INVESTIGATION OF THE STRUCTURAL DETERMINANTS STABILIZING THE OPEN CONFORMATION OF THE CLPP AXIAL CHANNEL

INVESTIGATION OF THE STRUCTURAL DETERMINANTS STABILIZING THE OPEN CONFORMATION OF THE CLPP AXIAL CHANNEL

BY

John A. Alexopoulos, B.Sc

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2013) McMaster University

(Biochemistry and Biomedical Sciences) Hamilton, Ontario

TITLE: Investigation of the structural determinants stabilizing the open conformation of the ClpP axial channel

AUTHOR: John Alexopoulos, B.Sc (McMaster University)

SUPERVISOR: Dr. Joaquin Ortega

NUMBER OF PAGES: x,50

ABSTRACT

Caseinolytic protease P (ClpP) is a compartmentalized bacterial protease tightly regulated by AAA+ proteins such as ClpA and ClpX within *Escherichia coli*. It is known that the amino terminus is required to gate the axial entry pores of ClpP however the conformation adopted during activation by ClpA and ClpX has not been properly characterized. Recently it has been determined that binding of a group of antimicrobials termed acyldepsipeptides induces the open conformation of the axial channel independent of ClpA or ClpX to mediate the translocation of unfolded proteins. To determine the structural determinates required to stabilize the open conformation of the axial channel during acyldepsipeptide binding we generated amino terminal variants by site directed mutagenesis. It was found that the formation and anchoring of a β-hairpin element at the amino terminus was crucial for the effective translocation of protein substrates. These results describe the structural requirements that mediate substrate translocation during acyldepsipeptide induced activation and provide a model for the structural requirements of the ClpP amino terminus during formation of the ClpAP and ClpXP holocomplexes.

ACKNOWLEDGEMENT

I would like to thank Dr. Joaquin Ortega for giving me the opportunity to work under his supervision and experience the amazing world of research science. I much appreciate the time you have taken to challenge and help me to develop as a scientist. I would also like to thank my committee members Dr. Gerry Wright and Dr. Marie Elliot for their guidance.

In addition I would like to thank members of the Ortega lab, especially Vivian for her advice and technical assistance. I would like to thank Dr. Alba Guarné and members of the Guarné for their help and scientific advice. I would also like to thank Franklin Chan for his friendship and discussions about how to take over the world.

Finally I would like to thank my parents for their support and understanding throughout my degree.

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AAA+	ATPases associated with various cellular activities
ADEP	acyldepsipeptide
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATPγS	adenosine 5’ (gamma-thio) triphosphate
ATPase	adenosine triphosphatase
ClpA	caseinolytic Protease A
ClpP	caseinolytic Protease P
ClpX	caseinolytic Protease X
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EGFP	enhanced green fluorescent protein
PDB	protein data bank
SDS-PAGE	sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SsrA	small stable RNA A

1.0 INTRODUCTION

1.1 ClpP is a structured self-compartmentalized bacterial protease

Energy dependent proteolysis is an important cellular function mediated by complex molecular assemblies within the cell. The bacterial proteome consists of functional networks that require the regulation of not only long term but also short lived polypeptides. In *Escherichia coli* there are four sets of energy-dependent proteases known as ClpP, HslV, Lon and FtsH (Gottesman, 2003) that provide the machinery to mediate this regulation. Over 90% of the regulatory or improperly folded proteins that are degraded in *E. coli* are mediated through these four proteases (Gottesman et al, 1997). While ClpP is structurally well characterized not much is known about the functional elements required to mediate the active translocation of substrates. The focus of this work will be on the ClpP system and characterization of the structural elements required to facilitate substrate translocation.

ClpP is highly conserved in bacteria, mediating much of the intracellular protein degradation (Wong et al, 2004). Through substrate trapping experiments, ClpP has been implicated in the degradation of proteins associated with DNA metabolism, cell division, cell motility, protein biosynthesis and transcription to name a few (Flynn et al 2003, Feng et al, 2012). Biochemical studies indicate that ClpP is involved in cell division, virulence responses during macrophage invasion and haemolysis (Raju et al, 2012, Gaillot et al, 2000), flagellum biogenesis (Tomoyasu et al, 2002) and amino acid biosynthesis (Gerth

et al, 2007). In agreement with these studies deletion of ClpP leads to an accumulation of protein cellular aggregates or inclusion bodies indicating that ClpP is responsible for the degradation of a wide range of intracellular proteins (Kruger et al, 2000).

While there is a limited knowledge in the literature of the specific substrates that are targeted by the ClpP system it is clear that the process is structurally regulated. Crystal structures of ClpP have been solved from 13 different organisms. These crystal structures include ClpP from Gram negative bacteria such as *E. coli* (Wang et al,1997; Szyk and Maurizi, 2006; Bewely et al, 2006) and *Helicobacter pylori* (Kim et al, 2008) as well as Gram positive bacteria such as *Bacilus subtilis* (Lee et al, 2011c; Lee at al, 2010b) and *Staphylococcus aureus* (Geiger et al, 2011, Zhang et al,2011). The ClpP structure has also been solved for eukaryotic organisms such as *Homo sapiens* (Kang et al, 2004) where ClpP is located in the mitochondria and *Plasmodium falciparum* (Bakkouri et al, 2010). These structures reveal that in monomeric form ClpP folds into a tertiary structure composed of three well described regions defined as an amino terminus, a handle region and head domain (Figure 1A). ClpP monomers are observed to associate into heptameric rings which stack co-axially to form a tetradecameric assembly (Wang et al, 1997) (Figure 1B, C). This configuration sequesters the catalytic residues inside an internal chamber from which access is mediated by apically facing axial channels.

