y(NO2) and K(Abz) FRET pair attached near the N terminal at position 26 and 43 respectively, all cysteines mutated to leucine, and all tryptophans mutated to phenylalanine
FRLN006
λN with a Y(NO2) and K(Abz) FRET pair attached near the N terminal at position 89 and 99 respectively, all cysteines mutated to leucine, and all tryptophans mutated to phenylalanine
GdnDCl
Guanidium Deuterochloride
HCl
Hydrochlooric acid
hClpXP
Human ClpXP protease
H/D exchange
Hydrogen/deuterium exchange
HXMS
Hydrogen/deuterium exchange mass spectrometry
hLon
Human Lon protease
IPTG
Isopropyl-β-D-thio-galactoside
kcat
Vmax/[E]
kcat/Km
Substrate specificity constant
Ki
Inhibition constant
Km
Michaelis constant equal to [substrate] required to reach ½ Vmax
kobs
rate/[E]
Kan
Kanamycin
KO Ac
Potassium acetate
KPi
Potassium phosphate
LB
Luria-Bertani medium
MeOH
Methanol
Mg(OAc)2
Magnesium acetate
MOPS
3-(N-morpholino)propanesulfonic acid
MS
Mass spectrometry
N-His-λN
N-terminal his-tagged λN
NaCl
Sodium chloride
NaPi
Sodium phosphate
NBT
Nitro blue tetrazolium
Ni-NTA agarose
Nickel-nitrilotriacetic acid agarose: resin used to purify 6xHistagged proteins
OD
Optical density
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO2</td>
<td>Nitro</td>
</tr>
<tr>
<td>P11</td>
<td>Phosphocellulose cation exchange resin used to purify Lon</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEI-cellulose</td>
<td>Polyethyleneimine-cellulose</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
</tr>
<tr>
<td>S3</td>
<td>10% FRETN8998, 90% nonfluorescent 8998Ac</td>
</tr>
<tr>
<td>S. Typhimurium</td>
<td><em>Salmonella enterica</em> serovar Typhimurium</td>
</tr>
<tr>
<td>SB</td>
<td>Super Broth</td>
</tr>
<tr>
<td>SBTI</td>
<td>Soybean trypsin inhibitor</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>StAR</td>
<td>Steroidogenic Acute Regulatory protein</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline containing 0.05% Tween 20</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultraperformance liquid chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>v</td>
<td>Rate</td>
</tr>
<tr>
<td>V_{mas}</td>
<td>Maximal rate</td>
</tr>
</tbody>
</table>
Develop Spectroscopic Approaches to Study Non-proteosomal ATP-Dependent Proteolysis

Abstract

NATALIE MIKITA

Lon is an ATP-dependent serine protease found ubiquitously in nature that degrades damaged, misfolded, and certain regulatory proteins in the cell while hydrolyzing ATP to ADP and inorganic phosphate. Using a physiological substrate of *Escherichia coli* Lon (ELon) protease known as the lambda N protein (λN), and truncated mutants, it was demonstrated that the N-terminus is not important for efficient degradation by ELon, however the C-terminus is important in binding and degradation. Using fluorescent substrates of Lon, steady-state and pre-steady-state kinetic techniques were employed to determine the timing of scissile sites approaching the protoelytic site of ELon and their subsequent cleavages in an ATP- and AMPPNP- dependent manner. Collectively, these results support a mechanism by which ELon delivers λN to the active site C-terminally first, followed by the N-terminal. Subsequent cleavage awaits the almost complete delivery of all the sites, which appears to lack a specific directionality. Using hydrogen-deuterium exchange mass spectrometry (HXMS), deuterium incorporation in ELon was compared to known crystal structures solved. These results identified regions that corroborated the crystal structure, or differed from it. Previously developed assays were used to characterize twelve mutants of human Lon (hLon) implicated in causing cerebral, ocular, dental, auricular, and skeletal syndrome. The results of these assays so far does not indicate a clear mode of disease.
CHAPTER 1

INTRODUCTION TO LON PROTEASE
Lon protease, also known as protease La, is an ATP-dependent serine protease found ubiquitously in nature. This protease is responsible for maintaining proper cellular functions by degrading damaged and short lived regulatory proteins while hydrolyzing ATP to ADP and inorganic phosphate, Pi.\(^{(1-6)}\) In prokaryotic cells, the enzyme is found in the cytosol, while in eukaryotes, it is found in the mitochondria.\(^{(7-12)}\) The enzyme acquired its name from the phenotype of Lon deficient *Escherichia coli* (*E. coli*) bacteria forming elongated structures under UV light.\(^{(13)}\) *E. coli* Lon (ELon) homologue shares 57% sequence identity with the *Bacillus Subtilus* (BsLon) and 42% sequence identity with the human form (hLon). As such, ELon and BsLon can be used as model systems for hLon.

**1.1 Structure of Lon**

Lon is thought to exist as a homo-oligomer, with each bacterial subunit being approximately 88 kDa, and each human subunit being approximately 100 kDa. They are both comprised of 3 domains; the N-terminal domain, the ATPase domain, and the protease domain (figure 1.1). The entire structure of the Lon monomer has not yet been determined; however x-ray crystal structures of the N-terminus, α subdomain of the ATPase domain, and the protease domain for ELon have been independently solved.\(^{(14-16)}\) The N-terminal of BsLon, and independently, part of the N-terminal domain, the ATPase domain, and the protease domain, albeit with ADP bound, has been solved.\(^{(17)}\) In contrast, only the protease domain of hLon has been determined.\(^{(18)}\)
Figure 1.1. Domain Layout of Lon protease monomer. The N domain is implicated in substrate recognition. The ATPase domain where ATP hydrolysis takes place is part of the AAA+ module which is highly conserved in members of the AAA+ family of proteins. The P domain contains the Ser-Lys catalytic dyad where peptide bond cleavage occurs.

The crystal structures of the N-terminal domain of ELon and BsLon are very similar, and align on top of each other very well. They both show two distinct regions: a compact β-sheet rich globular domain, connected by an extended loop to an α-helical domain. The N-terminal domain is poorly conserved amongst different Lon homologs, and the function is unknown, but has been proposed to be involved in oligomerization and substrate detection. The discrepancy in sizes between the bacterial and human homologs is due to a larger N-terminal domain in humans.

Lon, like other ATP-dependent proteases including FtsH, ClpAP, ClpXP, and HslUV, belongs to the AAA+ (ATPases Associated with various cellular Activities) family of proteins. These proteins are defined by a characteristic nucleotide binding domain whose conserved regions include Walker A and B motifs. Lon is unique from the other members, however, in that the ATPase and protease domains are located within each monomeric subunit, making it one of the simplest of ATP-dependent proteases. The ATPase domain, which is highly conserved among Lon homologues, consists of two subdomains, an α/β domain, and an α domain. The α/β domain contains the highly conserved Walker A and B nucleotide binding motifs, as well as the sensor-1 motif and the arginine finger. This domain has been solved in BsLon, but not ELon, and shows a characteristic Rossmann fold typical of proteins that bind nucleotides. The α domain has been solved for both ELon and BsLon, and both are
very similar containing mostly α helices. It contains the sensor-2 motif, which participates in the binding and hydrolysis of ATP.\(^{(19, 24, 27-30)}\)

The protoelytic domain contains the active site serine-lysine dyad used to catalyze peptide bond hydrolysis.\(^{(15)}\) The crystal structures for this domain have been solved in ELon, BsLon, and hLon. The ELon and BsLon homologs are compact and rich in β-sheets and superimpose very well onto each other.\(^{(15, 17)}\) The hLon crystal structure, in contrast, does not superimpose as well.\(^{(15, 17, 18)}\) The secondary structural elements between the three are widely similar, however they appear rotated and shifted from each other. A loop in hLon covers the active site, and this is considered to be a closed inactive form. In ELon and BsLon, this loop is shifted out of the way, leaving the active site in an open and active conformation.

The enzyme oligomerizes into a ring shaped structure, forming a central cavity through which protein substrates are unfolded and translocated to the protoelytic site for degradation. Analytical ultracentrifugation and cryo-electron microscopy studies have proven Lon from yeast, \textit{Saccharomyces cervisiae}, oligomerizes into a ring with seven subunits forming a heptameric ring.\(^{(31)}\) Previously, gel filtration studies indicated bacterial Lon to be a tetramer; however, the crystal structures of bacterial Lon show they oligomerize with six subunits, forming a hexameric ring.\(^{(8, 15, 17, 19)}\) Electron microscopy studies also reveals a hexamer.\(^{(32)}\) The crystal structures of BsLon also indicate formation of a hexamer.\(^{(17)}\) Homology modeling of hLon was compared with yeast Lon and BsLon.\(^{(33)}\) Results indicate hLon models better with BsLon than yeast Lon due to the presence of an extra 250 amino acids in the yeast homolog. As BsLon oligomerizes as a hexamer, it is proposed hLon oligomerizes as a hexamer as well.
AAA⁺ proteases have been shown to induce a conformational change upon ATP binding, including Lon.\(^{34, 35}\) In yeast, under cryo-electron microscopy, Lon exhibits a distinct asymmetry in the absence of nucleotide.\(^{31}\) However, after the addition of nucleotide, a conformational change takes place, forming more symmetric ring-shaped particles. This change has eluded characterization in the absence of full length crystal structures. Conformational changes upon protein or peptide binding have so far been undetected.

**1.2 Function of Lon**

As Lon is an ATPase, it catalyzes the hydrolysis of ATP to ADP and Pi (figure 1.2) as it hydrolyzes protein substrates into small peptides of 5-15 amino acids through a conserved serine-lysine dyad (figure 1.3).\(^{36, 37}\) Lon catalyzes the hydrolysis of ATP even in the absence of peptide or protein substrate, indicating intrinsic ATPase activity, however the rate is stimulated in the presence of substrate.\(^{9, 38, 39}\) Maximal peptide hydrolysis occurs in the presence of ATP, however, other nucleotide triphosphates have been shown to support this activity, as well as a nonhydrolyzable ATP analog, AMPPNP, albeit at a lower rate.\(^{34, 40, 41}\). This indicates only binding of nucleotide is required for minimal proteolysis, while hydrolysis increases proteolysis rate. The products resulting from the peptide cleavage are the same irregardless of nucleotide used.
Figure 1.2. *Hydrolysis of ATP to ADP and P$_i$ by Lon.* The α, β, and γ phosphates are indicated in orange.

Figure 1.3. *Proposed mechanism for peptide bond cleavage in Lon.* Upon binding substrate, the active site serine acts as a nucleophile and attacks the carbonyl carbon of the scissile bond, resulting in the first tetrahedral intermediate. This intermediate collapses and the C-terminal product is released, yielding the acyl-enzyme intermediate. An activated water molecule attacks the carbonyl carbon, resulting in the second tetrahedral intermediate. This intermediate again collapses, the N-terminal product is released, and the active site is regenerated to pick up a new substrate.
The coordination and stoichiometry between ATP hydrolysis and proteolysis is unknown. Figure 1.4 shows the structure and simplified mechanism of protein degradation. It is proposed that ATP binds first and activates the proteolytic site. A conformational change takes place to allow for unfolding and translocation of the protein substrate through the central cavity to the proteolytic site.\(^{9, 42}\) The peptide bond is then cleaved followed by ATP hydrolysis, which leads to inactivation due to ADP being bound. Binding of additional protein substrates promotes the release of ADP (the rate limiting step) allowing another ATP molecule to bind and the cycle to repeat.\(^{9}\)

**Figure 1.4. Structure of Lon and simplified mechanism of proteolysis.** In addition to ATP hydrolysis, Lon unfolds and translocates protein substrate through the central cavity to the proteolytic site where it is degraded into small peptide products.
1.3 Substrates of Lon

The ATPase activity of Lon protease is stimulated by protein substrates.\(^{43}\) ELon has been found to degrade many proteins including \(\lambda N\), RcsA, CcdA, SulA, as well as artificial substrates such as casein, and hemoglobin.\(^{8, 43-46}\) Many of these interactions have not been studied at the molecular level. Some of the substrates adopt secondary structures, while others are degraded by other proteases, indicating they are not specific for Lon. The \(\lambda N\) protein, however, is a bacteriophage protein which has been found to be a physiological substrate of ELon. It does not adopt any secondary structure in the absence of RNA.\(^{47, 48}\) The degradation profile of the \(\lambda N\) protein by ELon has been identified as shown in figure 1.5.\(^{43}\) Therefore, it has been identified as a desirable substrate for monitoring protein cleavage in Lon.

\textbf{Figure 1.5. Degradation profile of \(\lambda N\) protein.} Lon cleavage sites are indicated by red lines.\(^{43}\)

While hLon degrades some of the ELon substrates, albeit with different efficiencies, it also degrades its own substrates, including MPP\(\alpha\) and \(\beta\), StAR.\(^{49, 50}\) The MPP proteins form heteromeric complexes which are then resistant to degradation.\(^{49}\) For this reason, they are not good substrates to monitor hLon proteolysis. StAR, \textit{Steroidogenic Acute Regulatory protein}, is a monomeric protein responsible for
translocating cholesterol to the mitochondrial inner membrane. It is an endogenous substrate of hLon, and its cleavage sites have been determined.\(^{(49)}\) It has also been shown that hLon can degrade \(\lambda\)N.\(^{(51)}\) There has been no consensus sequence identified which allows the prediction of substrates and cleavage sites by Lon. It has been found in both of these substrates, however, that Lon generally cleaves between hydrophobic residues without apparent preference for secondary structure. It does not cleave between all hydrophobic residues, indicating a preference for which residues it does cleave. Therefore we are interested in determining a recognition site for cleavage, which could enable identification of other possible substrates.

1.4 Role of Lon in protecting the cell

Lon function is critical for maintaining the structure and integrity of mitochondria in eukaryotes. Mitochondria is a major place for oxidative stress to occur, which damages proteins.\(^{(52, 53)}\) Oxidatively damaged protein accumulation leads to mitochondrial dysfunction and cell death.\(^{(54-61)}\) Studies have indicated that Lon degrades oxidized aconitase at a much higher rate than it degrades native aconitase, implicating the ability of Lon to degrade oxidatively damaged proteins.\(^{(62)}\) Other studies in yeast have shown that cells without Lon accumulate more oxidized proteins than wild-type (WT) cells. As shown in figure 1.6, in yeast mitochondria that is deficient of Lon, large protein aggregate accumulates and damages mitochondria compared to normal mitochondria.\(^{(63)}\) In human cells deficient in Lon, an increase in oxidized mitochondrial aconitase was detected, as well as electron dense inclusion bodies, which suggests the presence of oxidized proteins.\(^{(64)}\) These cells also had an increased rate of apoptosis compared to WT cells. Cells treated with \(\text{H}_2\text{O}_2\) to induce oxidative stress showed an increase in the amount
of Lon. These studies indicate a possible role of Lon in protecting mitochondria from oxidative stress.

**Figure 1.6** *Normal and Lon-deficient mitochondria.*

Eukaryotic Lon is made in the cytosol as a precursor that carries an N-terminal mitochondrial targeting sequence. This sequence directs Lon translocation across the mitochondrial outer and inner membranes and into the matrix. Once in the matrix, this targeting sequence is cleaved off, resulting in a mature form of the enzyme. Lon is one of only three proteases within the mitochondrial matrix, along with ClpXP and m-AAA. It has been shown that Lon and ClpXP have different substrate specificities. As such, mutations within Lon that increase or decrease its ability to degrade protein could affect its ability to protect mitochondria, leading to mitochondrial dysfunction, and hence mitochondrial disease. Elucidation of the activities of mutations could provide insight.
into the structure and function of Lon, as well as the design of strategies to prevent mitochondrial related disease.