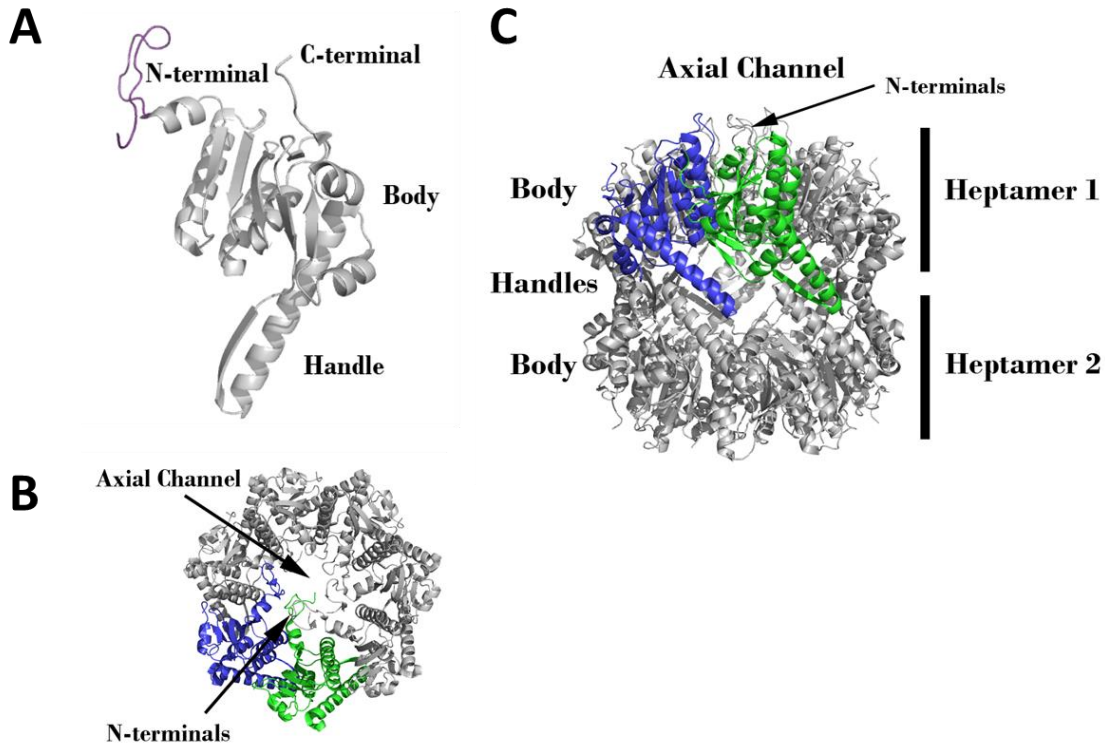

Figure 1. X-ray structure of *E. coli* ClpP

Different structural views are shown of wild type E. coli *ClpP (PDB 1YG6) solved without the ADEP compound A) Monomeric ClpP is shown indicating the location of the Handle and Head domains as well as the amino and carboxy termini B) Top view of heptameric ClpP showing the positions of each monomeric unit relative to the complex. Two separate monomeric units are shown in blue and green indicating the spacing between adjacent units. C) Tetradecameric ClpP, composed of two heptameric rings stacked co-axially between the handle regions. The handle regions are shown to intersect and pair with one another to allow for stacking of the two heptameric rings. The amino terminal residues are observed to group together and form a ring that lines the axial channels at each heptameric face.*

Substrate translocation through the axial channels of ClpP is mediated by the association of an ATPase (adenosine triphosphatase). In *E. coli* binding of the ATPases ClpA or ClpX regulates ClpP activation (Hoskins et al, 1998). The ClpP ATPases are part of the AAA+ (ATPases associated with various cellular activities) family, a group of proteins that assemble into hexameric rings and perform essential activities within the cell ranging from protein unfolding to DNA replication (Hanson et al, 2005, Ogura et al, 2001). ClpA and ClpX assemble into hexameric rings that bind to the axial ends of ClpP in an ATP-dependent manner, a process that can be replicated with non-hydrolyzable analogues of ATP such at ATPγS (Seol et al, 1995,Veronese et al, 2011). However only through rounds of ATP hydrolysis do ClpA and ClpX function to unfold and translocate cellular substrates through the axial channels of ClpP into the degradation chamber for hydrolysis (Baker et al, 2012).

Along with substrate unfolding the ATPase is responsible for targeting of substrates to the ClpP peptidase. Degradation tags termed degrons located at the N or C-termini of substrates are either directly recognized by the ATPase or indirectly recognized through adaptor protein (Sauer et al, 2011). One of the best characterized methods of substrate targeting and translocation that takes advantage of this process is the addition of an SsrA tag to the carboxyl terminus of polypeptides that are stalled on the ribosomes in order to mediate rescue of the ribosomal machinery and peptide recycling. While ClpA and ClpX can directly recognize the SsrA sequence AANDENYALAA in vitro (Gottesmen et al, 1998), SspB an adaptor protein functions to facilitate targeting of SsrA indirectly to ClpX (Flynn et al, 2001). Similarly ClpS functions as a recognition

component for ClpA-targeted substrates that follow the N-end rule pathway in *E. coli* (Schmidt et al, 2009). However ClpS binding alters ClpA substrate specificity preventing the degradation of SsrA tagged substrates and allowing for the targeting of aggregated proteins that expose hydrophobic residues (Dougan et al, 2002). While other substrate tags for ClpX such as the phage λ O protein and RepA for ClpA have been reported, much of the intracellular targeting networks are unclear (Hoskins et al, 1998).

1.2 The amino termini of ClpP protease gate access into the ClpP catalytic chamber

The amino terminal residues of ClpP have been implicated biochemically in the regulation of substrate translocation by mediating gating of the axial channels in the tetradecameric unit of ClpP. The axial channels of ClpP are wide enough to allow for the diffusion of small di or tri peptides into the catalytic chamber for hydrolysis (Maurizi et al, 1994). Peptides of this size fall below the 6-9 amino acid range of peptide products released from rounds of processive degradation (Choi et al, 2005). Conversely medium sized polypeptides ranging from 10 to 20 amino acids or longer have restricted access to the catalytic chamber. The close proximity of the amino terminal residues of ClpP at the axial channels suggests that their presence may physically gate entry of substrates. Deletion of the first 10-17 amino terminal residues from the mature monomer allows for the degradation of large unfolded polypeptides without requirement for ClpA activation although at a greatly reduced rate (Bewley et al, 2009; Jennings et al, 2008). It is also observed that site directed mutagenesis of residues along the ClpP amino terminal abrogates gating for medium sized polypeptides suggesting that not only the presence of

the amino termini but also the maintenance of functional contacts are required to stabilize a gated state (Lee et al, 2010a).

Limited information is available describing how the amino termini are structured in the open state. Comparison of the deposited crystal structures of ClpP indicates that the majority of the ClpP polypeptide folds in a well conserved arrangement maintaining the seven fold symmetric assembly, while the amino terminal regions adopt multiple conformations. In addition many of these structures do not show density for the first few residues of the amino termini as a result of poor electron density for this region suggesting that the amino termini are very motile and adopt multiple conformations during crystallization. The conformational diversity of the amino termini in these structures may be a reflection of the variable states that the amino termini naturally adopt during transition from a gated to an open state.

As first described by the Flannagan group (Bewley et al, 2006) two major orientations are presented in structures that have an observable electron density, where the amino termini are found to be in either an “up” or “down" conformation. The up conformation is characterized by the formation of an amino terminal loop mediated through residues 6-17 that reach out from the axial channel to the solvent accessible environment (Figure 2A). In this condition residues 1-7 fall back into the opening and line the axial channel. The down conformation is not as well characterized by a specific structural element or motif. In the down conformation the amino termini are not observed to extend into the apical environment above the axial pores and appear to fall back into the axial channel (Figure 2B). Comparison of the monomeric units from

published structures of ClpP reveal that the amino termini adopt conformations within these two distinct boundaries (Figure 2C). Although these static snapshots of the amino termini present models to define what the open and closed state of the axial channel may look like it is unclear what role the up or down conformations have in mediating substrate translocation during association with ClpA or ClpX.

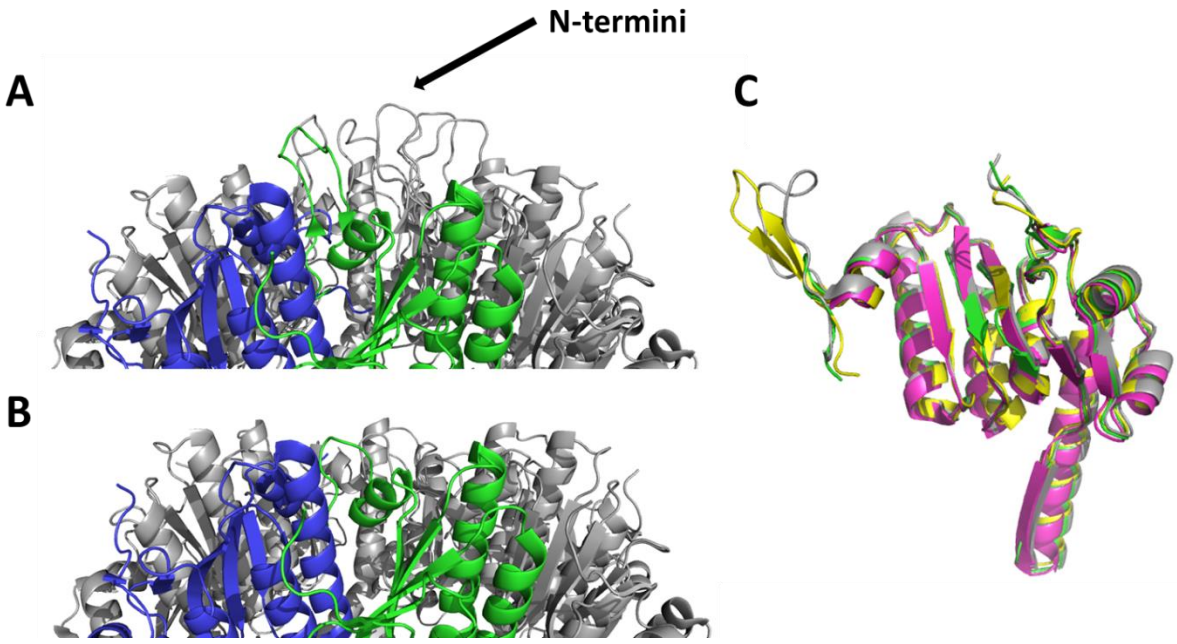

Figure 2. The amino termini of ClpP adopt structurally distinct conformations
A) Up conformation of the amino terminals are shown for E. coli ClpP (PDB 1YG6). The terminals are shown to a form a loop extending up towards the axial lumen. B) Down conformation of the amino terminals are shown for E. coli ClpP (PDB 1YG6) C) Alignment of the monomeric unit of ClpP is shown for four different structures depicting the range of conformations observed at the amino terminus. Grey:1YG6, Green: 1YTF, Purple: 2ZLO, Yellow: 3Q7H

1.3 ClpA and ClpX mediate activation of ClpP

While ClpA (Xia et al, 2002; Xia et al, 2004) and ClpX (Kim et al, 2003 ; Glynn et al, 2009) have been crystalized unassociated with ClpP a combined structural characterization has remained challenging. While ClpP is found to adopt a seven fold symmetry, ClpX and ClpA oligomerize into hexameric rings generating a symmetry mismatch upon association. This mismatch has made it difficult to elucidate how the ATPase mediates activation of ClpP during holocomplex formation as no assembled crystal structure has been solved. Although these individual structures have not directly indicated how the ATPase may structurally influence the amino terminals during association, they have aided in the identification of specific motifs present on the ATPase that are essential for ClpP activation.

Characterization of these motifs through molecular docking experiments and in vitro study has revealed the importance of two flexible extensions that are required for effective ClpP activation. It is clear that the ATPase specificity is facilitated by a highly conserved tri-peptide motif present on loops that extend outwards from the ATPase. This motif is conserved as an IGF/L consensus sequence where the last amino acid is most often allocated as a hydrophobic phenylalanine, or leucine residue (Kim et al, 2001, Singh et al 2001). It is through this IGF/L loop that ClpA or ClpX can make direct contact with ClpP and generate the formation of a holo-complex consisting of tetradecameric ClpP and hexameric ClpA/X. The IGF/L loops are suggested to dock into a hydrophobic pocket between two ClpP monomers upon ATPase binding (Joshi et al 2004). The IGF/L motif is absolutely essential for this communication as deletion of

even one IGF/L ligand on the hexameric ATPase has been reported to reduce binding affinity and ClpP activation (Martin et al 2007; Joshi et al 2004).

A second flexible region present on the ATPase has been characterized as essential for ClpP communication. The pore-2-loops which extend from the ATPase are described to come into close contact with the amino terminal residues of ClpP. Residues from the pore-2-loops can be crosslinked with the amino terminals of ClpP indicating that they orient themselves within close enough distance to associate with one another (Martin et al 2007). It has also been described that the ATP bound state of ClpX may influence up and down movements of the pore-2-loops facilitating transitional contacts between substrates with SsrA tags and the amino terminals of ClpP (Martins et al, 2008). In the absence of high resolution structures depicting static IGF/L or pore-2-loop contacts with ClpP limited information can be inferred on what effect these contacts have on the structure of the amino terminals.

Cryo electron microscopy (Cryo-EM) has been used to visualize these dynamic structural contacts with partial success. In work from the Steven's group a holocomplex structure of ClpAP indicated that extensions protruding from the C-terminal body of ClpA were sitting on the apical surface of ClpP representing the flexible IGF/L motif (Effantin et al, 2010a,b). In this structure when ClpA is associated with ClpP at a 1:1 ratio the ClpAP interface delineating the axial channel had much less density then the ClpA free ends. This agrees with biochemical studies which indicate that upon ClpA association the amino terminal regions of ClpP become more solvent exposed indicating that a structural change must occur at the amino terminus to unlock the gate and bring the

amino terminals away from the lumen or axial channel (Jennings et al 2008a). The ClpAP structure indicated that upon ClpA binding, the amino terminals of ClpP are likely oriented away from the axial channel. In the absence of an atomic resolution structure to provide a view between ClpAP and ClpXP interfaces the amino terminal structure in the active conformation could not be elucidated.

1.4 Structural elucidation of the open conformation of the ClpP axial channel

Recently a class of bacterial natural products termed acyldepsipeptides (ADEPs) have emerged aiding in the understanding of how ClpP mediates a transition from a gated to ungated state. The ADEPs were first isolated from the fermentation broth of a strain of the actinomycetes bacteria *Streptomyces hawaiiensis* (Michel and Kastner, 1985). ClpP was determined to be the target of the ADEPs by the Brotz-Oesterhelt group (Brotz-Oesterhelt et al, 2005).. In the presence of the ADEPs ClpP became activated to hydrolyze unfolded substrates *in vitro* that were previously not degraded unless ClpA was present (Brotz-Oesterhelt et al, 2005). It was unclear how the ADEPs were activating ClpP, however it was observed that these compounds associated with ClpP in a manner that excluded the association of ClpA or ClpX. It was also observed that in the case of *B. subtilis* ClpP, ADEP association promoted oligomerization into the tetradecamer form (Kirstein et al 2009). This observation indicated that the ADEPs interacted with ClpP through contacts that were either utilized by the ATPases or induced a structural change in ClpP that reduced or abolished ATPase binding. In the absence of a mode of action it

was postulated that the ADEPs provided some structural change at the amino terminus of ClpP upon ADEP binding to facilitate a transition from a gated to open axial channel.

Two crystal structures were solved of *E .coli* and *B. subtilis* ClpP bound with the ADEPs revealing the activation mechanism of ADEP binding (Li et al, 2010, Lee et al 2010b). ADEPs were observed to bind in the same hydrophobic pocket utilized by the IGF/L motif of ClpA. This finding validated that the ADEPs mimicked ATPase binding and suggested that ClpP activation occurred in a manner similar to that of ClpA or ClpX. Consequently the amino terminal regions of ClpP in the two structures revealed that ADEP binding induced a structural change at the axial regions of the enzyme. Both structures indicated that the axial channel widened upon ADEP binding. ADEP binding appeared to facilitate an enlargement of the axial channel from the gated state of 10-12Å to approximately 20Å in the open state. This structural observation explained why activated ClpP functioned to hydrolyze unfolded substrates and not complex folded proteins.