1.5 Monitoring peptide bond hydrolysis

Previous limitations in the study of Lon were partially due to the lack of a peptide substrate that interacts with the enzyme in a comparable manner to a protein substrate. Small synthetic peptides had previously been used to study the protease activity; however they lacked the ability to mimic the enhanced ATP hydrolysis observed in the presence of protein substrates. A synthetic peptide substrate was developed in our lab based on the cleavage profile of the λN protein by ELon. As seen in figure 1.7, this peptide, FRETN8998, derived from amino acids 89-98 of λN, is an 11 amino acid long peptide containing a single cleavage site and no secondary structure. The Lon cleavage site of Cys93 and Ser94 is contained in the middle of this peptide. The length of this peptide was made intentionally so that the products resemble the typical size of protein degradation products. This peptide also stimulated ATP hydrolysis comparable to a protein substrate. Fluorescent resonant energy transfer (FRET) was utilized such that a fluorescent donor, anthranilamide (abz), was engineered on the C-terminal, and the fluorescent quencher, 3-nitrotyrosine (Y-NO₂), was engineered on the N-terminal. The emission wavelength of the donor and the excitation wavelength of the quencher are similar, such that the emission of the donor is the correct energy to excite the quencher, thereby inhibiting the emission of the donor when they are in close proximity. The cleavage site was positioned between these groups, such that when cleaved, the donor and quencher separate, and the dyes are no longer close enough to be quenched, leading to an increase in emission of the donor over time. Therefore, the peptidase activity of
Lon can be measured in real time by excitation at 320 nm and emission at 420 nm using fluorescence spectroscopy. The fluorescent trace contains a short lag phase, followed by a linear phase, and then a plateau of fluorescence which indicates substrate depletion. The slope of the linear phase corresponds to the rate of peptide degradation. One complication resulting from the fluorescent peptide was the occurrence of inner filter effect. At high concentrations of FRETN8998, nitrotyrosine absorbs anthranilamide fluorescence through an inter-molecular mechanism rather than an intra-molecular mechanism. To overcome this deficiency, the use of a non-fluorescent peptide was incorporated, 8998Bz. This peptide substitutes a nonfluorescent benzoic acid moiety for the anthranilamide at the C-terminal, and a tyrosine for the 3-nitrotyrosine at the N-terminal. This peptide is degraded with identical kinetics by ELon. As such it was used to make a mixture of 10% fluorescent FRETN8998, and 90% nonfluorescent 8998Bz, allowing the monitoring of kinetics at high concentrations.
**Figure 1.7** *Explanation of the continuous fluorescent peptidase assay.* FRETN8998 peptide (residues 89-98 of λN protein) contains a fluorescent donor, anthranilamide (Abz), on the carboxy terminus and a fluorescent quencher, 3-nitrotyrosine (Y-NO2), on the amino terminus. Upon ATP-dependent cleavage by Lon between cysteine and serine, the donor and quencher separate, and a concomitant increase in fluorescence is seen.\(^{38}\)

![Diagram of FRETN8998 peptide](image)

<table>
<thead>
<tr>
<th>Time, sec</th>
<th>Relative intensity (I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>200</td>
<td>2</td>
</tr>
<tr>
<td>300</td>
<td>3</td>
</tr>
<tr>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td>500</td>
<td>5</td>
</tr>
</tbody>
</table>

Excitation = 320 nm
Emission = 420 nm

This small substrate has been used to probe the activity of ELon through fluorescence spectroscopy, providing a $k_{\text{cat}}$ of 9 sec\(^{-1}\) and $K_m$ of 102 μM. It has also been shown to enhance ATP hydrolysis similarly to that of the full length protein. Previously, peptides containing other ELon cleavage sites based on the λN sequence were also developed.\(^{71}\) Most of the peptides have similar $k_{\text{cat}}$ values, except for a peptide developed from the N-terminal end containing residues 11-21. This peptide showed a large reduction in $k_{\text{cat}}$, indicating difference in cleavage. Conversely, the peptides have a higher $K_m$ than the FRETN8998, indicating differences in binding. Therefore, it is the FRETN8998 that is routinely used to characterize ELon. However, this substrate only has
one cleavage site, and *in vivo*, Lon cleaves proteins that have multiple cleavage sites. Therefore, we want to probe the activity of Lon with a full length protein substrate.
CHAPTER 2

EVALUATING THE CONTRIBUTIONS OF THE N- AND C-TERMINAL OF λN TOWARDS NUCLEOTIDE-DEPENDENT DEGRADATION BY \textit{E. coli} LON PROTEASE
2.1 INTRODUCTION

The work presented in this chapter has contributed to a publication.\(^{(72)}\)

Lon is thought to be a processive machine that catalyzes the cleavage of protein substrates through coordinated repeated cycles of ATPase and peptidase without generating partially digested substrate intermediates. The connection between the ATPase and peptidase is still unclear. In a previous study, ELon degraded heat denatured CcdA faster than non heat denatured CcdA.\(^{(45)}\) This indicates cleavage of protein substrates by Lon requires unfolding and this is thought to be facilitated by the hydrolysis of ATP (figure 2.1). The unfolding step complicates the connection between ATPase and peptidase. By using an unstructured substrate, such as λN, the unfolding step is nonexistent, and thus characterization of the enzyme becomes less complicated.

**Figure 2.1** 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 translocated through the central cavity to the proteolytic site (step 3) where proteolysis takes place (step 4). By using and unstructured substrate, such as λN, the unfolding event is nonexistent and step 2 is bypassed.
In many other ATP-dependent proteases, there have been consensus sequence tags which allows for prediction of substrates, such as the SsrA tag in ClpXP.\(^{(73)}\) There has been no consensus sequence determined for Lon, but some preference has been found for cleavage after hydrophobic residues.\(^{(43, 45, 49, 74)}\) Previously, our lab utilized the FRET approach employed for the FRETN8998 to generate a library of peptides containing different cleavage sites in λN.\(^{(71)}\) All peptides were cleaved ATP-dependently by Lon, albeit with different kinetics. It was found, however, that FRETN8998 was the best substrate for Lon. Given that cleavage in all sites in λN occurs through the same mechanism, all the peptides should be degraded with the same kinetics. As they are not, the difference is assigned to specificity of substrates; Lon must recognize and bind the FRETN8998 peptide better than it recognizes and binds the others. Due to the position of the sites within the full length λN, it follows that something in the C-terminus, and not the N-terminus of λN leads to recognition. As such, λN mutants were generated to determine the effect of the C-terminal on protein degradation. It was found that truncated mutants were still degraded, however they were degraded slower than WT λN.\(^{(71)}\) This indicates that ELon recognizes something in the c-terminal; however there must be some other recognition site along the λN protein.

Lon degrades protein in an ATP-dependent manner. As seen in figure 2.2, AMPPNP, adenylyl imidodiphosphate, is an ATP analog that is nonhydrolyzable due to the position of an imido linker. As such, it was hypothesized that AMPPNP would not be able to support peptide hydrolysis. However, it has been shown that λN is still degraded in the presence of AMPPNP, and the same peptide products were generated as with ATP.\(^{(43)}\) This indicates that protein degradation requires the binding of nucleotide, and
not necessarily the hydrolysis. Using the fluorogenic peptide substrate, our lab has shown that the substitution of AMPPNP for ATP reduces the efficiency of cleavage\(^{(41)}\). As seen in figure 2.3, \(\lambda N\) was degraded faster in the presence of ATP compared to AMPPNP, indicating that AMPPNP slows down the reaction\(^{(72)}\). The band indicated by the star is a degradation product seen only in the AMPPNP-dependent reactions. It is proposed that this band corresponds to partially digested \(\lambda N\), indicating AMPPNP affects the processivity of degradation. However, figure 2.3 is unable to indicate where \(\lambda N\) is being cleaved to produce this product.

**Figure 2.2** *Structure of adenylyl imidodiphosphate, AMPPNP.* The presence of the imido linker in red between the \(\beta\) and \(\gamma\) phosphates renders it incapable of attack and subsequent hydrolysis of the \(\gamma\) phosphate.
Figure 2.3 Degradation of λN by ELon in the presence of ATP or AMPPNP. 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. Star indicates a degradation product only seen with AMPPNP.

Since the peptides generated showed differences in kinetics, it would follow that ELon has a preference for one site in λN over another. It is unknown if ELon cleaves all sites at once, or if it has a preference for one terminus before another. Up to this point, there have been no full length substrates generated that would allow for the elucidation of degradation. In order to characterize the interaction of ELon with protein substrates, full length λN needs to be generated and purified. Previously, this was done by employing the use of a 6x his-tag, which is a well-known technique in purifying proteins. It involves engineering six histidines to either the N-terminal or the C-terminal of the protein of interest, which can then bind to nickel on a nickel column. The column can then be washed to separate off unwanted impurities leaving only the protein of interest bound to the nickel. Protein can then be eluted by competition with imidazole. The extent to which the histidines affect the degradation of λN by ELon is unknown.
2.2 MATERIALS AND METHODS

2.2.1 Materials.

Restriction endonucleases were purchased from Promega or New England Biolabs. Oligonucleotides were custom-synthesized by IDT, Inc. Solvents, buffers, chromatography resin, antibiotics, and culture media were purchased from Fisher Biotechnology or Sigma/Aldrich. Plasmids used for protein expression and competent cells were purchased from Invitrogen and Novagen. λNΔ1-34 was synthesized by GenScript. [α\(^{32}\)P]ATP was purchased from Perkin-Elmer Life Science. PEI cellulose TLC plates were purchased from Fisher Biotechnology or Sigma/Aldrich.

2.2.2 General Methods.

WT ELon purification procedures were performed as described previously\(^{(38)}\). 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.

2.2.3 λN001, λN002, and λN006 plasmid construction.

A gene for λN001, λN002 and λN006 was synthesized by Genscript in pUC57 with a cysteine inserted at the 26 position for λN001, the 42 position for λN002, and 99 position for λN006, all other cysteines mutated to leucine and all tryptophans mutated to phenylalanine. This gene and the pCOLA-Duet vecter were digested with BamHI and HindIII restriction enzymes, purified and ligated using the Ligate-IT Rapid Ligation Kit (USB) and transformed into DH5α competent cells (Invitrogen). The plasmids, each containing an N-terminal 6x His-tag, were transformed into the BL21(DE3) cell strain.

2.2.4 Purification of His-tagged λN.
C-His-λN (λN with a c-terminal his-tag), N-His-λN (λN with an n-terminal his-tag), N-His-λNΔ99-107 (a c-terminal truncated mutant of λN lacking residues 99-107 and with an n-terminal his-tag), λN001, λN002 and λN006 were all purified as previously described.\(^\text{(71)}\) Cells with the plasmid for each in BL21(DE3) were grown to OD\(_{600}=0.6\) in 50 μg/mL kanamycin in SB (superbroth) and induced for 2 h with 1 mM IPTG. The cells were pelleted by centrifugation and lysed by adding 5 mL of lysis buffer per gram of cells [lysis butter: 100 mM sodium phosphate (pH 8.0), 10 mM Tris, 1 mM β-mercaptoethanol and 8 M urea] and stirring for 45 minutes. All cell debris was pelleted by centrifugation. His-tagged λN was purified using a previously published procedure.\(^\text{(71)}\) The protein was quantitated using the Bradford assay.

2.2.5 λN001, λN002 and λN006 dansyl labeling.

The purified λN001, λN002 and λN006 were then 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. The reaction was quenched the next day with β-mercaptoethanol, and dialyzed into λN storage buffer (20 mM Tris, 50 mM NaCl, 1 mM β-mercaptoethanol, 20% glycerol) to remove unreacted dye. The labeled proteins, dansylλN001, dansylλN002, and dansylλN006 were then quantitated using the Bradford assay.

2.2.6 Western blot analysis of λN degradation.

λN degradation assays contained 50 mM HEPES (pH 8), 75 mM KOAc, 15 mM Mg(OAc)\(_2\), 5 mM DTT, 65 μM C-His-λN or N-His-λN, 5 mM AMPPNP and the reaction was initiated with 6 μM WT ELon at 37°C. At 0 and 30 minutes, 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 hour. After extensive washing with TBST, the membrane was incubated for 1 hour in goat anti-mouse IgG alkaline phosphatase secondary antibody (1:3000, Sigma-Aldrich, St. Louis, MO, USA). Protein bands were visualized using BCIP/NBT.

2.2.7 Characterization of the AMPPNP-dependent degradation of dansyl\(\lambda\)N

\(\lambda\)N degradation assays contained 50 mM HEPES (pH 8), 75 mM KOAc, 15 mM Mg(OAc)\(_2\), 5 mM DTT, 65 \(\mu\)M dansyl\(\lambda\)N, 5 mM nucleotide and the reaction was initiated with 6 \(\mu\)M WT ELon at 37°C. At 0, 30, and 60 minutes, reactions were quenched with 5x SDS-PAGE loading dye and incubated at 100°C for 1 min. Aliquots were run on a 15% SDS-PAGE gel and viewed under UV transillumination using a Bio-rad Gel Doc 2000 to visualize undigested substrates and digested products.

2.2.8 \(\lambda\)N\(\Delta99-107\)NH plasmid generation.

Non-histagged \(\lambda\)N deletion mutant (\(\lambda\)N\(\Delta99-107\)NH) was generated using basic cloning techniques. PCR was used to amplify the \(\lambda\)N\(\Delta99-107\) insert from WT\(\lambda\)N DNA. The forward primer was Nde12N (5'-ATACATATGGATGCACAA-3') and reverse primer \(\lambda\)N\(\Delta99-107\)rev (5'-ATGCTATAAGCTTCTATTTCTGGCGTCCACT-3'). The PCR product was gel purified using a 1.5% agarose gel. The pET24c(+) vector (Novagen) and the purified PCR product was digested with NdeI and HindIII. The digested vector and insert were gel purified and ligated using the Ligate-IT Rapid
Ligation kit (Affymetrix) and transformed into XLI Blue supercompetent cells (Stratagene). The λN deletion mutant was verified by DNA sequencing analysis and transformed into BL21 (DE3) E. coli cell strain.

2.2.9 Purification of non-histagged λN proteins.

Non-histagged λN, λNNH and λNΔ99-107NH, in BL21(DE3) E. Coli cell strain, were grown to an OD$_{600}$=0.7 in 30 μg/mL kanamycin in SB (superbroth) and induced for 2 h with 1 mM IPTG. The cells were pelleted by centrifugation and lysed by adding cell lysis buffer (50 mM Tris pH 7.5, 5 mM EDTA, 0.2M sodium chloride, 2 mM beta-mercaptoethanol, 20% glycerol and 0.005% tween 20). All cell debris was pelleted by centrifugation. The cell pellet was then resuspended, washed with lysis buffer and pelleted by centrifugation. The resultant cell pellet was then resuspended in λN lysis buffer (100 mM sodium phosphate pH8, 10 mM Tris, 1 mM beta-mercaptoethanol, 8M urea). Cells were again pelleted and the cleared lysate was dialyzed into λN P11 buffer (50 mM Tris pH 8, 1 mM EDTA, 7 mM beta-mercaptoethanol) with 0.1M potassium acetate. A white precipitate was formed during dialysis and removed by centrifugation. The λN protein was then loaded onto an activated phosphocellulose P11 column and washed with λN P11 buffer with 0.1M potassium acetate, then washed with λN P11 buffer with 0.75M potassium acetate. The protein was then eluted with λN P11 buffer with 1M potassium acetate. The λN protein was then analyzed by SDS-PAGE on a 15% acrylamide gel and stained with Coomassie Brilliant Blue to assess purity. The purified fractions were pooled, and protein was quantitated using Bradford dye with his-tagged λN as a standard.

2.2.10 λN degradation assays.
λN degradation assays contained 50 mM Tris-HCl (pH 8.1), 15 mM Mg(OAc)_2, 5 mM DTT, 10 μM N-His-λN, λNNH, N-his-λNΔ99-107, λNΔ99-107NH or λNΔ1-34 (λN with no his tag but lacking residues 1-34), 5 mM ATP, and the reaction was initiated with 1 μM WT ELon. At 0, 4, 6, and 10 minutes, or 0, 5, and 10 minutes, 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 12.5% SDS-PAGE gel and stained with Coomassie Brilliant Blue to detect the protein. ELon and λN bands were quantified with the program ImageJ. The amount of λN remaining at each time point was normalized using the amount of ELon in each time point. The initial time point was then set to 1 and each remaining time point was normalized to this to determine the relative amount of λN remaining. Time points were plotted out and set to a single exponential decay. Each reaction and quantification was done at least in triplicate. Error bars represent experimental deviations among the different trials. Time points were plotted out and set to a single exponential decay (eq 2.1) to determine the rate constant of degradation.

\[ Y = \exp(-k_1 t) \]  

(eq 2.1)

where \( Y \) is the amount of λN, \( k_1 \) is the rate constant for degradation in sec\(^{-1}\), and \( t \) is the time in minutes.

2.2.11 Radiolabeled ATPase assay.

λN006 and N-His-λNΔ99-107 protein binding to WT ELon was detected by ATP hydrolysis at 37°C by radiolabeled ATPase assay as previously described.\(^{(24)}\) Briefly, each ATPase assay reaction mixture contained 50 mM Tris-HCl (pH 8.1), 5mM Mg(OAc)_2, 2 mM DTT, 500 μM ATP and varied concentrations of λN006 or N-His-λNΔ99-107 from 0.75-25 μM. Reactions were initiated by 100 or 200 nM WT ELon
monomer. Subsequently, 5 µL aliquots were quenched at various time points from 0 to 8 minutes 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 x 20 cm) and developed in 0.3 M potassium phosphate buffer (pH 3.4). Radiolabeled ADP 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, the concentration of ADP product obtained at each time point was corrected using an internal reference as shown in eq 2.2

\[
[ADP] = \left( \frac{ATP_{\text{div}}}{ATP_{\text{div}} + ADP_{\text{div}}} \right) [ATP] \quad (\text{eq } 2.2)
\]

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 \( k_{\text{obs}} \) values were fit to eq 2.3 where \( k_{\text{obs}} \) is the observed rate constant, \( k_{\text{obs,max}} \) is the maximal rate, B is the \( \lambda N \) concentration, \( K_d \) is the Michaelis-Menten constant and \( M \) is the start point.

\[
k_{\text{obs}} = \frac{k_{\text{obs,max}}[B]}{K_d + [B]} + M \quad (\text{eq } 2.3)
\]

2.3 RESULTS AND DISCUSSION

2.3.1 AMPPNP-dependent degradation of his-tagged \( \lambda N \).

As seen in figure 2.3, N-His-\( \lambda N \) was degraded better in the presence of ATP versus AMPPNP. Also deduced from figure 2.3 is the presence of partially digested N-his-\( \lambda N \) that was detected in the AMPPNP-dependent reaction, but not the ATP-dependent reaction. To determine what the partially digested N-his-\( \lambda N \) corresponds to, and to ensure the product generated was not due to the presence of the his-tag, full length \( \lambda N \) containing a his-tag at the C-terminal (C-his-\( \lambda N \)), and N-his-\( \lambda N \) were digested with ELon
in the presence of 5 mM AMPPNP and subjected to western blot analysis with anti-his-tag antibodies. Figure 2.4 reveals that a band was detected after 30 minutes when using C-his-\(\lambda\)N. As the antibody reacts with a his-tag, this indicates a his-tag is still present in this band. Due to the placement of the his-tag at the C-terminal, the band contains the C-terminal, indicating the N-terminal of \(\lambda\)N was cleaved off. Likewise, the presence of a band reacting with a his-tag placed at the N-terminal in N-his-\(\lambda\)N indicates the his-tag is still present and therefore the C-terminal has been cleaved off. The presence partially digested \(\lambda\)N generated regardless of the location of a his-tag at the N- or C-terminal of \(\lambda\)N indicates a lack of directionality in cleavage. Compared to the degradation profiles of \(\lambda\)N in the presence of ATP, the results presented in this section indicate that the catalytic efficiency as well as the extent of processivity of \(\lambda\)N 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 degradation of unstructured protein substrate. The fact that both his-tagged C- and N-terminal were detected under identical time points indicates that no obligatory order of peptide bond cleavage occur in the degradation of reactions.
**Figure 2.4** Western blot analysis of AMPPNP-dependent C-his-λN and N-his-λN degradation. Proteins were digested in the presence of AMPPNP and a western was run with an antibody against a his-tag. λN protein (65 µM) was digested with 6 µM ELon and quenched at the time points indicated. Arrows point to his tag labeled degradation products.