Closer inspection suggested two different mechanisms to explain how ADEP binding altered the amino terminal structure to facilitate opening of the axial channels. In the *B. subtilis* structure the amino terminals were absent suggesting that they became very motile and disorganized facilitating enlargement of the axial channel (Lee et al 2010b). The *E. coli* structure presented amino terminals in an upright β-hairpin conformation suggesting that the amino termini form an organized channel upon ADEP1 binding (Li et al, 2010). Formation of the β-hairpin appeared to be facilitated by hydrogen bonds along the carbon alpha backbone from residues 6-10 and 14-17. The hairpin also appears to be

stabilized by intramolecular (Glu 8, Arg 15, Lys 25) and intermolecular contacts (Glu 14, Arg 15) that appeared to keep the hairpin from falling back into the axial channel (Figure 3A, Figure 3B). Additionally a hydrophobic cluster of residues consisting of Phe 49, Leu 24 Ile 19 and Val 6 appeared to anchor the base of the hairpin in between the globular head domains of the heptamer (Figure 3 C). The hairpin conformation appeared to agree with the previous observations that the amino terminals can adopt either an "up" or "down" orientation.

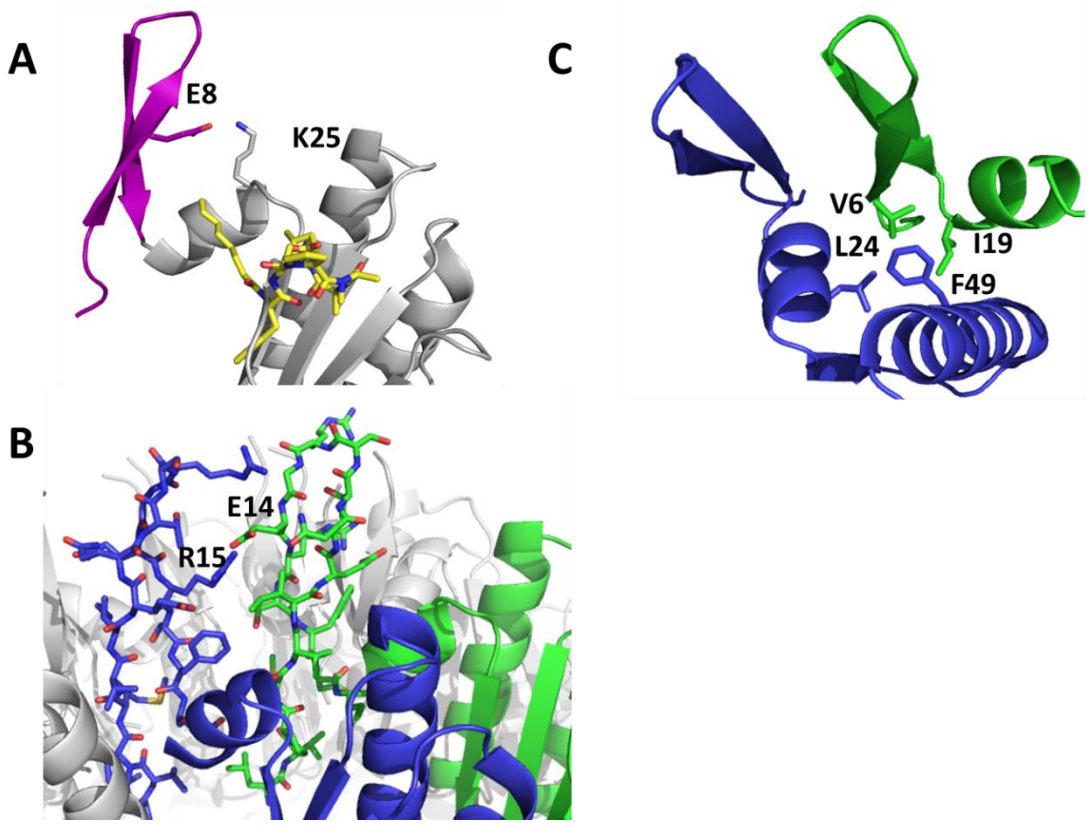

Figure 3. Structural elements of the amino termini observed to stabilize the open conformation of ClpP

The amino termini in the E. coli structure appear to coordinate with one another to form intra and intermolecular contacts that facilitate stabilization of the hairpin in an

upright conformation. A) Glutamic acid 8 is shown to pull the hairpin upright away from the axial channel through intramolecular interactions. B) Glutamic acid 14 and arginine 15 are shown to stabilize the upright hairpin by intermolecular interactions between adjacent monomers. Alignment of the carbon backbone also show positioning of the amino and carboxyl groups for secondary structure formation of the β-hairpin. C) The residues implicated in mediating hydrophobic interactions to stabilize the base of the amino terminal residues of ClpP are shown. Valine 6 is shown to orient into a cleft between isoleucine 19 on the same monomer and leucine 24 and phenylalanine 49 of the adjacent monomer.

Here we characterize the structural determinates of the amino terminus of ClpP required to stabilize the open conformation of the axial channel. We observed that a set of electrostatic interactions composed of glutamic acid 8, glutamic acid 14, arginine 15 and lysine 25 provided moderate stabilization of the open conformation observed to participate in the formation of the β-hairpin visualized in the ADEP1 bound structure of *E.coli* ClpP. Significant destabilization of the open conformation as measured by reduced substrate hydrolysis was achieved by the substitution of amino acids predicted to abolish secondary structure formation of the β-hairpin in this region. Destabilization of the anchoring contacts at the base of the β-hairpin was also observed to reduce substrate translocation. Partial to full recovery of substrate translocation by ClpA indicated that the ATPases also contributes to the structural stability of the open conformation. In addition targeting of the structural determinates observed to stabilize the open conformation of the axial channel were required for efficient gating of smaller peptides.

2.0 MATERIALS AND METHODS

2.1 Isolation of ADEP1 from *Streptomyces hawaiiensis*

ADEP1 was a kind gift from Dr. Yi-Qiang Cheng, University of Wisconsin-Milwaukee and was purified to 95% homogeneity from *Streptomyces hawaiienesis* strain NRRL 15010 according to the patent from (Michel and Kastner, 1985) with modification (Li et al, 2010).

2.2 Cloning

E. coli *clpP* in a pET9a expression vector was a gift from Dr. Walid Houry. Amino terminal mutants were generated from wild type *E .coli* clpP in a pET9a expression vector with Pfu turbo and designed primers following the QuikChange site-directed mutagenesis method (Stratagene). The *clpA M169T* gene hosted in a pET9a expression vector as a BamHI-NdeI insert was a kind gift from Dr. Walid Houry. *clpA M169T* encodes a mutation to stop internal translation initiation and that occurs in the wildtype variant (Seol et al, 1995). clpA M169T is referred to as *clpA* in the thesis. *clpA* was extracted from a pET9a vector with the restriction enzymes BamHI and NdeI, purified by gel extraction (Qiagen) and ligated into a pET15b expression vector. The amino terminal polyhistidine tag was removed from the pET15b vector by cloning a second NdeI site following the QuikChange site-directed mutagenesis method (Stratagene) to replace the NcoI restriction site 5 prime to the histidine tag. Removal of the histidine tag was performed with digestion by NdeI, and the ORF containing pET15b

vector was purified by gel extraction and ligated to itself. The final product was a pET15b-clpA which was used to purify ClpA without an affinity tag.

E. coli ftsZ cloned into a pCA24N vector was a gift from Dr. Eric Brown previously cloned as part of an ASKA library (Kitagawa et al, 2005).

EGFPSsrA (Clonetech) was cloned in a pProEX Htb vector with an amino terminal polyhistidine tag and a C-terminal SsrA (AANDENYALAA) tag according to Iwanczyk et al, 2007.