2.3.2 **Generation of fluorescent λN constructs to evaluate nucleotide dependent proteolysis.**

As the degradation of λN with a his-tag at either terminal showed degradation products, it is postulated that ELon has no directionality preference for cleavage. To investigate this possibility further, three λN constructs were generated as shown in figure 2.5. These three constructs all contained an N-terminal his-tag, but differed by the location of a cys inserted upstream or downstream of the ELon cleavage site at residues A30-K21, L40-N41, and L93-S94. Dansyl is a fluorescent dye that can be attached to the thiol side chain of cysteine through a reaction with dansyl aziridine (Invitrogen). The fluorescence of the dansylated protein can be visualized and quantified by UV transillumination on an SDS-PAGE gel. Capitalizing on this technique, the three constructs were generated and dansylated. DansylλN001 contains a dansyl label that is 6
residues upstream from the E Lon cleavage site at A30-K31, and is the furthest away from the C-terminal. DansylλN002 contains a dansyl label that is 1 residue downstream from the E Lon cleavage site L40-N41, and is 16 residues closer than dansylλN001 to the C-terminal. The fluorescent label of DansylλN006 is located 6 residues from the E Lon cleavage site at L93-S94, and is the closest to the C-terminal. Each dansyl label is located at the vicinity of an E Lon cleavage site, which differs by their respective locations from the C-terminal containing residues 99-107. All three constructs had the tryptophans mutated to phenylalanine so as to avoid false data due to FRET between tryptophan and dansyl, and all cysteines mutated to leucines to ensure the dansyl only reacted at one place along the protein. The $k_{cat}$ and $K_m$ values of the fluorogenic decapeptide analogs containing different regions of λN and the respective E Lon cleavage site have been previously determined (71). Despite differences in the peptide sequences and $K_m$ values, the three aforementioned sites have comparable $k_{cat}$ values (11.3 sec$^{-1}$, 9.5 sec$^{-1}$, and 9 sec$^{-1}$) (71). Therefore we anticipate that the kinetic parameters that limit the turnover of all three sites in λN will also be comparable such that any differences detected in the kinetic experiments described below will not be attributed to the intrinsic differences in the peptide bond cleavage efficiency. To ensure that the dansyl label or the placement of the his-tag did not interfere with substrate degradation, control experiments were conducted to verify that the rates of labeled and unlabeled λN were similar, as shown in figure 2.6.
Figure 2.5 *Amino acid sequences of the λN proteins used to evaluate the contribution of the C-terminal versus N-terminal.* All sequences contain the same dansyl fluorescent dye (labeled in blue) which can be used to view protein bands under UV illumination. Lon cleavage sites near dye locations are underlined and in red.

**Dansyl λN001**


**Dansyl λN002**


**Dansyl λN006**


Figure 2.6 *Graphical representation of amount of λN at each time point.* λN adducts dansylλN001 (red), λN001 (dark blue), dansylλN006 (light blue), λN006 (green), λN002 (gray), λN002, (pink), C-His- λN (black) and N-His- λN (orange) are degraded similarly. This ensures the placement and labeling of the Cys or the his-tag does not interfere with degradation.
2.3.3 Evaluation of the AMPPNP-dependent degradation of dansylated λN.

To further evaluate the contribution of each terminal of λN to AMPPNP-dependent degradation, the three dansylated λN proteins were digested with WT ELon in the presence of 5 mM AMPPNP (figure 2.7). Comparing the three time courses reveals that truncated λN lacking the N- or the C-terminal as well as fully digested peptide products were generated in each reaction time point. Since the fluorescent dye was attached to different regions of the λN, comparing the presence of fluorescent bands generated from each ELon digestion reaction reveals which region is cleaved. According to figure 2.7 fluorescence band 1 was detected in the reaction containing dansylλN001 or dansylλN002 but not in dansylλN006. Since dansylλN006 was labeled with the fluorophore at position 99 from the amino terminal, it follows that fluorescence band 1 corresponds to a partially digested λN band lacking the C-terminal. Fluorescence band 2 is detected in dansylλN006 and maybe slightly in dansylλN002 but not in dansylλN001. As dansylλN001 was labeled with the fluorophore at position 26 from the amino terminal, fluorescence band 2 corresponds to a partially digested λN product lacking the N-terminal. In addition to fluorescence bands 1 and 2, fluorescently labeled products corresponding fully hydrolyzed peptide products are also detected in each time point. The intensity of fluorescence bands 1 and 2 accumulated over 30 minutes and then decreased in 60 minutes, suggesting that the partially digested λN were ultimately degraded into smaller products. Taken together, these results indicate that in the presence of AMPPNP, λN could be degraded by ELon, albeit with partially digested intermediates, indicating a non-processive process. As partially digested λN lacking an N- or C-terminal
were detected independently at the 30 minute and 60 minute time point, this suggests that peptide bond cleavage in ELon does not adopt a directional or sequential order.

Figure 2.7 Degradation of dansyl-\(\lambda\)N001, dansyl-\(\lambda\)N002 and dansyl-\(\lambda\)N006 in the presence of AMPPNP. Three different regions of \(\lambda\)N were labeled with dansyl and degraded by WT ELon. Degradations were monitored by 15% SDS-PAGE and viewed under a UV transilluminator. Fluorescence bands 1 and 2 indicate degradation products seen from differently labeled regions of \(\lambda\)N.

2.3.4 Degradation profiles of WT and truncated \(\lambda\)N

As described above, truncated N-his-\(\lambda\)N lacking residues 99-107 were 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.\(^{(71)}\) By contrast, the contribution of the N-terminal of \(\lambda\)N is not known. Due to the perceived lack of directionality in cleavage, it is unknown if an N-terminally truncated mutant will also be degraded similarly to full length \(\lambda\)N. As such, the degradation profiles of ATP-dependent degradation of N-his-\(\lambda\)N, truncated \(\lambda\)N lacking the C-terminal (N-his-\(\lambda\)N\(\Delta\)99-107) and
truncated λN lacking the N-terminal and any his-tag (λNΔ1-34) were compared. Figure 2.8 shows the degradation profiles of ELon degrading full length N-his-λN, λNΔ1-34 and N-his-λNΔ99-107 in the presence of saturating [ATP] (5 mM) under identical conditions. Within 5 minutes into the reaction, full length N-his-λN and λNΔ1-34 were mostly degraded; but N-his-λNΔ99-107 was still detected. This indicates the C- but not N-terminal of λN contributes to the degradation efficiency of the substrate. As such, there is no recognition tag in the N-terminal that ELon uses to identify substrates. However, as the C-terminally truncated λN mutant was still degraded, there must be a recognition site somewhere between residues 34 and 99 of λN that has not yet been determined.

**Figure 2.8** *Compare the time courses of λN deletion mutant degradations by WT ELon.* Purified wild type N-his-λN and truncated λN lacking residues 1-34 (λNΔ1-34), or 99-107 (N-his-λNΔ99-107) were digested with ELon in the presence of 5 mM ATP and quenched at the times indicated. The progress of the degradation reactions was monitored by 12.5% SDS-PAGE as described in Materials and Methods.
2.3.5 Evaluate the effect of a his-tag on degradation of λN.

To ensure the degradation is not affected by the presence of a his-tag, full length N-his-λN and λNNH were degraded, as well as N-his-λNΔ99-107 and λNΔ99-107NH. According to figure 2.9 and table 2.1, the ATP-dependent degradation of the full length λN with and without a his-tag are comparable, as are the λNΔ99-107 with and without a his-tag. Therefore, the addition of a his-tag to λN does not affect protein degradation. However, the overall rates of degradation show the truncated mutant is two-fold lower than the WT λN, indicating the truncated mutant is degraded with less efficiency.

**Figure 2.9** Graphical representation comparing effect of a his-tag on amount of λN left over time. N-his-λN (red), N-his-λNΔ99-107 (blue), WTλNNH (green), and λNΔ99-107NH (black) were degraded by WT ELon in the presence of 5 mM ATP. Time points were plotted and set to eq 2.1 describing a single exponential to yield the rate constants shown in Table 2.1.
Table 2.1 Kinetic parameters for λN cleavage by ELon.

<table>
<thead>
<tr>
<th>λN variant</th>
<th>( k_1, \text{(sec}^{-1}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-his-λN</td>
<td>0.11 ± 0.008</td>
</tr>
<tr>
<td>N-his-λNΔ99-107</td>
<td>0.06 ± 0.006</td>
</tr>
<tr>
<td>WTλNNH</td>
<td>0.15 ± 0.004</td>
</tr>
<tr>
<td>λNΔ99-107NH</td>
<td>0.07 ± 0.002</td>
</tr>
</tbody>
</table>

2.3.6 Evaluate the contribution of residues 99-107 in the binding of λN.

Since the C-terminal containing residues 99-107 contributes to the degradation efficiency of λN, it is likely the binding of the truncated mutant N-his-λNΔ99-107 to ELon is reduced. As it has already been proven that the position of a his-tag and cysteine does not affect degradation, λN006 was used to determine full length protein binding to enzyme. To this end, both λN006 and N-his-λNΔ99-107 were titrated against ELon at saturating [ATP] to determine the concentration of each λN needed to attain 50% maximal ATPase stimulation. The ATPase activity was measured using a radioactive assay that quantified the amount of ADP generated over time using eq 2.2 shown in materials and methods. Dividing the observed rate of ATP hydrolysis by [monomeric Lon] yields the observed rate constant at the corresponding [λN]. As shown in figure 2.10, the \( k_{\text{obs}} \) values show a hyperbolic dependency against [λN], which upon fitting the data to eq 2.3 provides a \( K_{d,\lambda N006} \) of 1.4 ± 0.6 μM, and a \( K_{d,\lambda N\Delta 99-107} \) of 5.2 ± 1.7 μM. The \( K_{d,\lambda N\Delta 99-107} \) is ~3.7-fold higher than the \( K_d \) of full length λN006. Since the affinity as well as the degradation efficiency of the truncated substrate was reduced but not abolished, it is concluded that residues in the deleted C-terminal of λN contribute to
substrate degradation. However, additional recognition site(s) not found in either terminal of λN must be present to allow substrate recognition and degradation.

**Figure 2.10** Graphical representation of the stimulatory effect of λN on ATPase activity of ELon. λN006 (blue) and N-his-λNΔ99-107 (orange) were titrated against WT ELon. The $k_{\text{obs}}$ values of ATP hydrolysis were determined at 500 μM ATP containing α[$^{32}$P]ATP in λN006 or N-his-λNΔ99-107 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. Data was fit with eq 2.3 to yield $k_d,\lambda N006$ of 1.4 ± 0.6 μM and $k_d,\lambda NΔ99-107$ of 5.2 ± 1.7 μM.

2.4 CONCLUSION

Previously, it was unknown if ELon has a preference for directionality in cleavage. As degradations with ATP show no partially digested intermediates, it cannot be used to evaluate a preference. However, when comparing the effect of using AMPPNP versus ATP to cleave λN, as shown in figure 2.3, it was shown that AMPPNP distorts processivity, as partially digested intermediates were identified. This provides evidence that AMPPNP can be used to detect directionality. To characterize these
intermediates, a western showed bands that corresponded to a product that containing a
his-tag regardless of the terminal where it was placed. This indicates that ELon cleaves
\( \lambda N \) at both the C-terminal and the N-terminal without a preference for directionality. To
investigate this further, a set of fluorescent substrates were developed, dansyl\( \lambda N \)001,
dansyl\( \lambda N \)002 and dansyl\( \lambda N \)006, which was used spectroscopically to visualize cleavage.
As shown by the data, AMPPNP-dependent cleavage occurred at both the N-terminal and
the C-terminal, further confirming the lack of a preference for a sequential order of
degradation.

A C-terminally truncated mutant of \( \lambda N \) was degraded less efficiently than WT\( \lambda N \)
or an N-terminally truncated mutant, indicating the N-terminus does not play a role in
recognition. The C-terminally truncated mutant of \( \lambda N \) did not stimulate ATPase as well
as full length \( \lambda N \), indicating reduced efficiency in binding. As the FRETN8998 peptide,
which was developed from the C-terminus of \( \lambda N \), was cleaved with a lower K_m than other
peptides developed from the same \( \lambda N \) protein, it follows that proteins containing the C-
terminal should be degraded more efficiently than proteins without it. The data here
supports this hypothesis of the C-terminal being used for recognition and binding of
substrate. However, given the fact that the C-terminally truncated mutant was still
degraded, there must be some other recognition element not yet determined.

The tools generated here show that it is possible to generate fluorescent labels on
protein substrates. The fluorescent analogs generated show better sensitivity than staining
on a gel, as bands not visible on the gel were visible under UV light. This provides
evidence for using fluorescently labeled substrates for characterization of this and other
enzymes in the future.
CHAPTER 3

EVALUATING THE COORDINATION BETWEEN SUBSTRATE
TRANSLOCATION AND PEPTIDE BOND HYDROLYSIS
3.1 INTRODUCTION

The work presented in this chapter has contributed to a paper.\(^{(72)}\)

Fluorescence resonance energy transfer, or FRET, occurs when one molecule is excited (donor), and transfers its emission to excite another molecule (acceptor) when the two are close in proximity. The interaction between the two can be seen when the donor is excited, its emission will decrease as it donates its energy to the acceptor. Likewise, the emission of the acceptor will increase as the two come together. In contrast, the emission of the donor will increase and the emission of the acceptor will decrease when the two separate.\(^{(76, 77)}\) Our lab exploited this technique in the generation of the FRETN8998 peptide substrate utilizing the FRET of an abz/NO\(_2\)Y pair. This technique worked well in determining the activity of ELon on the steady-state timescale, with a \(k_{\text{cat}}\) of 9 sec\(^{-1}\), and a \(K_m\) of 102 μM.

Important aspects of the kinetic mechanism of an enzyme are obtained from steady-state kinetic analysis. From these studies, kinetic parameters can be determined such as \(k_{\text{cat}}, K_m,\) and \(k_{\text{cat}}/K_m\), however they are limited in detecting the first initial interactions of enzyme with substrate. Therefore, pre-steady-state methods, such as stopped-flow fluorescence, have been employed to monitor the reaction on the millisecond time scale, and hence evaluate the timing of the initial events including translocation. Here, these instruments employ two syringes, one filled with enzyme and λN, and one filled with nucleotide. A motor pump is used to rapidly mix the reactants in an observation cell where fluorescence is monitored immediately.

To fully understand the mechanism of Lon protease and how it processes protein substrates, it is important to determine how the translocation of full length substrate and
subsequent peptide bond hydrolysis events are linked. As illustrated in figure 3.1, there are two possible models for λN degradation. In model 1, the whole protein is delivered to the active site before subsequent peptide bond cleavage of all sites. In this model, only fully digested products are generated. The delivery of all sites should occur before any peptide bond cleavage. In model 2, the cleavage of each bond is flanked by a translocation event so that partially digested substrate remains bound to Lon until enough delivery/cleavage events occur to digest the entire protein. These two models can be distinguished by detecting whether peptide bonds in a protein substrate are translocated and cleaved with similar (model 1) or different (model 2) kinetic parameters. As discussed previously, it is also unknown if Lon has a directionality preference to deliver one site before another.
Figure 3.1 *Cartoon illustration for two possible models of protein degradation.* In model one, the whole protein is translocated before subsequent cleavage of all sites (concerted model). In model two, cleavage of each site is flanked by a translocation event while partially digested substrate remains bound during the reaction.

Pre-steady-state experiments of FRETN8998 cleavage with ATP showed a lag phase in the fluorescent time course, indicating the buildup of at least one reaction intermediate. This buildup is thought to involve translocation.\(^{(41)}\) By substituting AMPPNP in for the reaction, the length of this lag phase increases, indicating this buildup is slower. However, Lon cleaves multiple sites within one substrate. The presence of a lag phase has not been determined due to the absence of substrates to monitor it.

As discussed earlier, Lon utilizes a serine-lysine dyad to cleave peptide bond. It has been proven that replacement of the active site serine at 679 in ELon, which is responsible for attacking the scissile peptide bond in a substrate, to an alanine (S679A
ELon), inhibits peptide cleavage; however ATPase enhancement in the presence of the small peptide substrate is similar to WT ELon enhancement.\(^{(78, 79)}\) This provides evidence that S679A is able to bind peptide without cleaving it. Previously, our lab capitalized on this and mutated the active site to a tryptophan (S679W ELon), which showed the same ATPase kinetic characterization as S679A.\(^{(79)}\) By adding a dansyl dye to the small peptide substrate 8998Bz already developed, the well-known FRET between a tryptophan residue (donor) and dansyl (acceptor) was exploited to monitor the translocation of the peptide to the active site.\(^{(77, 79)}\)

Capitalizing on the techniques already generated for the peptide, it was hypothesized that substrates can be developed to monitor the N-terminal versus C-terminal translocation and subsequent cleavage in a full length substrate. As λN is only 107 amino acids and has no secondary structure, it can chemically synthesized with modifications. To this end, two λN substrates were developed to monitor cleavage at both terminals independently, one with an abz/NO\(_2\)Y FRET pair flanking a cleavage site at the N-terminal and one with an abz/NO\(_2\)Y flanking a cleavage site at the C-terminal. To monitor the translocation, the three dansylated λN products generated previously can be used to monitor translocation as the dansyl moiety on the λN FRETs with the tryptophan at the active site of the proteolytically inactive S679W. Using AMPPNP as the nucleotide will slow down the reaction and allow for an evaluation of timing at a slower rate.
3.2 MATERIALS AND METHODS

3.2.1 Materials.
FRλN001 was synthesized by GenScript (Piscataway, NJ), and FRλN006 was synthesized by LifeTein (Hillsborough, NJ).

3.2.2 General Methods.
S679A, S679W and WT ELon purification procedures were performed as described previously. All enzyme concentrations were reported as ELon monomer concentrations.

3.2.3 Monitoring the ATP-dependent degradation of fluorescently labeled λN (FRλN) by steady state kinetics.
Proteinase 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 FRλN001, or FRλN006, (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 3.1.

$$k_{obs} = \frac{k_{cat, \lambda N}[S]}{K_m + [S]} \quad (eq \ 3.1)$$

Where $k_{obs}$ is the observed rate, $k_{cat, \lambda N}$ is the max rate constant of product formation at saturating substrate, $K_m$ is the Michaelis-Menten constant, and [S] is the substrate concentration.
3.2.4 Pseudo-first order time course of FR\(\lambda\)N 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 β. The sample syringes were maintained at 37°C by a circulating water bath. Syringe A contained 10 μM WT ELon, a 10% (90% unlabeled \(\lambda\)N001 or \(\lambda\)N006) mixture of FR\(\lambda\)N001 or FR\(\lambda\)N006 (8 μM for ATP, 10 μM for AMPPNP), and reaction buffer (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 a 10% mixture of FR\(\lambda\)N001, or FR\(\lambda\)N006 (8 μM for ATP, 10 μM for AMPPNP), reaction buffer and 1 mM ATP, AMPPNP, 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 seconds for AMPPNP, 10 seconds 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 micromolar peptide due to complete digestion by trypsin under identical conditions on the stopped-flow. The lag equation, eq 3.2, was fit to the averaged time courses:

\[
F = v_f t - \frac{v_f}{k_{lag}} \times \{1 - \exp(-k_{lag}t)\} \quad (eq\ 3.2)
\]

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

3.2.5 Fluorescence emission scans of dansyl\(\lambda\)N interaction with S679W ELon.

Emission spectra were collected on a Fluoromax 4 spectrofluorometer (Horiba Group) with excitation of the Trp residues at 295 nm. All assays were performed in
microcuvettes (Hellma) with a 3-mm path length. Reactions contained 50 mM Tris-HCl (pH 8.1), 5 mM Mg(OAc)$_2$, 1 mM ATP, 5 µM S679W ELon and varying concentrations of dansyl-glutamic acid, dansylλN001, dansylλN002, dansylλN006, or nonfluorescent protein.

3.2.6 Pseudo first order translocation of dansylλN by fluorescent stopped flow.

Experiments to monitor the translocation of DansylλN with ELon (S679W and S679A) was performed on a KinTek Stopped Flow controlled by the data collection software Stop Flow version 7.50 β with a 0.5 cm path length. The samples 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 (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 2 mM ATP or AMPPNP, 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 80 seconds for AMPPNP, 20 seconds for all others. In addition to monitoring the emission with a 450 nm long-pass filter, emission using a 340 nm band-pass filter monitor to view the changes in Trp fluorescence was monitored. 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 averaged time courses of S679W ELon with AMPPNP and dansylλN were fitted with eq 3.3 describing a double exponential

\[ F = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + C \]  

(eq 3.3)

where \( F \) is relative fluorescence, \( A_1 \) and \( A_2 \) are amplitudes in relative fluorescence units, \( t \) is time in seconds, \( C \) is the endpoint, \( k_1 \) 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.

3.3 RESULTS AND DISCUSSION

3.3.1 Fluorescently labeled λN as a substrate of ELon.

In this study, the abz/NO2Y FRET pair strategy discussed above was utilized to monitor the cleavage of the N-terminal 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 3.2) such that the fluorescence emission signal of abz (λex=320 nm, λem=420 nm) was internally quenched by the nitrotyosine due to proximity of the two moities.\(^{80}\)

Upon cleavage, the two chromophores separate, leading to an increase in the abz fluorescence over time, thereby allowing for the determination of kinetic parameters. These were generated by chemical synthesis without any his-tag. Since tryptophan also internally quenches the fluorescence of abz, the intrinsic tryptophan residues in λN were replaced with phenylalanine to ensure the observed changes in fluorescent signal was attributed to peptide bond cleavage. The cysteines were replaced by leucines to ensure the amino acid sequences were similar to the previously constructed λN adducts used in
dansyl labeling. The resulting N-terminal versus C-terminal 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 $k_{cat}$ and $K_m$ of each ELon cleavage site in λN were determined in fluorogenic decapeptides containing λN sequence flanking each site. The cleavage sites at A30-K31, L40-N41 and C93-S94 have comparable $k_{cat}$ values.

**Figure 3.2** Design of fluorescent λN protein used to monitor peptide bond cleavage by Lon.

Using the fluorogenic assay described above, the $k_{cat,\lambda N,ATP}$ and $K_m$ values for the cleavage of the N-terminal in FRλN001 and C-terminal of FRλN006 at 1 mM [ATP] were determined by fitting the data that related the rate constant of peptide bond cleavage ($k_{obs,\lambda N,ATP}$) with the indicated [substrate]. The plots shown in figure 3.3 fit well with eq 3.1 to provide the values summarized in Table 3.1. Within experimental deviation, the $k_{cat,\lambda N,ATP}$ and $K_m$ values of both substrates are comparable. The detection of comparable
$k_{\text{cat,N,ATP}}$ and $K_m$ values for the cleavage of both sites indicated the same rate-limiting step occurred in both degradation reactions. The $k_{\text{cat}}$ and $K_m$ of ATP-dependent protein cleavage are lower than the $k_{\text{cat}}$ and $K_m$ of the respective fluorescent peptides. The lower $k_{\text{cat}}$ could be attributed to extra time needed to cleave the longer substrate, either due to more time to translocate or to the multiple sites needing cleaving. The difference in $K_m$ could be due to the length of substrates as well, as $\lambda N$ has multiple regions which can interact with ELon compared to the shorter sequences in the peptides substrates.

**Figure 3.3** Steady-state kinetics of ATP-dependent hydrolysis of FR$\lambda N001$ versus FR$\lambda N006$ cleavage by WT ELon. The steady-state rate constants ($k_{\text{obs}}$) of protein hydrolysis with varying concentrations of FR$\lambda N001$ (green), and FR$\lambda N006$ (purple) were determined using the continuous fluorescence based proteinase assay as described in Materials and Methods. The $k_{\text{obs,cleavage}}$ 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 3.1 to yield the kinetic parameters summarized in table 3.1. 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 3.1 Kinetic parameters for protein degradation of FR\(\lambda\)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},N,\text{ATP}}) (sec(^{-1}))</td>
<td>(K_m) ((\mu)M)</td>
<td>(k_{\text{obs},N,\text{ATP}}) (sec(^{-1}))</td>
</tr>
<tr>
<td>FR(\Lambda)N001</td>
<td>0.22 ± 6.12 ±</td>
<td></td>
<td>0.11 ± 0.77 ±</td>
</tr>
<tr>
<td></td>
<td>0.006 ± 0.53 ±</td>
<td></td>
<td>0.02 ± 0.88 ±</td>
</tr>
<tr>
<td>FR(\Lambda)N006</td>
<td>0.15 ± 5.55 ±</td>
<td></td>
<td>0.08 ± 0.87 ±</td>
</tr>
<tr>
<td></td>
<td>0.001 ± 0.50 ±</td>
<td></td>
<td>0.01 ± 0.12 ±</td>
</tr>
</tbody>
</table>

#### 3.3.2 Pre-steady-state stopped flow time courses of peptide bond cleavage in \(\lambda\)N under pseudo first order conditions.

One technical issue associated with the fluorogenic assay described here is the presence of inner filter effect at high substrate concentrations\(^{(70)}\). To overcome this, a substrate cocktail containing 10% of the fluorescently labeled \(\lambda\)N was mixed with 90% of unlabeled \(\lambda\)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\(^{(71)}\). 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 (data not shown) using a substrate concentration that did not suffer from inner filter effect. The substrate cocktail is referred to as \(\lambda\)N001A and \(\lambda\)N006A, respectively.

To gain insights into the rate-limiting step in the ATP- and independently AMPPNP-dependent degradation of \(\lambda\)N001A and \(\lambda\)N006A, the cleavage of the NO\(_2\)Tyr/Abz-labeled sites in \(\lambda\)N001A and independently \(\lambda\)N006A were monitored under excess [substrate] over [Lon] (~2-fold \(K_m,\lambda\)N, \(\lambda\)N; > 10-fold \(K_d,\lambda\)N, \(\lambda\)N) in the presence of
1 mM ATP (figure 3.4 A) or AMPPNP (figure 3.4 B) using a stopped-flow apparatus. An increase in fluorescence with nucleotide indicates cleavage. For control, fluorescence was also monitored without nucleotide and with the inhibitor ADP; no change was detected indicating there was no cleavage. Lag kinetics were detected in all reactions containing nucleotide. Fitting the respective time courses with eq 3.2 yielded the kinetic parameters summarized in table 3.1. The $k_{\text{obs}}N,\text{ATP}$ 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 $k_{\text{lag}}$ values reflect the rate constant for the transition of the slow to the fast phase of the time course. The $k_{\text{lag}}N,\text{ATP}$ values for the ATP-dependent cleavage reactions are between 20- to 35-fold higher than the $k_{\text{lag}}N,\text{AMPPNP}$ obtained for the AMPPNP-dependent reactions. A ~10-fold lower ratio in the $k_{\text{lag}}N,\text{AMPPNP}$ of AMPPNP- versus the $k_{\text{lag}}N,\text{ATP}$ ATP-dependent cleavage of a decapeptide containing residues 89–98 of $\lambda N$ (the labeled site in FR$\lambda N006$) was previously detected.\(^{(41)}\) 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 decapeptide 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 (data not
shown). The lag kinetic profiles detected in the cleavage of λN001A and λN006A 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 λN (41). The reciprocal of $k_{lag}$ provides an estimate for the duration of the lag phase ($\tau$) which is 1.3 sec for λN001A and 1.15 sec for λN006A in the presence of ATP (81). In the presence of AMPPNP, the $\tau$ values are 40 sec and 24 sec for λN001A and λN006A, respectively, agreeing with the observation that AMPPNP slows down the reaction. As further discerned in table 3.1, the $k_{lag}$ and $k_{obs}$ of the ATP-, and independently AMPPNP-dependent cleavage of λN001A are comparable to the values obtained for λN006A, indicating that both terminals of the λN protein are cleaved at the same rate and therefore ELon has no preference for one terminal over the other. This indicates that sites in λN are cleaved simultaneously rather than sequentially pointing towards a more processive method of degradation.
**Figure 3.4** Stopped-flow analysis of nucleotide-dependent FRλN cleavage by WT ELon under excess FRλN conditions. (A) Five micromolar WT ELon was incubated with 8 μM λN001A with no nucleotide (red), 1 mM ADP (purple) or 1 mM ATP (green), or 8 μM λN006A with no nucleotide (dark blue), 1 mM ADP (light blue), or 1 mM ATP (orange). (B) Five micromolar WT ELon was incubated with 10 μM λN001A (green) or 10 μM λN006A (orange) 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 in color is an average of 4 traces, and the fitted curve is shown in black. The traces with ATP and AMPPNP were set to equation 3.2 to determine the kinetic parameters listed in table 3.1. No change in fluorescence was observed without nucleotide or with ADP.

### 3.3.3 Dansylated λN as reporters to monitor translocation of N- versus C-terminal of λN and the utilization of S679W ELon.

In a previous study, the translocation/delivery of the synthetic peptide substrate to the proteolytic site of ELon was detected by FRET, where the tryptophan in S679W ELon served as the donor and the dansylated peptide substrate acted as the acceptor. By using the proteolytically inactive S679W mutant, the system is simplified as proteolysis is decoupled from substrate binding. 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. In this study, the three dansylated proteins from figure 2.6 were utilized to monitor the delivery of the full length λN to the proteolytic site S679W.

To ensure any fluorescence generated when S679W ELon was incubated with dansylated λN was due to the FRET between the two, S679W ELon was incubated with nondansylated λN, and excited at 295 nm. As seen in figure 3.5, no change in fluorescence was detected at the Trp wavelength of 350 nm. However, S679W ELon incubated with 1 mM ATP and 4 μM dansylλN causes a reduction in the fluorescence of Trp when excited at 295 nm (figure 3.6). As there are 4 other Trps present in ELon at W297, W303 and W603, it is possible the changes detected are from the dansyl interacting with these residues. Figure 3.6 shows that the Trp fluorescence was non-specifically quenched by dansyl glutamic acid; however, more decrease was detected in reactions containing any one of the dansylated proteins. To further ensure the changes detected are due to the FRET of S679W ELon and dansyl protein, enzyme and protein were incubated with 1 mM ATP and increasing concentrations of dansylλN. For simplification, figure 3.7 shows S679W interacting with only dansylλN001, however similar results were obtained for dansylλN002 and dansylλN006. Increasing concentrations show a reduction in the fluorescence of Trp with a concomitant increase in the dansyl moiety at 520 nm when excited at 295 nm. These results indicate the interaction between S679W and dansylλN in the presence of nucleotide can be
quantitatively characterized by monitoring the increase in dansyl fluorescence using stopped-flow spectroscopy.

**Figure 3.5** Fluorescence emission scan of S679W ELon with unlabeled λN. Five micromolar S679W ELon was incubated without (blue), or with 4 μM λN001 (green), λN002 (purple), or λN006 (orange) and 1 mM ATP. The sample was excited at 295 nm and the fluorescence emission spectrum was monitored. No changes in tryptophan fluorescence is detected.
Figure 3.6 Fluorescent emission scans of S679W ELon interacting with dansyl\(\lambda\)N. Five micromolar S679W ELon was incubated without (blue), or with 4 \(\mu\)M dansyl\(\lambda\)N001 (green), 4 \(\mu\)M dansyl\(\lambda\)N002 (purple), 4 \(\mu\)M dansyl\(\lambda\)N006 (orange), or 4 \(\mu\)M dansylated glutamic acid (red) and 1 mM ATP. The sample was excited at 295 nm and emission was monitored from 300 to 420 nm. A decrease in tryptophan fluorescence at 350 nm is observed with dansylated glutamic acid; however there is a greater decrease with the dansyl\(\lambda\)N.
**Figure 3.7** Fluorescent emission scans of S679W interacting with dansyl\(\text{N001}\) at increasing concentrations. Five micromolar S679W ELon was incubated with 1 mM ATP and 0 (blue), 1 \(\mu\)M (purple), 2 \(\mu\)M (green), 3\(\mu\)M (orange), or 4 \(\mu\)M (red) dansyl\(\text{N001}\). The sample was excited at 295 nm and the fluorescence emission spectrum was monitored. A decrease in the Trp fluorescence is detected at 350 nm with a concomitant increase in the dansyl fluorescence at 520 nm.

![Fluorescent emission scans of S679W interacting with dansyl\(\text{N001}\) at increasing concentrations.](image)

Figure 3.8 shows the stopped-flow time courses for the increase in dansyl and concomitant decrease in Trp fluorescence generated from preincubation of 5 \(\mu\)M S679W ELon with 8 \(\mu\)M dansyl\(\text{N001}\) (A and B), dansyl\(\text{N002}\) (C and D), or dansyl\(\text{N006}\) (E and F) and rapidly mixed with 1 mM ATP. To determine the extent to which the 679W contributed to the FRET signal, the stopped-flow fluorescence time courses of dansyl\(\text{N}\) interacting with S679A were also monitored. For control, the time courses of dansyl\(\text{N}\) mixed with S679W ELon in the absence of any nucleotide, and in the presence of 0.5 mM ADP were also obtained, which shows negligible amount of changes. The samples
were excited at 295 nm and the fluorescent signals were detected using a 350 nm bandpass filter to monitor Trp fluorescence, and a 450 nm cutoff filter to monitor dansyl fluorescence. According to figure 3.8, a small amount of changes in fluorescence were detected in the S679A ELon reactions. This could be the dansyl approaching one of the other internal Trp residues, or the result of a conformational change in which the Trp residues are more accessible to interact with the dansyl moiety. In all cases, the signal generated from the S679W ELon interacting with dansyl\(\lambda\)N showed the most noticeable changes. Taken together, these results suggest that protein binding to ELon can be monitored by FRET between the 679W and dansyl moiety.
Figure 3.8 Representative time courses for S679W interacting with dansylλN through fluorescence stopped-flow. Five micromolar S679W was preincubated with dansylλN001 (a and b), dansylλN002 (c and d), or dansylλN006 (e and f) were rapidly mixed with 1 mM ATP (blue), 1 mM ADP (green), or no nucleotide (orange). Five micromolar S679A was preincubated with dansylλN and 1 mM ATP (red). The experimental time courses were excited at 295 nm and monitored at the dansyl fluorescence wavelength using a 450 long pass filter (a, c, and e), and at the Trp fluorescence wavelength using a 340 bandpass filter (b, d, and f). The most noticeable changes were detected in reactions containing S679W ELon and ATP.
3.3.4 Pre-steady-state FRET time course for the delivery of a specific dansylated site in λN 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. (80, 82-85) To evaluate the existence of directionality in the delivery of substrate to the proteolytic site of ELon, the order by which dansyl\(λ\)N001, dansyl\(λ\)N002, and dansyl\(λ\)N006 interacted with S679W ELon was determined through monitoring the duration needed for each construct to obtain maximum FRET signal between the dansyl label and 679W Trp upon incubation with ATP. The Förster distance is the distance where FRET efficiency is at 50%. Therefore beyond this distance, fluorescence is at a minimum. The Förster distance for the Trp donor and dansyl acceptor is between 21-24 Å. (86) According to the crystal structure of *Thermococcus onnurineus* Lon, which shows sequence homology with the protease domain of ELon, the active site in each subunit is 32 Å apart from each other, and 28 Å from the center of the translocation channel. (87) Therefore, the FRET approach employed here detected the kinetic events occurring
within the Förster distance set by the donor-acceptor pair, which was from the end of the translocation channel to the proteolytic site of ELon. Kinetic information concerning the initiation of substrate delivery to the active site that occurred beyond the specified Förster distance would not have been recorded. Given this constraint, the conclusion drawn from this study reveals the timing and kinetic coordination between the final stage of substrate delivery to the proteolytic site and peptide bond cleavage.