2.3 Protein expression and purification

All proteins were expressed in E. coli BL21 (DE3) cells (with the exception of FtsZ). First cells were grown in a 50 ml starter cultures overnight at 37^{o}C with appropriate antibiotics. Once saturated overnight a 1 in 100 inoculation was prepared in 1 L culture volumes at 37^{o}C with appropriate antibiotics until an OD_{600} of 0.6 was reached. Cells were induced for 3 hours (5 hours for EGFPSsrA) and harvested by centrifugation at 3,700 g for 10 minutes at 4^{o}C. The cell pellets were washed with 1X phosphate buffered saline, pelleted again at 3,700 g for 10 minutes at 4 ^{o}C and flash frozen with liquid nitrogen before being stored at -80^{o}C. On the day of purification the cell pellets were suspended in the appropriate lysis buffer on ice and lysed three times with a chilled French Press at 20,000 lb/in^{2}. Lysates were centrifuged at 30,000 g for 40 minutes at 4^{o}C to clear cellular debris and supernatants were filtered through a 0.45 µM syringe to remove particulates before loading on the appropriate column.

Cells overexpessing wild type or variants of ClpP were lysed in buffer A (50 mM Tris-HCl pH 7.5, 150 mM KCl, 10% glycerol and 1 mM dithiothreitol (DTT)). Filtered cellular lysates were loaded onto a QHP (GE Healthcare) column (3 times 5ml) previously equilibrated with buffer A. The column was washed with 5 % buffer B (50 mM Tris-HCl pH 7.5, 1000 mM KCl, 10% glycerol and 1 mM DTT) and ClpP was eluted with an increase of buffer B concentration to 25%. Fractions containing ClpP were pooled and diluted by one fold in buffer containing 50 mM Tris-HCl pH 7.5 and 10% glycerol before being loaded onto an 8 ml MonoQ 10/100 (GE Healthcare) pre-equilibrated in buffer A. ClpP was eluted with a gradient from 0-25% buffer B. Fractions containing the purest (greater than 95% ClpP) amounts of ClpP were pooled as determined by analysis with SDS-PAGE and Coomassie blue staining. Pooled fractions were concentrated to a 500 µl volumes with a 10 kDa molecular weight cutoff filter (Amicon) and loaded onto an S200 (GE Healthcare) size exclusion column pre-equilibrated with buffer C (50 mM Tris-HCl pH 7.5, 200 mM KCl and 10% glycerol). ClpP was eluted observed to elute at approximately 12 ml corresponding to a 300 kDa protein. Elutions were verified for purify by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. Pure fractions were concentrated using a 10 kDa molecular weight cutoff filter (Amicon), flash frozen in liquid nitrogen and stored at -80^{o}C.

Proteolitically inactivated variants of ClpP (ClpPin) were purified as above and treated with carbobenzoxy-Leu-Tyr-chloromethylketone (Bachem) adapted from Singh et al (Singh et al, 2000). Twenty milligrams per millilitre of the chemical inactivator was

prepared in dimethyl sulfoxide (DMSO) and added to a solution of ClpP at a 5 molar excess in relation to $ClpP_1$ as a monomer. Solutions were kept at room temperature for approximately 40 minutes with slight agitation to promote mixing, followed by centrifugation at 13,000 g for 10 minutes at 4°C. Excess chemical inactivator was removed by diluting approximately 300 µl of sample into 5 ml of buffer C and concentrating in a 10 kDa molecular weight cutoff filter (Amicon) for a total of 3 times to ensure removal of the carbobenzoxy-Leu-Tyr-chloromethylketone reagent.

ClpA containing cells were lysed in buffer D (50 mM Tris-HCl pH 7.5, 100 mM KCl, 1 mM DTT and 5% glycerol). ClpA was precipitated with a drop wise addition of 100% saturated ammonium sulfate to a final concentration of 50% ammonium sulfate. After 1 hour of spinning at 4°C the lysate was cleared by centrifugation at 3,700 g for 20 minutes and the pellet was suspended in buffer D and dialysed overnight. The dialysate was filtered through a 0.45 syringe filter and loaded onto a 5ml sulfopropyl high performance column (GE Healthcare) pre-equilibrated with buffer D. ClpA was eluted with a linear gradient from 0% buffer D to 65% buffer E (50 mM Tris-HCl pH 7.5, 1000 mM KCl, 1 mM DTT and 5% glycerol), concentrated and loaded onto an S75 column (GE Healthcare) equilibrated in buffer D. ClpA containing fractions were pooled, loaded onto a MonoS (GE Healthcare) equilibrated with buffer D and eluted with a linear gradient from 0-65% buffer E over 8 column volumes. Elutions were verified for purify by SDS-PAGE and Coomassie staining. Pure fractions containing ClpP were flash frozen in liquid nitrogen and stored at -80°C.

Cells overexpressing FtsZ in *E. coli* AG1 were lysed in buffer F (50 mM Tris-HCl pH 7.5, 500 mM NaCl and 5% glycerol) and loaded onto a 5 ml Ni-NTA column (GE Healthcare). The column was washed with increasing concentrations of buffer G (50 mM Tris-HCl pH 7.5, 500 mM NaCl , 300 mM imidazole and 5% glycerol) and eluted with 80% buffer G. Fractions containing FtsZ were pooled, dialyzed in buffer A and frozen at -80^{o}C.

EGFPSsrA containing cells were lysed in buffer F (50 mM Tris-HCl pH 7.5, 500 mM NaCl and 5% glycerol) and loaded onto a 5 ml Ni-NTA column (GE Healthcare) previously equilibrated with buffer F. The column was washed with with increasing concentrations of buffer G (50 mM Tris-HCl pH 7.5, 500 mM NaCl , 300 mM imidazole and 5% glycerol) and eluted with 80% buffer G. Elutions were diluted five to one in buffer H (50 mM Tris-HCl pH 7.5, 100 mM NaCl and 5% glycerol) and loaded onto a previously equilibrated 5 ml quaternary ammonium high performance column (GE Healthcare). The column was washed with increasing concentrations of buffer I (50 mM Tris-HCl pH 7.5, 1000 mM NaCl and 5% glycerol) and eluted with a gradient from 0-60% buffer I. Pure fractions of EGFPSsrA as verified by SDS-PAGE and Coomassie staining were concentrated with a 10 kDa molecular weight cutoff filter (Amicon), flash frozen in liquid nitrogen and stored at -80^{o}C.

2.4 Peptides and protein substrates

Fluorescein isothiocyanate (FITC) labeled α-casein (bovine) was purchased from Sigma. In this system FITC is the fluorophore labeled on primary amines of α-casein.

Upon excitation at 490 nm neighbouring FITC molecules act as a quencher through Forster Resonance Energy Transfer. Separation by cleavage of α-casein can be monitored by the emission of light at 520 nm. N-Succinyl-Leu-Tyr-7-amido-4-methylcoumarin (Suc-LY-AMC) was purchased from Sigma. The 11mer peptide Abz-KASPVSLGDY^{NO2} was synthesized chemically (Genscript). In this system Abz (2-aminobenzoic acid) is the fluorophore while Y^{NO2} (3-nitrotyrosine) is the quencher (Lee et al, 2010a). Cleavage of the 11mer peptide results in separation of the fluorophore quencher pair as measured by an increase in fluorescence at 420 nm when excited at 320 nm.