In theory, the amplitude of the changes in FRET signal could be used to calculate the Förster distance between a donor and acceptor. However, both Trp and dansyl are environmentally sensitive dyes. Therefore, the FRET efficiency between the dansyl group and Trp will vary due to different peptide sequences surrounding each dansyl probe in the three dansyl λN constructs, thereby obscuring the utilization of the FRET amplitude to obtain distance information. Moreover, once bound to the active site of ELon, conformational changes in enzyme may occur to cause additional changes in the orientation of dansyl and Trp, thereby producing further changes in FRET kinetics. Each kinetic step could be attributed to the translational movement or spatial orientation of the fluorescent probes due to enzyme conformational changes. As pointed out in materials and methods, in the acquisition of the FRET time courses, different detector sensitivity settings were used to optimize signal detection in each individual dansylλN reaction. Given these considerations, the amplitudes of the FRET time courses cannot be used to extract any translocation or distance data. Only kinetic parameters were extracted from the FRET time courses to deduce the order of terminal delivery in λN to 679W of the ELon mutant.
Figure 3.9 presents the standardized stopped flow time courses (representative of at least three independent experiments) of S679W ELon mixed with near-saturating concentration (~2-fold $K_m$, and > 10-fold $K_d$) of dansylated $\lambda N001$, $\lambda N002$ and $\lambda N006$, at saturating [ATP] (10-fold $K_m$). Reactions were excited at the Trp wavelength (295 nm) and changes in fluorescence intensity were recorded at the dansyl emission wavelength. The stepwise increases in the dansyl fluorescence is assigned to the FRET signal generated from the delivery of the respective dansylated site to 679W. Attempts to fit the data to a standard single or double exponential were unsuccessful due to the presence of “steps”. These steps are defined as a quick increase in fluorescence followed by a short plateau. Subsequent slow increases in fluorescence are assigned to dansyl tumbling within the active site. Instead a more qualitative approach to analyzing the data was undertaken. The initial quick increase in fluorescence is attributed to the pre steady state translocation of the probe to the active site. In dansyl$\lambda N001$, this spans $\sim 1.5$ sec and takes three steps, in dansyl$\lambda N002$, this takes $\sim 1$ sec and spans two steps, while in dansyl$\lambda N006$, this occurs in one step and takes $\sim 0.5$ sec.
**Figure 3.9** Substrate delivery to S679W ELon active site can be monitored using fluorescence stopped-flow with dansylλN and ATP. Five micromolar S679W ELon was incubated with 8 μM dansylλN001 (green), dansylλN002 (blue), or dansylλN006 (red). The left y-axis shows relative FRET signal between the S679W ELon and dansylated λN. Reactions were excited at 295 nm and dansyl fluorescence was monitored using a 450 long pass filter. Steps are areas of rapid fluorescence increase followed by a short plateau. Cleavage of λN001A by WT ELon with ATP is also shown for comparison (black). The right y-axis shows relative fluorescence due to peptide bond cleavage.

As extrapolated from the $k_{\text{lag λN,ATP}}$ of $λN006A$ and $λN001A$ found in table 3.1 and discussed earlier, the duration of the lag phase ($τ$) in peptide bond cleavage amounts to 1.15- to 1.3-sec. This lag phase is attributed to the buildup of an enzyme intermediate needed to initiate peptide bond cleavage. The termination of the 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 dansyl probes in dansylλN001, dansylλN002 and dansylλN006, as seen in
figure 3.9, which also includes the fluorescence generated due to peptide bond cleavage, reveals a maximum increase in signal was observed in the reactions containing dansylλN002 or dansylλN006 within the first 1.15 - 1.3 sec (τ). An “almost completed” increase in FRET signal was detected in the time course containing dansylλN001, whose dansyl label was the furthest away from the C-terminal of the substrate. As the lag phase of the cleavage of peptide bond in λN006A or λN001A spans approximately the duration needed to complete the delivery of dansylλN001, it is proposed that the kinetic data supports a mechanism in which the initiation of peptide bond cleavage occurs after almost complete delivery of the scissile sites in λN substrate to the active site in ELon.

It is possible that the observed FRET signal could also be partially attributed to changes in the orientation of the dansyl label with respect to 679W once it is delivered into the proteolytic chamber. As such, one or more of the steps detected in the reactions with dansylλN001 and dansylλN002 could be caused by conformational changes in the proteolytic site that leads to changes in the time courses; however this does not change the analysis of the data. As ELon is a homohexamer, it is possible that once the dansylated substrate is delivered into the proteolytic chamber, the fluorescence label is close enough to two active site 679W (one on each enzyme subunit) to allow significant FRET to occur. The kinetic experiments described could not eliminate such possibility. As each 679W is at the proteolytic site, the interaction of one dansyl between two 679Ws does not change the kinetics of the translocation; the FRET time courses recorded in this study still allows for the comparison of the kinetics by which different regions of λN (labeled with dansyl) approaches the proteolytic site prior to their cleavages.
3.3.5 AMPPNP-dependent Delivery of λN to 679W.

As ELon catalyzed the delivery of the synthetic decapeptide containing residues 89-98 of λN to the active site of ELon in the presence of AMPPNP (79), the kinetics of ELon delivering dansylλN001, dansylλN002 and dansylλN006 in the presence of this non-hydrolyzable ATP analog were also examined by FRET technique. As discussed earlier, AMPPNP slows down the reaction, allowing elucidation of kinetic parameters. Figure 3.10 shows the time courses of the respective dansylated λN approaching 679W (representatives of at least three independent experiments). For comparison, the time course of the AMPMP-dependent degradation of λN001A was also included. The y-axis reports the FRET signal intensity generated from each reaction. Unlike the ATP-dependent time courses shown in Figure 3.9, the AMPPNP-dependent time courses are much simpler and devoid of steps. They are able to be fit to a double exponential function (eq 3.3) to yield the rate constants shown in table 3.2. Both the $k_{1,FRET,AMPPNP}$ and $k_{2,FRET,AMPPNP}$ values follow the same descending order in which dansylλN006> dansylλN002> dansylλN001, suggesting that C-terminal end of λN approaches and reaches 679W first, agreeing with the proposed delivery order discerned from the ATP dependent reactions.
Figure 3.10 Representative time courses for dansylated substrate delivery to S679W with AMPPNP using fluorescent stopped-flow. Five micromolar S679W ELon was incubated with 10 μM dansylλN001 (green), dansylλN002 (blue), or dansylλN006 (red) and 1 mM AMPPNP. The reaction was excited at 295 nm and dansyl fluorescence was monitored using a 450 long pass filter. The left y-axis shows relative FRET signal between the S679W ELon and dansylated λN. Time courses shown in color were fit with equation blueberry to yield the kinetic parameters summarized in, and the fitted lines are shown in black Table pepper. Cleavage of λN001A (black) by WT ELon with AMPPNP is also shown for comparison. The right y-axis shows relative fluorescence due to peptide bond cleavage.

Table 3.2 Kinetic constants for AMPPNP-dependent dansylλN interaction with S679W ELon

<table>
<thead>
<tr>
<th></th>
<th>( k_{1,\text{FRET,AMPPNP}} ) (sec(^{-1}))</th>
<th>( k_{2,\text{FRET,AMPPNP}} ) (sec(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>DansylλN001</td>
<td>0.337 ± 0.029</td>
<td>0.028 ± 0.003</td>
</tr>
<tr>
<td>DansylλN002</td>
<td>0.404 ± 0.059</td>
<td>0.038 ± 0.006</td>
</tr>
<tr>
<td>DansylλN006</td>
<td>0.633 ± 0.035</td>
<td>0.083 ± 0.009</td>
</tr>
</tbody>
</table>
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. Previously, the ATP- and AMPPNP-dependent delivery of the dansylated peptide substrate was observed to have a two-step delivery as well. This involved the formation of an 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.\(^{(79)}\) As revealed by the standard errors of the fits, the \(k_{\text{lag}}\) is smaller than the \(k_{1,\text{FRET,AMPPNP}}\), indicating that translocation occurs before peptide bond cleavage. The \(k_{2,\text{FRET,AMPPNP}}\) of dansyl\(\lambda\)N001 and dansyl\(\lambda\)N002 are comparable to the \(k_{\text{lag,AMPPNP}}\) of peptide bond cleavage in FR\(\lambda\)N001 and FR\(\lambda\)N002 (see Table 3.1), suggesting \(k_{2,\text{FRET,AMPPNP}}\) of dansyl\(\lambda\)N001 or 002 constitutes the lag of peptide bond cleavage in the FR\(\lambda\)N constructs. By contrast, the \(k_{2,\text{FRET,AMPPNP}}\) of dansyl\(\lambda\)N006 is approximately 2- to 3-fold higher than the \(k_{\text{lag,AMPPNP}}\) of the FR\(\lambda\)N constructs and the \(k_{2,\text{FRET,AMPPNP}}\) of dansyl\(\lambda\)N001 or dansyl\(\lambda\)N002, indicating that the step corresponding to \(k_{2,\text{FRET,AMPPNP}}\) in dansyl\(\lambda\)N006 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\(\lambda\)N006, approaches the proteolytic site of ELon before the N-terminal, which is represented by dansyl\(\lambda\)N001 and dansyl\(\lambda\)N002. The similarity in the \(k_{2,\text{FRET,AMPPNP}}\) of dansyl\(\lambda\)N001 and dansyl\(\lambda\)N002 could be attributed to the location of these two probes being too close to detect noticeable differences in the kinetic experiment.

As S679W ELon is a proteolytically inactive mutant, it does not have peptide product to release. It is unknown, therefore if the enzyme shuttles product back out of the
active site after it cannot be cleaved, or if it stays bound in the active site waiting for proteolysis. As discerned from figures 3.9 and 3.10, fluorescence increases without a subsequent decrease; indicating dansyl\(\lambda N\) binds and stays bound. Otherwise, fluorescence would decrease as the donor Trp and acceptor dansyl separated once again.

3.4 CONCLUSION

Lon is a serine protease that translocates and hydrolyzes peptide bonds. In this chapter, it was demonstrated that the fluorescent tools developed earlier can be used to monitor translocation and subsequent cleavage. Using these tools identified comparable kinetics in the steady-state and pre-steady-state analysis of cleavage. The presence of a lag phase as seen in peptide cleavage was also seen in protein cleavage. Comparable kinetics of this lag phase in the FR\(\lambda N001\) and FR\(\lambda N006\) cleavage indicates the different scissile sites were cleaved with comparable timing. This indicates the presence of the same intermediate buildup, and no preference for directionality in cleavage.

To evaluate the events that lead to this cleavage, translocation was monitored by delivery of three dansylated sites in \(\lambda N\) to a 679W active site in ELon. This detected an almost completely delivery of the three sites in \(\lambda N\) to the active site coinciding with the length of the lag phase in the ATP- as well as AMPPNP-dependent reactions. These results indicate all sites are delivered to the active site before subsequent cleavage, showing processive degradation of \(\lambda N\) by ELon occurs via model 1 shown in figure 3.1. By comparing the kinetic time courses of the delivery of different regions of \(\lambda N\) in ATP- and AMPPNP-dependent reactions, it is discerned that the delivery of the C-terminal occurred first, followed by the N-terminal.
Additional experimentation will be required to further characterize the mechanistic basis for the stepwise changes in FRET signals upon incubation with ATP. The kinetic approach described here will provide the framework for completing these studies. Additional kinetic studies which involve determining the entire kinetic mechanism of λN translocation will be required to further define the molecular natures of the two kinetic steps detected in the AMPPNP-dependent reactions.
CHAPTER 4

PROBING THE STRUCTURAL DYNAMICS AND CONFORMATIONAL
CHANGES OF *E. COLI* LON PROTEASE USING HXMS
4.1 INTRODUCTION

4.1.1 Homology of Lon

Due to their sequence homology, Lon proteases are classified as either Lon A, typically found in bacteria, or Lon B, mainly found in archaea. \(^{(88)}\) Lon A members have three functional domains: an N-terminal domain, an ATPase, which contains two subdomains, an \(\alpha/\beta\) domain and an \(\alpha\) domain, and a protease domain, as seen in figure 4.1. \(^{(17)}\) As both ELon and BsLon belong to Lon A, it is predicted that they will have similar sequences, and indeed their sequences align with 56% similarity. Alignment of the individual domains shows a 47% similarity in the N-terminal domain, 63% similarity in the ATPase domain, and 61% similarity in the protease domain.

Figure 4.1 Domain composition for ELon (top) and BsLon (bottom). The crystal structures determined for each homolog are indicated with the start and end amino acids under each domain.
4.1.2 Crystal structures of BsLon and ELon elucidated so far.

The function of a protein is closely tied to its structure and dynamics. While there is structural information available for ELon, there has been no full length unbound crystal solved, and thus a clear picture of how its function and structure are linked has yet to emerge. Current crystal structures of ELon include ELon-N, the N-terminal domain spanning residues 1-245 (figure 4.2, PDB 3ljc), ELon-A, the AAA\textsuperscript{+} \(\alpha\) domain spanning residues 491-584 (figure 4.3, PDB 1qzm), and ELon-P, the protoelytic domain spanning residues 585-784 (figure 4.4, PDB 1rr9).\textsuperscript{(14-16)} Compiling these crystal structures together allows the elucidation of a large portion of the protein, however there is still a large span missing, from residues 245-491.

Figure 4.2 View of the crystal structure of ELon-N from amino acid 7 to 245. Figure constructed using PyMOL.\textsuperscript{(16)}
**Figure 4.3** Crystal structure of the α subdomain of the ATPase domain of WT ELon from residues 491-584. Figure constructed using PyMOL.\(^{(14)}\)

![Crystal structure of the α subdomain of the ATPase domain of WT ELon from residues 491-584.](image1)

**Figure 4.4** Crystal structure of the protease domain of WT ELon containing residues 593-784. Figure was constructed using PyMOL.\(^{(15)}\)

![Crystal structure of the protease domain of WT ELon containing residues 593-784.](image2)
Crystal structures of BsLon include BsLon-N, the N-terminal domain spanning residues 1-209 (PDB 3m65) and BsLon-AP, containing a small part of the N-terminal domain, the ATPase domain, and the protease domain, spanning residues 240-774 with ADP bound (PDB 3m6a). Mapping the crystal structures of ELon and BsLon to their aligned sequences (data not shown), and overlapping the crystal structures of the two homologs shows a high degree of similarity. Due to this, it is highly possible the 3D structure of the unsolved region of ELon coincides with the crystal structure of BsLon. The N-terminal is the most poorly conserved region amongst Lon homologs, however it is still highly similar in ELon and BsLon, albeit the lowest conservation among the three domains. Combining this with the fact that the crystal structures are very similar in the N-terminal provides further proof that the missing pieces of ELon can be mapped based on the known pieces of BsLon.

The N-terminal of ELon contains two subdomains with the boundary between the two around residue 120. As seen in figure 4.2, the first subdomain consists predominately of β-strands forming intermixing β-sheets with an α-helix in between. The second subdomain is all helical, and encompasses a large helix from residues 189-245, thought to be involved in a coiled coil region. It has been proposed that this region is involved in substrate recognition, as removal of the N-terminus by our lab and others leads to a loss of ATP-dependent protein degradation without affecting the ATPase or peptidase activities. It has been postulated that this helix works to disrupt local structural elements in protein aggregates and thus help untangle and solubilize aggregated proteins before degrading them. In this case, this helix would need to be solvent exposed and flexible to be able to move around and locate substrate. Following this long
helix, it is suggested there is a flexible linker, around residue 233-240, due to proteolysis by various different proteases cleaving around this site.\(^{(34, 91, 93)}\)

The rest of the N-terminal domain, and the \(\alpha/\beta\) subdomain of the ATPase domain has been solved in BsLon, but not ELon. The second half of the N-terminal domain forms a helical bundle from 3 helices. Following this is a loop that separates the N-terminal domain from the ATPase domain. The \(\alpha/\beta\) subdomain contains the well conserved Walker A (residues 356-363) and Walker B motifs (residues 421-427), the sensor 1 residue (N473), and the arginine finger (R484). The sensor 1 is responsible for orienting a nucleophilic water close to the \(\gamma\) phosphate of ATP\(^{(27)}\). The arginine finger is located on the surface of the \(\alpha/\beta\) subdomain, and reaches inside the nucleotide binding site of a neighboring subunit to detect nucleotide. The following \(\alpha\) subdomain is mainly \(\alpha\)-helical as seen in figure 4.3, and contains the sensor 2 residue (R542) which is proposed to contact the nucleotide and regulate activity.\(^{(14, 21)}\) In BsLon-AP, the \(\alpha\) domain forms an interface with the \(\alpha/\beta\) domain, forming a groove where nucleotide is cradled between them.\(^{(17)}\)

The ATPase domain is connected through the protease domain with another flexible loop. The protease domain contains many \(\beta\) strands making up a large \(\beta\) sheet along with smaller \(\beta\) sheets, with 6 \(\alpha\)-helices scattered throughout (figure 4.4).\(^{(15)}\) The protease domain oligomerizes to form a solvent accessible pore, which contains the conserved Ser-Lys dyad at residues 679 and 722 respectively.

4.1.3 Theory of hydrogen/deuterium exchange
In order to fully characterize Lon, we must gain insight into its solution structure with substrates bound. Due to the deficiencies in obtaining crystals for proteins, hydrogen/deuterium exchange is quickly becoming a dominant method for probing protein structure, dynamics, and interactions. As seen in figure 4.5, all polypeptides have three groups of hydrogens: those attached to carbons are tightly bound and therefore do not exchange quickly, while those attached to the side chains of amino acids can exchange too fast to be trapped, but the backbone amide hydrogens have exchange rates with deuterium in solution suitable for detection. Since all residues except proline have a hydrogen at its amide linkage, theoretically exchange can happen along the entire polypeptide chain. Hydrogens not involved in bonding, and hydrogens that are very solvent exposed exchange very rapidly. As hydrogen bonding is involved in the formation of secondary structure, these hydrogens only exchange when the bonding is disrupted, usually due to unfolding and refolding of the protein structure, meaning they are slower to exchange. These disruptions can be schematically described by eq 4.1:

\[
\begin{align*}
F_H & \quad \overset{k_{\text{unfold}}}{\rightleftharpoons} \quad U_H \quad \overset{k_2}{\longrightarrow} \quad U_D \quad \overset{k_{\text{fold}}}{\rightleftharpoons} \quad F_D \\
F_H & \quad \leftarrow \quad U_H \quad \leftarrow \quad U_D \quad \leftarrow \quad F_D \\
\end{align*}
\] (eq 4.1)

Where F and U are the folded and unfolded states of the protein respectively, \(k_{\text{unfold}}\) and \(k_{\text{fold}}\) are the rates of unfolding and refolding respectively, and \(k_2\) is the intrinsic exchange rate.\(^{(94)}\)
Figure 4.5 *Three groups of hydrogens in proteins*. Hydrogens bonded to carbons are too tightly bonded to exchange, while hydrogens along the amino acid side chains are too labile to be trapped and analyzed. Amide hydrogens, however, have exchange rates suitable for detection.

The exchange of hydrogen is also influenced by temperature and pH, and has been shown to be a minimum at 0°C and pH of 2.5. Exploiting these conditions allows for deuterium to be “trapped” into the peptide backbone, which can then be analyzed. Many spectroscopic techniques can be utilized to analyze the deuterium uptake of the protein. Among these, NMR and IR were early popular choices, however they require a significant amount of protein, and is problematic for proteins that tend to aggregate. Mass spectrometry, however, has proved to be a powerful tool due to its low concentration requirement, as well as its ability to study a large range of proteins and protein assemblies under a variety of solution conditions. Mass spectrometry measures the mass to charge ratio (m/z) of a molecule. As deuterium is an isotope of hydrogen with a heavier mass, the mass spectrum of the protein shifts to a higher mass as more
deuterium is incorporated. After deuterium incorporation has been quenched, peptides are digested with a protease that can cleave proteins at a low pH, such as pepsin, resulting in peptic fragments. This enables localization of data to specific regions rather than the protein as a whole, which provides much more useful information. The fragments are separated by high performance liquid chromatography, HPLC, or more recently ultra performance liquid chromatography, UPLC, before injection into a mass spectrometer.

Under normal physiological conditions, most proteins are very stable, meaning the normal unfolding/refolding events are much slower than the rate of exchange, $k_{\text{fold}} \gg k_2$, and the protein must undergo many unfolding events before it can exchange amide hydrogens. In this case, the observed exchange serves as a measure of the structural dynamics of the secondary structure within the peptide. This is known as EX2 exchange.\(^{(96)}\)

When the refolding of the structure is slower than the intrinsic exchange, $k_2 \gg k_{\text{fold}}$ and the protein is said to have EX1 exchange. The resulting MS spectra are distinguishable, as shown in figure 4.6, as EX2 exchange produces a gradual increase in the average mass, while EX1 exchange results in the appearance of two distinct mass envelopes separated on the m/z scale\(^{(96)}\). These two envelopes correspond to two different states of the region; a more folded, protected state that has less deuterium uptake (ie lower m/z value), and a more unfolded, less protected state that has more deuterium uptake (ie higher m/z value). The fluctuations in the local structure are slower than the rate of hydrogen exchange, meaning that the region unfolds and all the exchangeable hydrogens exchange quickly before the region can fold again. The two envelopes correspond to the unfolded state and the folded state. Since each individual molecule
undergoes unfolding and refolding at different times, a decrease in the lower m/z peak over time correlates with an increase in the higher m/z peak as all molecules visit the unfolded state and thus become deuterated.

**Figure 4.6** *The MS spectra resulting from two exchange kinetics, EX2 and EX1.*\(^{(96)}\)

![Figure 4.6](image)

4.1.4 Experimental Process.

The basic HXMS experiment is schematically described in figure 4.7.\(^{(94)}\) This normally consists of five steps: labeling, quench and rapid proteolysis, fragment separation, MS measurement, and data analysis. After a period of incubation to ensure the protein has reached equilibrium, the protein is labeled by dilution into deuterated buffer containing greater than 95% deuterium. Labeling is quenched at various time points by adjusting the pH to 2.5 and a temperature of 0°C, followed by injection onto a pepsin column at 20°C to generate the peptic fragments. A peptide trap is used to concentrate fragments and wash under quenching conditions to remove salts before being loaded onto a UPLC column. The peptic fragments are separated based on hydrophobicity due to a water/acetonitrile gradient. The separated fragments are then
subjected to electrospray ionization mass spectrometry, and the centroid of peptide fragments are determined to be used in data analysis.

**Figure 4.7 Overview of the experimental procedure for monitoring deuterium uptake.**

Before the HXMS experiments can be analyzed, the peptide fragments must be identified, and the mass of the undeuterated fragment, \( D_0 \), must be determined. Previously, this was done by running tandem MS experiments on undeuterated proteins. The experiment is set up the same as for deuterated experiments, except that the protein is not diluted in any amount of deuterium. Peptide fragments are eluted off a long slow HPLC gradient followed by tandem mass spectrometry. The mass spectrometer determines the \( m/z \) of a peptic fragment, isolates it and fragments it again by splitting the peptide backbone, resulting in pairs of \( b \)- and \( y \)-ions, and determines the mass of these ions. The total mass of pairs adds up to the mass of the original peptic fragment.\(^{(97)}\)
a series of b- and y-ions has been determined and the mass is compared to the expected mass, peptides are scored and chosen based on their score. In this way, the sequence of each peptide is determined, and the resulting mass is used as the undeuterated mass. However, recent advances in mass spectrometry using high and low collision energy, known as MS^E, generate both precursor and product ions in a single analytical run.\(^{(98)}\)

The benefit of MS^E over tandem mass spectrometry is that the peptides can be eluted on the same gradient as the deuterated samples. Automated programs, such as Protein Lynx Global Server, are then used to identify and score the peptides from the mass spectrum, greatly reducing the amount of time it previously took to identify the peptides.

### 4.1.5 Data Analysis

The amount of deuterium incorporated over time can be followed. As such, the labeling is quenched at various times before subjection to the proteolysis, separation, and mass spectrometry. Previously, the peptide fragments identified from the tandem mass spectrometry had to be manually identified from the mass spectrum in each of the time points run. However, due to MS^E and its ability to be run on the smaller UPLC gradient, analysis programs allows the tracking of the peptides over time via the elution time off the UPLC. Since the peptides are separated based on hydrophobicity, and the hydrophobicity of each fragment is unchanged upon exchange, the fragments will elute from the column at similar times regardless of the amount of deuterium exchange. DynamX (Waters, Milford, MA) is one program that tracks the mass spectrum of each peptide due to its elution time. This automated software tracks information using retention time, intensity, fragment ions, drift time, and mass accuracy. After manual inspection of the mass spectrum for each peptide to ensure proper assigning, the centroid
mass for each peptide at each time point is generated and used to determine the amount of
deuterium uptake over time via eq 4.2:

\[
\% \text{ incorporation} = \frac{D_t - D_0}{D_F - D_0} \times 100 \quad \text{(eq 4.2)}
\]

Where \(D_t\) is the centroid mass of the peptide at time \(t\), \(D_0\) is the centroid mass of the
undeuterated peptide (when \(t = 0\)), and \(D_F\) is the centroid mass of the fully deuterated
peptide.

Even though exchange is at a minimum after quenching, it is not completely
stopped and back exchange happens during digesting and loading. This is corrected by
running a fully deuterated reference sample. This sample is obtained by denaturing the
protein causing all hydrogen bonding to fall apart. By using deuterated buffer containing
guanidinium deuterochloride (GdnDCl), the protein will be denatured under deuterium
conditions, allowing all hydrogens to exchange with deuterium. The sample is allowed to
sit for 3 hours to ensure full deuteration, and then analyzed normally. The resulting mass
spectrum is used to determine the centroid for the fully deuterated (\(D_F\)) peptide.

Peptides with high deuteration at early time points indicate hydrogens that are
very labile, not involved in secondary structure, or very solvent exposed because they
exchange quickly. Peptides that have low deuteration at early time points, but high
deuteration at later time points are thought to be buried in the structure where it takes the
solvent some time to reach, or have tightly bonded secondary structure. Over time,
deuterium is able to diffuse through the protein to exchange, or they undergo local
fluctuations. These local fluctuations cause the hydrogens to briefly break their bonding,
allowing them to exchange with the solvent, and then reform. When this happens, the
region is said to be unfolded, and hence flexible. Peptides that show low deuteration
through all time points are considered tightly bonded, rigid, and inflexible, because the hydrogens do not briefly break their bonding, and thus do not undergo any local unfolding.

As many proteins bind substrates with a conformation change, this change can be tracked via hydrogen/deuterium exchange. Peptides with differing amounts of deuterium uptake over time can indicate regions that shift. This can involve creation or disruption of secondary structure, changes in local fluctuations and flexibility, or changes in solvent exposure, indicating burying or unburying of peptide regions. Here HXMS was utilized to evaluate the secondary structures of WT ELon proposed from reported crystal structures.

4.2 MATERIALS AND METHODS

4.2.1 Materials.

Materials were purchased as described in previous chapters, and proteins were purified as described in previous chapters. D₂O was purchased from Cambridge Isotope Laboratories, Inc (Andover, MA).

4.2.2 Peptide mapping.

A total of 100 pmol (10 μM) of WT ELon in 10 μL of Lon buffer [50 mM Tris (pH 8), 60 mM Mg(OAc)₂, 2.5 mM tris(2-carboxyethyl)phosphine] was mixed with quench buffer [300 mM sodium phosphate (pH 2.3), 10 mM tris(2-carboxyethyl)phosphine, 10% 1M HCl] and injected into a 20°C pepsin column for digestion of protein into peptic fragments. Mass spectra of desalted peptic fragments purified by a gradient of 5%-35% acetonitrile in 7 minutes was acquired by Waters HDX with nanoACQUITY reverse-phase ultraperformance liquid chromatography (RP-UPLC)
with HDX technology (Waters) in resolution mode (m/z 100-2000) on a SynaptG2 mass spectrometer with a standard electrospray ionization source. Peptide identities were confirmed by MS^E (mass spectrometry elevated energy) analysis. Data was processed using Protein Lynx Global Server 2.5 and manually confirmed.

4.2.3 Deuterium labeling.

Deuterium labeling was performed at room temperature by first incubating Lon for 5 minutes in Lon buffer, and then dilution in deuterated Lon buffer with a pD of 8. Samples were quenched at 5, 10, 50, 500, 1000 and 3180 sec with quench buffer at 4°C and immediately injected. Fully deuterated samples were prepared as described above except with 6M guanidine deuterochloride added to the deuterated buffer and quenched after 3 hours at room temperature. Each time point was run at least in duplicate, and data was averaged.

4.2.4 Data Analysis

DynamX software (Waters) was used to select peptides for analysis and extract each centroid mass as a function of labeling time. Percent deuterium incorporation was determined using eq 4.2

\[ \% \text{ incorporation} = \frac{D_t - D_0}{D_F - D_0} \times 100 \text{ (eq 4.2)} \]

Where \( D_t \) is the centroid mass of the peptide at time \( t \), \( D_0 \) is the centroid mass of the undeuterated peptide (when \( t = 0 \)), and \( D_F \) is the centroid mass of the fully deuterated peptide. Heat maps were created using HXMS software using the amino acid sequence and percent deuterium incorporation.\(^9^9\)

HX-Express2 was used for EX1 exchange analysis.\(^{100}\) Mass spectrum for each time point under the different conditions were uploaded, and data was fit to a double
The weight of each envelope in the data output was used to determine percentage of folded state at each time point with eq 4.3:

\[
\% \text{ folded} = \frac{W_{\text{folded}}}{W_{\text{folded}} + W_{\text{unfolded}}} \times 100 \quad \text{(eq 4.3)}
\]

Where \( W_{\text{folded}} \) and \( W_{\text{unfolded}} \) are the weights of the folded and unfolded state respectively.

### 4.3 RESULTS AND DISCUSSION

#### 4.3.1 Peptide mapping and coverage.

Tandem mass spectrometry experiments with native wild-type ELon identified 185 peptic fragments. These fragments cover 94% of the entire ELon sequence, and in many regions, have multiple overlaps. The fragments were narrowed down to eliminate redundancy to a total of 66 peptides. A few noticeable gaps encompass residues 1-6, 488-496, and 633-641, and 779-784.

#### 4.3.2 H/D exchange data for the unbound ELon.

Deuterium incorporation was followed for each peptic fragment over a period of one hour. Representative MS spectra for the doubly charged peptide 253-260 are shown in figure 4.8. The shift in mass upon deuterium incorporation is easily seen as the spectra shifts from a lower mass to a higher mass. By measuring the centroid mass of the deuterated peptide and subtracting the centroid mass of the undeuterated peptide, the total deuterium uptake as a percentage can be determined as shown in eq 4.2.
Figure 4.8 MS spectra of the double-charged peptide 253-260 illustrating time-dependent deuterium uptake.

Percent exchange at various incubation times is mapped onto the sequence of the WT ELon as seen in the heat map in figure 4.9. The lighter green indicates a low percentage of deuterium, and as deuterium levels rise, the color changes from green to purple.
One of the downfalls of HXMS is that inferences can only be made about the whole peptide detected and not individual amino acids. If there is a helix of interest, for example, but the only data that covers this area covers a piece longer than the helix, additional data not pertaining to the helix will also be obtained. The exchange data, therefore has to be inferred with caution, and cannot be used to assign specific secondary structures. However, it is able to give insight into what areas are dynamic, and which areas of the protein are interesting to study further.
The majority of the β-sheets in the first subdomain of the N-terminus are stable, as evidenced by the low deuterium incorporation through all time points. The exception is the β-strand spanning residues 110-123, which shows little protection from exchange. The helices in the second subdomain show little protection from exchange, as evidenced by the large amount of deuterium incorporation. In particular, the long α-helix in the second subdomain spanning residues 189-245 shows a large amount of exchange, indicating solvent exposure and little protection in solution. This region is thought to be involved in a coiled coil region, and make contact with other monomers, as seen in the BsLon-N crystal structure. These hydrogens would be involved in secondary structure and hence not available for exchange. This is in contrast with what is seen here. However, it has been proposed that this area is involved in substrate sensing. It is believed that this helix is dynamic and possibly helps to position substrate.\textsuperscript{(14, 91, 101)} If this is the case, it needs to be flexible in solution in order to move around, which would agree very well with the exchange data.

The area of 233-244 is thought to be solvent exposed due to the proteolysis experiments discussed in the introduction. Inspection of the heat map in figure 4.9 would indicate a low level of deuterium at shorter time points with more deuterium incorporation at higher time points. Manual inspection of the mass spectrum indicates this area exhibits EX1 kinetics. Figure 4.10 shows the +2 charge state spectra of this peptide at 0, 5, 10, 50, and 500 seconds of incubation in D₂O, as well as the fully deuterated spectrum. The 5-50 s spectra shows a double isotopic envelope, where the lower m/z envelope corresponds to a folded, less exchangeable form, and the higher m/z envelope corresponds to an unfolded more exchangeable form. The percent of each peak
was determined as described in materials and methods, and listed in table 4.1. Attempts to fit the data at 500 s and beyond to a double bimodal were unsuccessful, suggesting all Elon has undergone the unfolding/refolding and only the higher m/z envelope is present. This agrees with the ability of proteases to cleave this region, as it unfolds and becomes solvent exposed long enough for the exchangeable hydrogens to exchange before refolding happens.
**Figure 4.10** Mass spectra of peptide 233-244 (QMKAiQKELGEM) exhibiting EX1 kinetics. ELon was incubated in D$_2$O buffer to determine deuterium incorporation. Undeuterated and fully deuterated are shown in the top panel and bottom panel respectively. In the HXMS time points, the whole envelope is shown with a red line. The two isotopic peaks detected with the double bimodal are shown in red for the folded state and blue for the unfolded state, indicating the existence of EX1 kinetics. The weight of each peak was used to determine the percent of each state as listed in table 4.1.
Table 4.1 Percent of the folded state envelope at each time point for peptide 233-244 QMKAIQKELGEM.

<table>
<thead>
<tr>
<th>Time, sec</th>
<th>Folded state</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 s</td>
<td>78.3</td>
</tr>
<tr>
<td>10 s</td>
<td>74.6</td>
</tr>
<tr>
<td>50 s</td>
<td>46.2</td>
</tr>
<tr>
<td>500 s</td>
<td>0.0</td>
</tr>
<tr>
<td>1000 s</td>
<td>0.0</td>
</tr>
<tr>
<td>3180 s</td>
<td>0.0</td>
</tr>
</tbody>
</table>

In BsLon-AP, the carboxy-terminal end of the N-terminal domain consists of three helices that form a helical bundle. These span residues 253-260, 266-277, and 289-301 of ELon. As deduced from figure 4.9, these regions are protected from exchange, indicating the presence of these same secondary structures in ELon. The N-terminal domain is connected to the ATPase domain through residues 302-312 of ELon. Peptides encompassing this region are highly exchanged through all time points, indicating it is very flexible and solvent exposed and unlikely to be involved in secondary structure, agreeing with a proposed flexible loop in BsLon-AP.

The N-terminus of the α/β subdomain shows many helices in BsLon-AP. These regions in ELon are largely protected from exchange, indicating the possibility of their existence. There is a helix in BsLon-AP that spans residues 316-323 in ELon. This region shows almost complete exchange even at the early time points, indicating low protection, which is in contrast with the possibility of a helix here. There are three possibilities that could account for this discrepancy. As the BsLon has ADP bound, and
ELon has no nucleotide bound, it is possible that this region is more accessible without nucleotide. It is also possible that in solution, this helix is more flexible and solvent exposed than in the crystal structure. A third possibility is the helix that exists in BsLon-AP does not exist in ELon. The data presented here cannot distinguish between these possibilities, however due to the overwhelming evidence that BsLon and ELon have similar secondary structures, it is most likely due to the helix being more flexible and solvent exposed in solution.