2.5 Peptidase and protease activity assays

Kinetic reactions were performed at 30^{o}C and components were incubated for 5 minutes at 30^{o}C before starting. Reactions were started with the addition of substrate of addition of ClpA in the case of ClpA assays. Reactions containing ADEP1 were added from a stock solution in DMSO to a final concentration of 10 µM. Reactions not containing ADEP1 were supplemented with an equivalent amount of DMSO. Reactions containing ClpA contained a mixture of 4 mM ATP and utilized an ATP regeneration mixture containing 3.2 mg/ml creatine phosphate and 0.4 mg/ml creatine phosphokinase (Sigma). When present ClpP or variants were at a final concentration of 0.14 µM ($ClpP_{14}$) and when required ClpA was utilized at a final concentration of 0.56 µM ($ClpA_6$).

Assays utilizing Suc-LY-AMC as a substrate contained final concentrations ranging from 50-1000 µM for steady state kinetic analysis in a final volume of 75 µL. 11mer peptide assays utilized a substrate concentration of 1000 µM with a final volume of 75 µL. Reactions were performed in a buffer containing 25 mM HEPES (pH 7.25), 100 mM KCl, 10 mM $MgCl_2$, and 10% glycerol. Reactions utilizing FITC-α-casein and EGFPSsrA were performed in a volume of 50 µL and contained a final substrate concentration of 2 mg/ml and 10 µM respectively. Reactions for these two substrates contained a buffer consisting of 25 mM HEPES (pH 7.25), 100 mM KCl, 10 mM $MgCl_2$, and 10% glycerol respectively.

Reactions were monitored by changes in fluorescence (Suc-LY-AMC: excitation = 345 nm, emission = 440 nm, 11mer peptide: excitation 320 nm, emission = 420 nm, FITC α-casein : excitation 490 nm, emission 520 nm, EGFPSsrA: excitation 488, emission 510 nm) with a Tecan Safire spectrophotometer. Reactions were assembled in a transparent polystyrene 96 well plate. Values for K_m, and V_{max} utilizing Suc-LY-AMC were calculated by non-linear regression analysis using the program GraFit (7.0, Erithacus software).

FtsZ assays and ClpP stability were assembled in 100 µl reaction volumes and contained 0.14 µM $ClpP_{14}$ in buffer containing 25 mM Hepes pH 7.25, 100 mM KCl, 10 mM $MgCl_2$, 10% glycerol. Reactions were incubated on ice with a 5 M excess of ADEP1 (10 µM) solubilized in DMSO. Control reactions contained equivalent amounts of DMSO. Reactions were started with the addition of 4 µM FtsZ or added to a water bath in the case of stability assays, incubated at 37^{o}C and quenched with hot SDS at

various timepoints. Proteins were resolved by SDS-PAGE on 15% polyacrylamide gels and stained with Coomassie brilliant blue.

2.6 Kinetic analysis

All mutants were characterized by calculating the initial rates of product formation (or product disappearance in the case of EGFPSsrA) from 100-300 seconds in triplicate. The Relative Fluorescence Units (RFU) were plotted per second for this timeframe and normalized by subtraction of the RFU value at 100 seconds from the whole data set. To determine the initial rate for each replicate the RFU at 300 seconds was subtracted from the RFU at 100 seconds and divided by the difference in time.

3.0 RESULTS

In the *E. coli* ClpP structure activated with ADEP1 the amino termini were observed to be stabilized in an upright conformation consisting of a β-hairpin comprised by residues 6-17. Three primary elements appeared to be important in mediating a structured axial channel. Firstly the hairpin element appeared to be stabilized by a set of intramolecular and intermolecular electrostatic interactions. The intramolecular interactions appeared to be facilitated by hydrogen bonds between glutamic acid 8 and lysine 25 while the intermolecular interactions appeared to be facilitated by hydrogen bonds between glutamic acid 14 and arginine 15. Secondly the β-hairpin secondary structure appeared to be stabilized by a network of hydrogen bonds along the backbone of the Cα carbons. The β-hairpin appeared to be anchored into a hydrophobic cluster

formed by residues valine 6, isoleucine 19, leucine 24 from one monomer and phenylalanine 49 from an adjacent monomer. In order to determine the requirements of these observed contacts to mediate stabilization of the open conformation of the axial channel a series of mutants were generated and assayed with various substrates.

3.1 Electrostatic interactions contribute modestly to the stabilization of the open conformation of the axial channel

To determine the role of the intramolecular and intermolecular electrostatic interactions in mediating the open conformation of the axial channel, amino terminal point mutants were generated by site directed mutagenesis and assayed for the degradation of FITC α-casein. FITC α-casein is an unfolded substrate that is degraded by ClpP when activated with ADEP1, and does not depend on ClpA for unfolding. When the electrostatic charges of the intramolecular interactions were altered by point mutation of glutamic acid 8 to lysine (E8K) or lysine 25 to glutamic acid (K25E) ClpP was observed to degrade FITC α-casein at a reduced hydrolysis rate in the presence of ADEP1 to about 50 percent that of wild type (Figure 4A). In an attempt to determine the contributions mediated by the intramolecular interactions glutamic acid 14 and arginine 15 were mutated to alanine as a double mutant (E14A+R15A). The E14A+R15A double mutant was observed to degrade FITC α-casein at a hydrolysis rate indistinguishable from wild type in the presence of ADEP1 (Figure 4A). However the triple mutant E14A+R15A+K25A was observed to degrade FITC α-casein at a hydrolysis rate around 50 percent relative to wild type (Figure 4A) suggesting that the intramolecular contacts

were utilized during stabilization of the open conformation while the intermolecular contacts were dispensable.

3.2 Electrostatic interactions contribute to the stabilization of the closed conformation of the axial gate

It had been observed in the literature that mutation of the amino terminal residues unlock the gating mechanisms that functions to seclude access of polypeptides to the ClpP catalytic chamber (Lee et al, 2010). The electrostatic interaction targeting mutants were tested for background degradation of FITC α-casein in the absence of the activator ADEP1. It was observed that while E8K had partial degradation amounting to approximately 6 percent the hydrolysis rate of wildtype activated with ADEP1, the gating mechanism of the other mutants was not visibly disrupted (Figure 4A). This observation disagreed with previously published results utilizing a smaller 10mer substrate which indicated that peptides of this length have enhanced access to the catalytic chamber during amino terminal mutation (Lee et al, 2010). FITC α-casein requires a significant structural rearrangement of the amino terminals by either ADEP1 or ClpA activation, and it was postulated that such a large substrate cannot be utilized as a tool to study small changes in the gating of the axial channel mediated by the amino terminus.

In order to probe for defects in gating of the axial channel an 11mer peptide was assayed which has minimal access to the catalytic chamber when the amino terminals stabilize a closed conformation. The basal hydrolysis rate of the 11mer peptide by wild type is barely detectable and observed to be at approximately 4 percent that of wild type

activated with ADEP1 (Figure 4B). Interestingly the mutants (K25E,E8K, E14A+R15, K25A+E14A+R15A) were observed to mediate a background hydrolysis rate of 11mer degradation from approximately 2 to 20 times the basal rate of wild type without ADEP1 (Figure 4B). ADEP1 was however observed to activate each of the mutants to degrade the 11mer peptide at a hydrolysis rate higher then background indicating that ADEP1 mediated activation was likely of a different structural quality than the gate disrupted state (Figure 4B). These results indicated that the electrostatic interactions were required to stabilize the closed conformation of the axial gate.