There is a β strand spanning residues 351-354, directly preceding the highly conserved Walker A motif which spans residues 356-363. Both the strand and the motif show little protection. As this area is involved in nucleotide binding, and there is no nucleotide present, these results are not surprising. This area must be very solvent exposed without nucleotide bound. The Walker B motif, on the other hand, shows protection from exchange. As this area is a β sheet, the exchange indicates a rigid β sheet.

The peptides encompassing residues 453-467 show little exchange, indicating high protection through all time points. As this region does not map to any secondary structure in BsLon-AP, it gives rise to why it is so protected. It is possible that it is buried inside the structure, and not able to exchange freely with the solvent. However, even a buried region should exchange over time, and this region stays protected. A second possibility is that there is secondary structure here in ELon that is not present in BsLon, however as discussed above, the overlap in secondary structures in other parts of Lon suggest otherwise. These hydrogens must be bound so tightly, they cannot exchange under these conditions, however they do not appear to be bound in a secondary structure.
BsLon-AP shows two internal flexible loops which span residues 385-402 and 430-436 of ELon. The peptides that encompass these regions show almost complete deuteration in the early time points, agreeing with the proposed flexibility and solvent accessibility of these loops.

The arginine finger, which is located on the surface of the α/β domain, is thought to reach inside the nucleotide binding site. It is thought to be involved in inter-subunit communication. As such, its structure should be somewhat flexible to give it the ability to interact with a separate subunit. The peptide encompassing this finger in ELon, Arg484, shows protection at early time points, but little protection at later time points. This confirms the flexibility of this arginine finger.

The α subdomain (residues 496-570) of the ATPase domain of ELon shows little deuterium incorporation, indicating tightly bound secondary structure. This matches well with the reported ELon crystal structures showing mainly α-helices with a small β sheet inbetween. The peptides between the proposed helices show low deuterium incorporation at early time points, with almost complete exchange at later time points. This could suggest a buried but flexible region, which agrees well with the hypothesis that the α domain of one ELon monomer makes contact with the α/β domain of an adjacent ELon monomer. These peptide regions might not be involved in secondary structure, but sandwiched between two ELon monomers, making it harder for deuterium to reach it at early time points.

The connection between the ATPase domain and the protease domain, residues 582-593, is a flexible loop in BsLon-AP, which is not covered by either the ELon-A or ELon-P crystal structures. The area is covered by 2 peptides one of which contains a
helix before the loop, and the other contains a β sheet after the loop. Both of these peptides show little deuteration at early time points, with near complete exchange by 500 s, indicating a region with either low solvent exposure or with secondary structure, but flexible, matching well with the crystal structure.

The crystal structure of the protease domain shows another subunit interaction between a β sheet at the N-terminal part of the domain on one subunit, and a helix encompassing residues 632-649 on another subunit. The β sheet is made up of many β strands, some with high deuterium levels, indicating this sheet is unstable and very flexible in solution. However, the first β strand shows protection from exchange early, with only moderate protection in later time points. Only half of the helix involved in the interaction is covered by HXMS, however it shows protection from exchange. The protection of these two regions agrees with the possibility that they are involved in binding, as their exchange should be minimal.

The S679 active site is found in a long peptide, spanning residues 659-681. This peptide shows little deuterium incorporation in early time points, with more incorporation in later time points. This is consistent with the crystal structure, as it shows the S679 active site is not involved in secondary structure, but instead is a loop connecting a β strand to an α helix. The active site needs to be somewhat protected from solvent so it does not proteolyze every protein it comes in contact with, however it also needs to be somewhat accessible to the substrates it does need to cleave. The pattern of exchange is consistent with this. However, within the length of the peptide, are two proposed β strands, and the deuterium incorporation could also be from these strands. The crystal structure shows the second active site, K722 is in a helix encompassing residues 719-730.
Although this helix runs along the surface, it is protected from exchange through all the time points, indicating it is very rigid.

A small β sheet is formed from β strands encompassing residues 700-705, 732-737, and 756-761. These β strands are protected from exchange through all time points. In the crystal structure they are sandwiched by 2 α helices, one of which is the protected 719-730 peptide. The other is a helix encompassing residues 762-772. This second helix shows moderate deuterium incorporation, in agreement with it being a solvent exposed secondary structure.

Of the crystal structures of the protease domain determined, only one molecule had the last 9 residues visible. They adopt an extended conformation, which reaches into the active site of another molecule. The exchange data shows high deuterium incorporation here, indicating solvent exposure, which is in agreement with the crystal structure.

4.4 CONCLUSIONS

In this chapter, it was shown that HXMS can be used to probe the structural dynamics of proteins. As shown by the heat map in figure 4.9, the amount of deuterium incorporation matches with the proposed secondary structures obtained from crystal structures.

For proof of principle, areas of interest can be mutated to remove regions which do not match with the crystal structure, and activity assays as described in the preceding chapters can be utilized to determine activity. It is expected that these mutants will exhibit different activities compared to WT, which can help in elucidating the function of the areas mutated out. While it is expected that specific areas will completely remove
activity, such as the binding sites for nucleotide, or the active sites for protein degradation, some of the other regions not directly involved should provide insight into their function. For example, the highly protected yet unstructured region of 453-465; as this region has not been shown to be directly involved in secondary structure, the observance of low deuterium levels is interesting. Removal of this region followed by activity assays could lead to insights into why it is so protected. Our lab has proven this is an effective strategy, as mutating out regions surrounding the 233-250 area based on limited tryptic digestions and the HXMS data present here showed a change in activity of ELon.\(^{(90)}\)

It has been indicated that Lon undergoes a conformational change upon nucleotide binding. Characterization of this change has been difficult in the absence of crystal structures. HXMS has been proven to be a powerful tool for probing the structure of Lon. Therefore, it is proposed that incubating ELon with ATP and following the deuteration pattern will result in a different HXMS pattern, indicating regions will structural differences. Current studies are underway to evaluate this HXMS pattern.
CHAPTER 5

CHARACTERIZING 12 HUMAN LON MUTATIONS IMPLICATED IN CODAS
5.1 INTRODUCTION

As discussed earlier, human Lon, hLon is synthesized as a precursor in the cytosol and imported into mitochondria, where an N-terminal sequencing tag is cleaved off. It has been proposed to protect the mitochondria by degrading regulatory proteins, as well as oxidatively damaged proteins. As mitochondria are known as the “workhouse” of the cell, disruption of mitochondria has been implicated in many diseases such as Parkinson’s and Alzheimer’s. Therefore mutations in Lon that affect its ability to degrade proteins could be a factor in mitochondrial diseases.

One of these diseases, known as CODAS, cerebral, ocular, dental, auricular and skeletal anomalies syndrome, is a rare disease generated from mutations in hLon. The phenotype of this disease is marked by developmental delay, congenital cataracts, unusual dental findings, skeletal and facial anomalies, and mental retardation. Recently, in unpublished results, there have been 12 variants of hLon that have been implicated in this disease: W464*, E476A, S631Y, A670V, R672C, P676S, R679H, R721G, A724V, P749S, G767E, and ΔIle927. This numbering system is based off the precursor form. All CODAS is recessive, meaning both parents have one copy of healthy WT hLon and one copy of the mutated hLon. Offspring patients must have two copies of dysfunctional lon genes inherited from the parents to manifest symptoms. The P676S and R721G are homozygous, meaning both of their dysfunctional lon genes carry the same mutation. The other patients are heterozygous, and pair up as follows: W464*/R679H, P749S/G767E, E476A/R672C, A670V/ΔIle927, and S631Y/A724V. Unpublished results from the Jinks lab have indicated the precursor mutant hLon is regulated normally to mitochondria where the mitochondrial tracking tag is theoretically cleaved to yield the
mature form. Therefore disease is not due to improper import into mitochondria. As such, the extent to which these mutations cause disease is unknown. Due to the success of generating and characterizing recombinant Lon in our lab previously, these mutant hLon variants can be studied utilizing the kinetic techniques previously developed. Elucidating the extent to which these mutants function could provide insights into how WT hLon functions to maintain mitochondrial integrity. This would help in developing tools to rescue mitochondria from disease as well as prevent disease from occurring.

As ELon and hLon are homologs that operate similarly in their respective organisms, it is believed that they will be able to degrade the others substrates. And indeed, it has been shown that hLon degrades λN. Therefore, it was proven that hLon degrades the FRETN8998 peptide substrate developed, albeit with lower kinetics. As such, the FRETN8998 peptide substrate can be used to evaluate the activity of hLon.

As discussed earlier, a cocktail containing FRETN8998 and a nonfluorescent version, 8998Bz, has been used to avoid the inner filter effect (figure 5.1). This nonfluorescent version has a benzoic acid moiety on the epsilon amino group of lysine instead of the fluorescent anthranilamide. As there is no fluorescent moiety in the nonfluorescent analog of 8998 peptide, its degradation needs to be monitored in another way to ensure it is degraded similarly. Due to the absorption of the peptide backbone at 220 nm, peptide degradation can be viewed under UV. By using HPLC, the substrate can be separated from the product based on hydrophobicity using a gradient of increasing acetonitrile. The relative amount of substrate and product can be monitored over time. The amount of the fluorescent versus non fluorescent substrates after degradation by Lon can be compared. This has been used previously to show that the fluorescent and
The nonfluorescent versions of peptide are degraded similarly by Lon.\(^{(38)}\) The synthesis of the nonfluorescent peptide requires the addition of a lysine with the side chain amino group protected, and this side chain needs deprotecting without affecting any other residues already attached. Subsequent addition of the benzoic acid moiety onto the side chain follows. In order to eliminate this step for ease of synthesis, the side chain of lysine would need to be protected by the benzoic acid before addition to the peptide sequence. As there is not a commercially available benzoic acid protected lysine, different versions of the 8998 peptide substrate with a different moiety on the side chain of lysine were sought to be developed and tested. A lysine with the side chain amino group protected by an acetyl group is commercially available, and therefore could be used in the synthesis of the 8998 peptide to make 8998Ac (figure 5.1). This peptide can be ordered from external companies in small quantities to test before being used in activity assays.

**Figure 5.1** Structures of nonfluorescent 8998Bz and 8998Ac substrates. Arrow indicates Lon cleavage site.
This will serve as a basis for evaluating activity of hLon mutants involved in CODAS. The activity of the mutants can be compared to WT to ascertain how the mutation affects activity.

5.2 MATERIALS AND METHODS

5.2.1 Materials.

Fmoc-protected amino acids, Boc-Abz, Fmoc-protected Lys Wang resin, and HBTU were purchased from Advanced ChemTech and NovaBiochem. All cloning reagents were purchased from Promega (Madison, WI), New England BioLabs, Inc (Ipswich, MA), Invitrogen (Carlsband, CA), and Affymetrix (Santa Clara, CA). Tris buffer, cell culture, media, IPTG, chromatography media, DTT, Mg(OAc)$_2$, trypsin, kanamycin, ampicillin, chloramphenicol, ATP, and all other materials were purchased from Fisher, Sigma, and VWR (Radnor, PA). Primers were purchased from Integrated DNA technologies. Peptide substrate 8998Ac was purchased from Genscript.

5.2.2 General Methods.

Peptide synthesis of FRETN8998 was performed using Fmoc solid-phase synthesis methodologies as described previously.$^{(38)}$

5.2.3 HPLC peptidase analysis.

Peptidase activity was monitored with HPLC. Reactions were performed at 37°C and contained 50 mM Hepes pH 8.0, 5 mM magnesium acetate, 2 mM DTT, either 100 μM 8998Ac, 100 μM 8998Bz, or 50 μM 8998Ac and 50 μM 8998Bz, 300 nM hLon, and the reaction was initiated with 1 mM ATP. Reaction aliquots were quenched with 0.02% TFA at 0, 10, and 20 minutes. Quenched time points were analyzed on an analytical C-18 HPLC column (Vydac, Deerfield, IL) with a Jasco (Oklahoma City, OK) HPLC
system using a linear gradient of 5–30% acetonitrile/water with 0.05% TFA in 25 minutes. Absorbance was monitored at 220 nm and peaks corresponding to substrate and products were identified. The area for peaks corresponding to substrate was plotted against time.

**5.2.4 Plasmid Construction.**

The plasmid for the mutants A670V, P676S, R672C, P749S, and G767E in pENTR/D-TOPO/LONP1 DNA, and R271G, S631Y, R679H, and A724V in pcDNA3.2_LONP1 was provided by Dr. R. Jinks. The mutant human Lon genes were cloned into the mature form of WT human Lon by swapping the region containing the mutation with the same region containing the WT coding information. The mature form of human Lon is a truncated version from the protein sequence encoded by the plasmids provided by Dr. R. Jinks, which excludes 114 amino acids from the N-terminal region of the full length precursor proteins. In this study, all amino acid numberings are assigned based on the precursor form unless specified. The mutants A670V, P676S, R672C, P749S, G767E, and R271G, S631Y, R679H, A724V, as well as WT Lon in pET24c(+) vector (pHF002) were digested with SacII and NcoI. The digested DNA reactions were purified by agarose gel electrophoresis. The large DNA fragment in pHF002 was ligated with the smaller DNA fragment in the mutant vector digestions.

To generate the E476A hLon encoding plasmid, a gene for Lon encoding amino acids 114 through 512 containing E476A was synthesized by Genscript in pUC57. This E476A/pUC57 plasmid and pHF002 were digested with NdeI and SacII. The digestions
were purified by agarose gel electrophoresis. The DNA fragment encoding E476A was ligated into the pHF002 DNA fragment lacking the same region.

To generate the ΔIle927, a gene for Lon encoding amino acids 867 to 959 containing a deletion at 927Ile was synthesized by Genscript in pUC57. This ΔIle927/pUC57 and pHF002 were digested with NcoI and BamHI. The digestions were purified by gel electrophoresis. The DNA fragment containing the mutation was ligated into the pHF002 DNA fragment lacking the same region to generate the final expression vector for ΔIle927.

The mutants E476A, A670V, P676S, P749S, G767E, R271G, and ΔIle937 were originally cloned into pET24c(+) without any his-tag. To generate expression vectors that over-express N-terminal his-tagged proteins, these hLon mutant genes were ligated into the pProEx-1/hLon plasmid, digested with NdeI and BamHI to remove the WT hLon gene.

To insert a histag into the R672C subcloned in pET24c(+), two oligonucleotides were synthesized by IDT, (5’- TATGCACCACCACCACCACCACCA-3’, and 5’- TATGGTGGTGGTGTTGTTGCA-3’). These oligonucleotides were gel purified and annealed by heating 20 μM of each to 100°C and cooling slowly to room temperature. The oligonucleotide was then phosphorylated with T4 polynucleotide kinase. The R672C in pET24c(+) was cut with NdeI and subsequently dephosphorylated with calf intestinal alkaline phosphatase. The resulting oligonucleotide was then cloned
into the R672C in pET24c(+) to create an N-terminal histagged R672C Lon in pET24c(+).

To generate the expression vector for the W464* mutant, a gene for Lon encoding amino acids 358 to 464 with a mutation W464* was synthesized by Genscript in pUC57. This W464*/pUC57, R679H in pET24c(+), A724V in pET24c(+), and the histagged R672C in pET24c(+) were digested with Bsp14071 and BamHI. The DNA fragment containing the respective mutation was subcloned to the larger DNA fragment of histagged R672C in pET24c(+) pre-digested with Bsp14071 and BamHI to generate N-terminal histagged Lon with containing a W464*, R679H, or A724V mutation.

To generate The S631Y expression vector, the S631Y and the histagged R672C in pET24c(+) were cut with SacII and BamHI and the resulting band from the S631Y mutant was subcloned into the histagged R672C pET24c(+) plasmid to create N-terminal histagged Lon with only an S631Y mutation.

All ligations were transformed into DH5α cells and plasmid propagations were selected by Amp or Kan. All DNA sequences were verified by DNA sequencing.

5.2.5 Purification of WT hLon and mutants.

Lon mutations E476A, A670V, P676S, R721G, P749S, and G767E were overexpressed in Rosetta (DE3) (Novagen), while Lon mutations R672C, W464*, S631Y, R679H, and A724V were overexpressed in Rosetta 2 (DE3). Cells were grown in superbroth (SB, per L: 30 g tryptone, 20 g yeast, 10 g mops, pH 7.5, 34 μg/mL cam,
and either 100 μg/mL amp for E476A, A670V, P676S, R721G, P749S, and G767E, or 30 μg/mL kan for R672C, W464*, S631Y, R679H) at 37°C and induced with 1 mM IPTG at OD_{600} = 0.6 for 1 hour. Cells were then harvested with Lon lysis buffer (50 mM Tris, 5 mM EDTA, 0.3M NaCl, 20% glycerol, 0.005% Tween 20, 1 mM BME, pH 7.5), and the resulting lysate was loaded on a preequilibrated phosphocellulose column. The column was then washed with wash buffer (50 mM Tris, 0.5M NaCl, 10% glycerol, 0.005% Tween 20, 1 mM BME, pH 7.5), and then eluted onto a nickel column with elution buffer (50 mM Tris, 1M NaCl, 10% glycerol, 0.005% Tween 20, 1 mM BME, pH 7.5). The nickel column was washed with 0.05M imidazole buffer (50 mM Hepes-KOH, 0.3M NaCl, 20% glycerol, 0.1M MgCl₂, 1 mM TCEP, 0.05M imidazole, pH 8). Lon was then eluted off with a step-wise gradient of 0.1M, 0.2M and 0.4M imidazole buffers. Lon positive fractions were pooled, concentrated, and initially quantified with Bradford assays using BSA as a standard, and subsequently verified by UV absorbance at 280 nm using eq. 5.1
\[ \varepsilon_{280} = W(5500) + Y(1490) + C(125) \]  
(5.1)

where \( \varepsilon_{280} \) is the molar absorptivity at 280 nm, \( W \) is the number of tryptophans, \( Y \) is the number of tyrosines, and \( C \) is the number of cysteines present in the protein. \(^{107, 108}\) An SDS-PAGE gel of each mutant showed differences in band intensity after coomassie staining. To account for these differences, the band intensities of each variant were determined using the program ImageJ and compared to WT Lon. \(^{109}\) Mutant concentrations were then corrected based on the comparison.
5.2.6 Steady-State Peptidase Activity Assay.