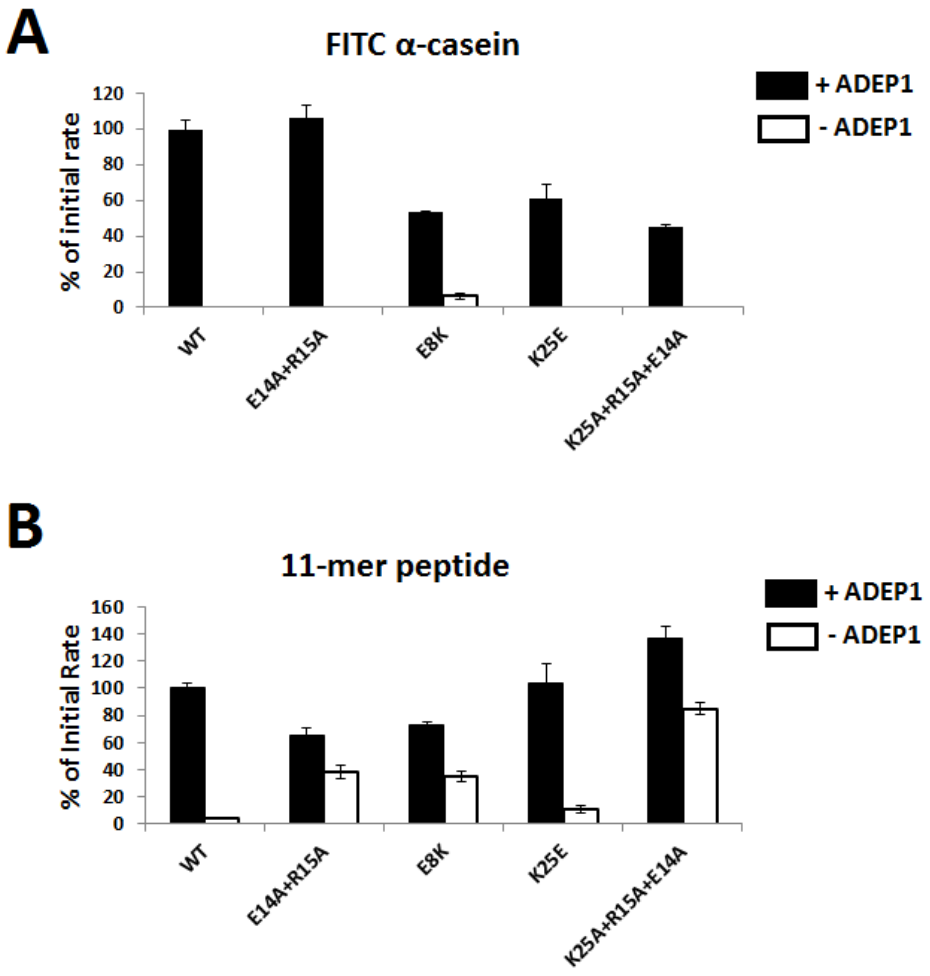

Figure 4. The contribution of electrostatic interactions in stabilization of the open conformation and gating of the axial channel

Initial rates of substrate degradation with ClpP amino terminal mutants plotted relative to wild type representing 100% activity. A) Rate of FITC α-casein hydrolysis in the presence and absence of ADEP1. Reactions contained 0.14 μM of $ClpP_{14}$, 10 μM of ADEP1 and 2 mg/ml of FITC α-casein. B) Rate of 11mer peptide hydrolysis in the presence and absence of ADEP1. Reactions contained 0.14 μM of $ClpP_{14}$, 10 μM of ADEP1 and 1000 μM of 11mer peptide. Bars represent a mean ± standard deviation of three replicates.

3.3 Maintenance of the β-hairpin element is essential for the stabilization of the open conformation of the axial channel

A second set of contacts were present in the *E. coli* ClpP ADEP1 activated structure that appeared to be equally responsible for stabilization of the open conformation. Hydrogen bonding along the backbone carbons of residues 6-17 mediate the formation of the structured β-hairpin element which retracted the amino-terminals away from the lumen of the axial channel (Figure 3B). To determine the role of secondary structure formation in mediating efficient substrate translocation a series of glycine point mutants were generated along the β-hairpin region.

Mutation of glutamic acid 8 to glycine (E8G) and lysine 25 to glycine (K25G) was observed to moderately reduce the hydrolysis rate of FITC α-casein to around 70 percent relative to the wild type hydrolysis rate (Figure 5A). The contribution of these residues in stabilizing the hairpin element appeared to be comparable to the effects of targeting the electrostatic interactions. However mutation of glutamic acid 14 and arginine 15 to glycine (E14G+R15G) in the form of a double mutant significantly

reduced the rate of FITC α-casein degradation, a result that was not observed when these same residues were mutated to alanine which displayed a wild type phenotype (Figure 5A). When both of the contacts stabilizing the electrostatic interactions were substituted by glycine in the form of a K25G+R15G+E14G mutant, the hydrolysis rate of FITC α-casein was reduced to below 90 percent in the presence of ADEP1. This result indicated that glycine incorporation along the amino terminal region compromised the functionality of the open conformation.

Having observed that secondary structure formation was likely important in mediating stabilization of the open conformation, two amino terminal mutants were generated in order to severely disrupt formation of the β-hairpin element. The first mutant was generated with glycine residues from positions 9 to 15 (9-15G) to interrupt the hydrogen bonding network along the backbone carbon. The second consisted of an isoleucine 7 to proline mutation in order to produce a bend in the first portion of the hairpin and disrupt its formation. It was observed that both of these mutants degraded FITC α-casein at a hydrolysis rate significantly below the wild type hydrolysis rate in the presence of ADEP1 with no observable background degradation (Figure 5B). Interestingly these secondary structure targeting mutations also disrupted the gating mechanism resulting in up to a 24 fold increase in basal 11mer peptide degradation but required ADEP1 to achieve maximal degradation rates (Figure 5B). These results suggested the β-hairpin is required for the open conformation of the axial channel to efficiently translocate protein substrates.