Peptidase activity was measured using a Fluoromax-4 spectrofluorimeter (Horiba Group). Reactions containing 50 mM HEPES (pH 8.0), 5 mM Mg(OAc)$_2$, 1 mM DTT, 250 nM WT Lon or mutants, and 0.5 mM S3. S3 is a mixed substrate of 10% fluorescently labeled peptide FRET$N_{8998}$ (Y(NO$_2$)RGITCSGRQK(ABz)) with 90% nonfluorescent analog of the peptide 8998Ac (YRGITCSGRQK(Ac)) to avoid problems from the inner filter effect. After equilibrating at 37°C for 1 min, the reaction was initiated with 1 mM ATP. The amount of hydrolyzed peptide was measured by determining the maximum fluorescence generated per micromolar peptide after complete digestion by trypsin. The steady-state rate of the reaction was determined from the tangent of the linear portion of the time course. This rate was converted to an observed rate constant ($k_{obs}$) by dividing the rate by the enzyme concentration. All assays were performed at least in triplicate.

5.3 RESULTS AND DISCUSSION

5.3.1 Cleavage of 8998Ac versus 8998Bz

Human Lon degrades the nonfluorescent peptide 8998Bz similarly to the fluorescent peptide FRET$N_{8998}$. As such, a substrate that is degraded similarly to the 8998Bz will be degraded similarly to FRET$N_{8998}$, and therefore can be used as a nonfluorescent analog to overcome inner filter effects. To this end, 8998Bz and 8998Ac were degraded separately by hLon and the products of which were viewed with HPLC as seen in figure 5.2. As the two sequences differ in the side chain of lysine, which changes the hydrophobicity, they should elute off the HPLC at different retention times, as seen by the difference in times of the green arrow versus the red arrow. As Lon cleaves in the
middle of the peptide, and the only difference is the lysine residue on the C-terminal, they should both have a similar N-terminal product, which elutes off the HPLC column with similar retention times. As seen by the orange arrows in figure 5.2, after 20 minutes of cleavage, both HPLC traces show the appearance of a peak with a retention time around 14.4 minutes, indicating this is the N-terminal product. The appearance of this peak, and concomitant disappearance of the substrate peaks around 17.6 min for the 8998Bz or 13.1 min for the 8998Ac indicate peptide is being cleaved.

**Figure 5.2** HPLC traces of (A) 8998Bz and (B) 8998Ac degradations by hLon viewed at 220 nm. Reactions contained 100 μM peptide, 1 μM hLon, and initiated by 1 mM ATP at 37°C, and quenched at times indicated. Green arrow represents 8998Bz peptide substrate, red arrow represents 8998Ac peptide substrate, blue arrow represents the C-terminal SGRQK(Bz) peptide product, and orange arrow represents the N-terminal YRGITC peptide product.
As the elution pattern of the two peptide substrates do not overlap, the degradation of both at once can be monitored. Figure 5.3A shows the HPLC trace of a degradation reaction containing both 8998Bz and 8998Ac quenched at 0 and 20 minutes. As discerned from the figure, the peak at the green arrow corresponding to 8998Bz gets smaller after 20 minutes than the peak at the red arrow corresponding to 8998Ac. This indicates 8998Bz gets degraded better than 8998Ac. To further confirm these results, the area integrations of each substrate peak were plotted versus time as seen in figure 5.3B. While there are not enough data points to obtain a $k_{obs}$, it is enough to identify differences in degradation efficiencies. The 8998Ac peptide shows a lower efficiency in degradation, indicating it will not be cleaved similarly to the FRET8998 peptide, and thus cannot be used to replace the 8998Bz in determining kinetic parameters for hLon. However, it is still degraded by hLon, and as such was used in the kinetic assays described below to compare the activity of mutant hLon to wild-type hLon. To overcome the inner filter effect, it was used in a cocktail of 90% 8998Ac with 10% FRET8998, referred to as S3.
Figure 5.3 Degradations of \textit{8998Ac} versus \textit{8998Bz} by WT \textit{hLon}. (A) HPLC traces of 8998Ac/Bz degradation by \textit{hLon} viewed at 220 nm. Reaction contained 50 μM 8998Ac substrate and 50 μM 8998Bz, 1 μM \textit{hLon}, initiated by 1 mM ATP at 37\textdegree{}C, and quenched at times indicated. Green arrow represents 8998Bz peptide substrate, red arrow represents 8998Ac peptide substrate, blue arrow represents the C-terminal SGRQK(Bz) peptide product, and orange arrow represents the N-terminal YRGITC peptide product. (B) Graphical comparison of the areas of 8998Bz (red) versus 8998Ac (blue) over time.

5.3.2 Purification of recombinant human Lon (\textit{hLon}) and mutants.

Human Lon is a homohexamer \cite{110} in which each enzyme subunit is expressed as a precursor in the cytosol and then processed into a shorter mature form when imported into the mitochondrial matrix, where Lon resides. \cite{10} In this study, the mature form of \textit{hLon} and mutants lack the first 114 amino acid residues found in the precursor protein; this truncation is chosen as the mature form based on the work by Fu and Markovitz. \cite{111} Non-his-tagged enzymes were initially purified and characterized; however, the mutants
lost activity rapidly upon storage in conditions that stabilized WT hLon. Consequently, N-terminal his-tagged enzymes were generated that allowed for purification in less than 24 hours of cell lysis. These enzymes retained ATP-dependent peptidase and proteolytic activity for at least 8 months after purification when stored at -80°C.

5.3.3 ATP-dependent peptidase activity of hLon and mutants.

The steady-state parameters for peptide degradation of the 8998 peptide by hLon have previously been determined. These parameters were used to set up the peptidase assay using the peptide substrate S3 for comparing the ATP-dependent peptidase activities of WT hLon with twelve hLon mutants that are found in CODAS patients. Figure 5.4 shows the observed rate constants (k_{obs}) for the peptidase activity of WT hLon and mutants that were assayed presumably as homo-oligomers under identical reaction conditions. Using the k_{obs}, which is the steady-state rate of peptide cleavage divided by [Lon monomer], of WT hLon as a reference point, as indicated on the graph by an orange line, any mutant above this line is more active than WT, any mutant around this line is comparably as active, and any mutant below this line is less active. It is discerned that E476A and P749S have higher activities than WT hLon whereas R679H, R672C, ΔIle927, and A670V have comparable ATP-dependent peptidase activities as WT h Lon within in experimental deviation. In contrast, R721G, P676S, W464* (a C-terminal deletion of hLon that ends at W464), G767E, S631V and A724V are noticeably less active than WT hLon.
Figure 5.4 *ATP-dependent degradation of the fluorogenic peptide S3 by WT hLon and CODAS mutants.* In each assay, 250 nM of hLon monomer (WT or mutant) was mixed with 0.5 mM of S3 (0.5-fold Km) and 1 mM MgATP. The cleavage of S3 was monitored by an increase in fluorescence at 420 nm through excitation at 320 nm. The reactions were conducted at 37°C for 600 to 900 sec. All reactions were conducted at least in triplicate on different days. The rate of peptide cleavage in each reaction was calculated from the reaction time course as described in materials and methods. Dividing the rate of peptide cleavage with monomeric enzyme concentration yield the k\text{obs} value of the respective protease. Orange line represents WT hLon activity. Blue bars represent mutants that are more active than WT, green bars represent mutants that are comparable as active as WT, red bars represent mutants that are less active than WT.

5.4 CONCLUSIONS

CODAS is a disease marked by cataracts, skeletal abnormalities, mental retardation and dental features among other various malformations. Unpublished results
indicate 12 mutations in hLon that lead to the phenotypes exhibited. Characterizing the activity of these mutations can lead to elucidation of how they cause disease, which can in turn be useful in correcting the abnormalities, and prevention of the disease in the future. Although the 8998Ac peptide does not exhibit the same degradation efficiency as the FRETN8998 peptide, it can still be used to compare the activity of mutated hLon to WT.

The variants that lead to CODAS do not show an overall trend with respect to activity. Some of the mutants exhibit more activity than WT, while others exhibit lower activity. As CODAS is a heterozygous disease, it is interesting to note that the more active variants pair up with the less active variants. As Lon oligomerizes, the oligomerization state of these mutants is unknown. It is therefore possible that two mutants oligomerize together, and in this case, the reduced activity of one monomer could reduce the activity of another monomer. This could also be true in reverse, where the increased activity of one monomer increases the activity of the other. Initial studies in our lab indicate the former; however more studies need to be undertaken to identify how these mutants oligomerize in vivo before any concrete hypothesis can be postulated. As discussed earlier, the parents of the diseased offspring contain one wild-type hLon, and one of the mutant hLon variants. The parents do not show any signs of disease, therefore it is possible the WT hLon rescues the activity of the mutant hLon. Although this would indicate the more active mutants should rescue the less active mutants, and thus not cause disease.

An overall mode of disease is still unclear, however the work presented here provides a framework for elucidating activity in other medically relevant hLon mutations.
identified. As hLon degrades full length proteins, and hydrolyzes ATP to ADP and P_i, future studies are underway to monitor the steady state kinetic parameters associated with these activities, and therefore elucidate a mode of disease.
CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS
In chapters 2-5, I discussed experiments to develop spectroscopic tools to study Lon, and the results of those tools developed. These tools will be useful in studying and characterizing Lon to elucidate structure and function. This chapter serves to sum up the experiments performed, and the data acquired, as well as to outline my current status and future directions.

In chapter 2, I determined that the his-tag does not have an effect on the degradation efficiency of λN, therefore his-tagged λN can be used to develop spectroscopic tools. I found that the N-terminal of degradation is not necessary for efficient degradation, however the C-terminal is. Due to the reduced binding of a C-terminally truncated λN mutant as discerned from figure 2.6, I propose that the reduced degradation efficiency is due to the reduced ability to bind to ELon and not a reduced ability to cleave protein. However, given that the C-terminally truncated λN was still cleaved, I propose there is another recognition sequence somewhere throughout λN that ELon uses to bind and cleave protein. Future studies will be directed towards elucidating this recognition sequence. As evidenced by the ATPase assays, the binding of λN can be studied through ATPase enhancement. Previously, our lab sought to develop an inhibitor for Lon. The peptide products of λN cleavage were used as a starting point. This inhibitor, DBN93, is only 6 acids long and contains no cleavage site, however it still stimulated ATPase similar to the peptides that do contain cleavage sites (unpublished results), indicating binding.\(^{(50)}\) This provides evidence that Lon does not need a cleavage site for recognition. Therefore if Lon is recognizing the C-terminal of λN, a peptide containing residues from the C-terminal should stimulate ATPase as well, even without a cleavage site. Previously, our lab discovered that only three amino acids in the 8998
sequence seemed important for cleavage.\(^{(71)}\) Given this, and the fact that the small DBN93 peptide stimulates ATPase, brings up an interesting question of how small a peptide can be and still stimulate ATPase. Making peptides developed from the \(\lambda N\) cleavage profile with different lengths should provide insight into this, determining if even a single amino acid can stimulate ATPase. This should help in elucidating a recognition sequence.

Peptides that do not stimulate ATPase will not be important for recognition. Fluorescent peptide substrates developed previously corresponding to the N-terminal of \(\lambda N\) (residues 11-21) showed a reduction in \(k_{\text{cat}}\), compared to peptides developed from the C-terminal. Given this, and the comparable degradation efficiency of the N-terminally truncated \(\lambda N\) compared to WT \(\lambda N\), it is anticipated that peptides developed from the N-terminal of \(\lambda N\) without a cleavage site will not stimulate ATPase to the same level as those from the C-terminal. Residues from the middle region of \(\lambda N\), from residues 35-99, must contain some kind of recognition sequence as well. Making peptides derived from this sequence with varying lengths will again help in elucidating a recognition sequence due to their ability to stimulate ATPase. Likewise, making various mutants of full length \(\lambda N\) with different residues left out and monitoring their degradation efficiency should indicate regions required for binding and hence degradation. Once these regions or residues are identified, they can be used to help determine other possible substrates of Lon. This will also help in getting a better understand of Lons role in overall cell health.

I have shown that substituting AMPPNP as the nucleotide instead of ATP disrupts the processivity of degradation, as partially digested intermediates are visualized with AMPPNP but not ATP. By using AMPPNP, the reaction is slowed down. I have
developed fluorescent substrates that exploit this slower reaction to allow for elucidation of intermediates. Based on figures 2.7 and 2.8, I propose that ELon has no preference for directionality in the cleavage of substrates.

In chapter 3, I identified the presence of a lag phase in the cleavage of a full length protein substrate with ATP and independently, AMPPNP, however it was longer with AMPPNP. Regardless of nucleotide used, the duration of this lag phase was similar for the cleavage of both terminals of λN. This further confirms that AMPPNP slows down the reaction, and also further confirms Lon has no preference for directionality in cleavage. I propose this lag phase indicates a step before cleavage. As a previous study showed this step to be translocation in a small peptide substrate, I propose the step seen here to be translocation as well. To monitor this step, the fluorescent substrates generated in chapter 2 were used to FRET with a proteolytically inactive S679W ELon mutant. Upon excitation of the Trp, the fluorescence of the dansyl moiety increases as it approaches the Trp active site. Using this assay shows fluorescence reaches a maximum before the end of the lag phase, confirming the hypothesis that the step before cleavage is translocation. This indicates protein degradation in ELon occurs through model 1 in figure 3.1, where the different sites in λN are delivered at different times, but their subsequent cleavages encounter the same rate-limiting step.

The fluorescent assay also allows for determination of directionality with respect to translocation to the active site. Based on the fluorescent time courses with ATP and independently AMPPNP, I propose the C-terminal of λN is delivered to the active site first, followed by the N-terminal. This agrees with earlier proposal that the C-terminal has some recognition elements. It is possible the C-terminal is recognized and bound and
pulled in through the central pore to the active site. If the C-terminal is not present, there would be nothing to bind and pull the rest of the protein through. Conversely, if the C-terminal is present, but not the N-terminal, ELon would bind to the C-terminal and pull it through the central pore to the active site to cleave. If the N-terminal is not present, this would not cause any problems; ELon would pull any residues that are attached to the C-terminal through to the active site. If this is the case, the recognition of the C-terminal would likely play a role in directionality. To investigate this, the tools developed here can be applied to different mutants of λN. A C-terminally truncated mutant with a dansyl moiety attached near each terminal will show translocation to a 679W active site. If it plays a role in directionality, the fluorescence would show a similar plateau regardless of the placement of the dansyl moiety. Conversely, an N-terminally truncated λN mutant should still show similar trends to the data shown in chapter 3 when a dansyl moiety is placed on either end of the mutant.

In chapter 4, I demonstrated the use of HXMS to probe the structural dynamics of ELon in solution. The crystal structures determined thus far were compared to the results of the deuterium exchange, and regions with corroborating and conflicting results were identified. Future studies of these areas are required for characterization of the HXMS results. This includes mutating the regions, either by excising out regions or mutating residues to different amino acids, and characterizing the mutants using the assays described previously. This shows that HXMS can be used as a tool to probe the structure of ELon. This tool can be used in the future to probe substrate bound ELon, as well as mutated versions of ELon and determine if the HXMS pattern is similar, and current studies are aimed at analyzing these deuteration patterns. This could indicate if the
mutations will cause a change in the structure of ELon, together with characterization techniques, the impact of these regions on ELon function could be identified. Likewise, this technique can also be applied to other homologs of Lon, including hLon with and without ATP to identify regions of interest.

In chapter 5, I demonstrated that the replacement of the benzoic acid lysine with an acylated lysine in the 8998 peptide substrate reduces efficiency of cleavage. This indicates that the side chain of lysine is important for efficient cleavage. This side chain is smaller, and less hydrophobic than the benzoic acid. This could account for the reduced efficiency. Therefore, a lysine with a side chain more similar to the size and hydrophobicity of benzoic acid, but also commercially available could be used instead in the degradation. A carboxybenzyl contains a carbonyl and a benzene ring which mimics the size of benzoic acid more closely than the acetyl group. This also makes it more hydrophobic than the acetyl group, again closer to mimicking the benzoic acid group. As such, I propose that the 8998 peptide with the side chain lysine protected by a carboxybenzyl (8998Cbz, figure 6.1) will be degraded similarly to the benzoic acid moiety. In this case, the 8998Cbz can be synthesized easier than 8998Bz, and used to avoid inner filter effect. Using the techniques already developed to identify degradation efficiency of the 8998Ac, the degradation efficiency of 8998Cbz can be monitored.
Figure 6.1 *Structure of nonfluorescent 8998Cbz.* Arrow indicates Lon cleavage site.

Using the 8998Ac substrate in peptidase assays, I have probed ATP-dependent activities of 12 mutant hLon variants implicated in CODAS. The results of this assay have not provided a similar trend overall, therefore it is unclear how the different mutations all cause the same phenotype. Future studies will be directed towards studying the ATP-dependent protein degradation activity, ATPase activity, as well as the oligomerization state of each mutant. Since CODAS is a heterozygous disease with respect to hLon, determining how the different variants communicate with each other in the cell could be important for determining the mode of disease. It is unknown if the oligomerization includes an equal number of individual monomers or if one monomer is present more than another. The best route to study the heterozygous nature is by allowing the monomers to oligomerize *in vivo* before purification.
In summary, I have developed chemical tools to monitor ATP-dependent proteolysis in Lon. These tools help provide pertinent information for elucidating the structure and function of Lon. Discovery of this structure and function leads to insights into how mutations of Lon could cause disease, and hopefully one day in the future develop strategies to rescue these mutations.
BIBLIOGRAPHY