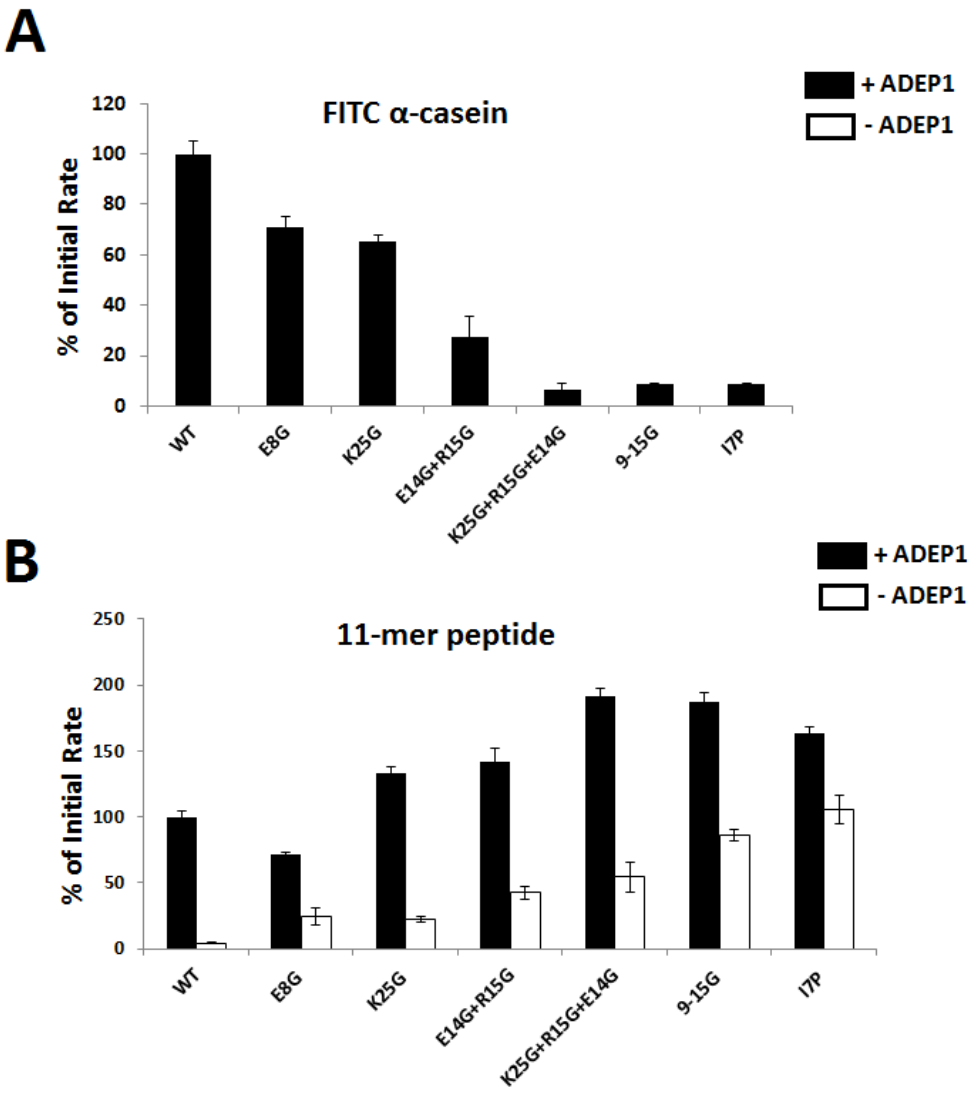

Figure 5. Contribution of an amino terminal β-hairpin in stabilization of the open conformation and gating of the axial channel

Initial rates of substrate degradation with ClpP amino terminal mutants plotted relative to wild type representing 100% activity. A) Rate of FITC α-casein hydrolysis in the presence and absence of ADEP1. Reactions contained 0.14 μM of $ClpP_{14}$, 10 μM of ADEP1 and 2 mg/ml of FITC α-casein. B) Rate of 11mer peptide hydrolysis in the presence and absence of ADEP1. Reactions contained 0.14 μM of $ClpP_{14}$, 10 μM of

ADEP1 and 1000 μM of 11mer peptide. Bars represent a mean ± standard deviation of three replicates.

3.4 Anchoring of the β-hairpin is an essential element at the amino termini during the stabilization of the open channel

A third set of stabilizing contacts were observed at the base of the hairpin. This stabilizing region appeared to be formed by the association of hydrophobic residues composed of valine 6, isoleucine 19, leucine 24 and phenylalanine 49 between two adjacent monomers (Figure 3C). It was hypothesized that this region functioned to act as an anchoring point for the base of the hairpin mediating stabilization of the β-hairpin element perpendicular to the axial lumen.

Mutation of isoleucine 19 to alanine appeared to significantly reduce the degradation rate of FITC α-casein in the presence of ADEP1 (Figure 6A). While there was no observed background degradation of α-casein in the absence of ADEP1, isoleucine 19 was also observed to be responsible for mediating locking of the axial gate. Mutation to alanine resulted in an increase in background degradation of the 11mer peptide to approximately 20 times that of wild type without ADEP1 (Figure 6B). This indicated that like the other mutants disruption of the structural elements mediating the stabilization of the open conformation had an alternate consequence of disrupting substrate gating.

While I19A was observed to disrupt the stability of the open conformation it was unclear whether formation of the β-hairpin element was compromised during targeting of this region of ClpP. Interestingly when either the 9-15G or I19A mutants were incubated

with ADEP1 at 37°C for 24 hours and analyzed by gel electrophoresis a degradation fragment was observed to appear in a time dependent manner (Figure 6C). Production of a lower fragment was not observed in the control reaction without ADEP1 or with wild type ClpP (Figure 6C). This observation suggested that removal of the hairpin stabilizing contacts facilitated a structural instability at the amino terminal region of ClpP evident by enhanced auto-processing in these mutants in the presence of ADEP1.

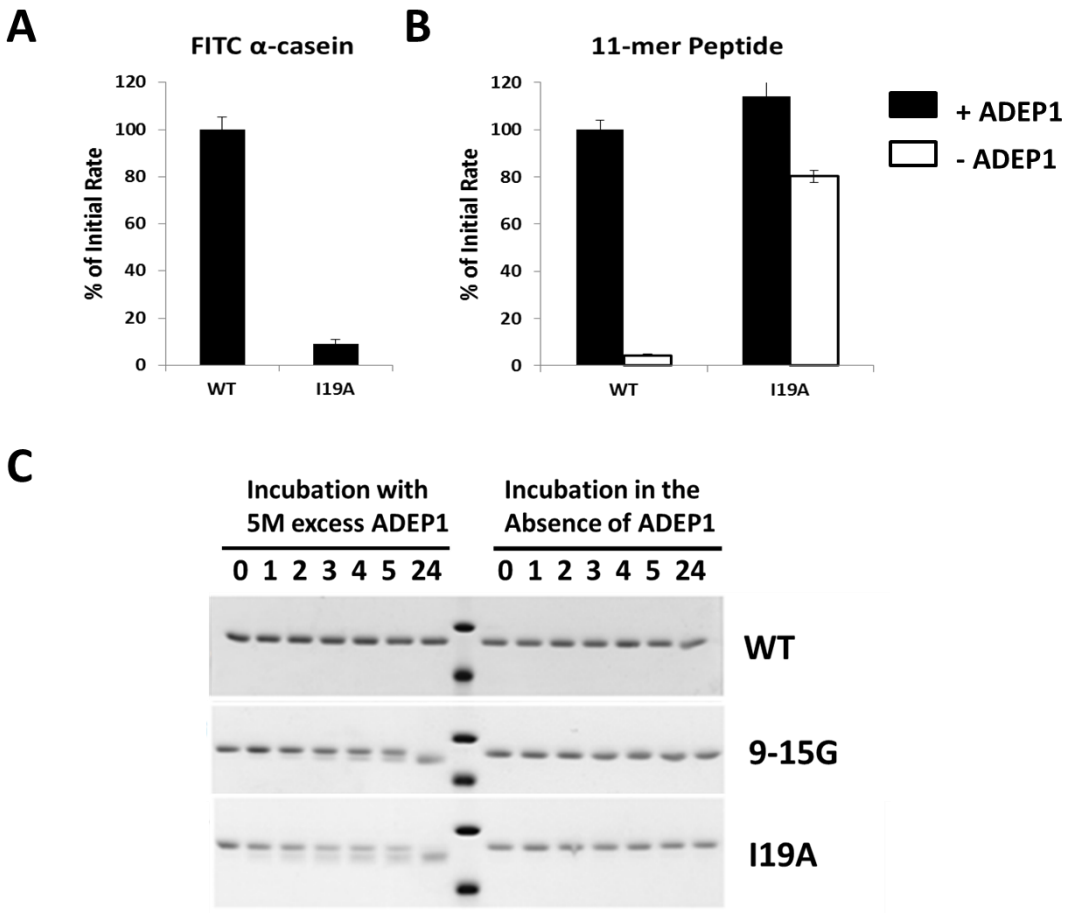

Figure 6. Contribution of anchoring of the β-hairpin in stabilization of the open conformation and gating of the axial channel.

A) Rate of FITC α-casein hydrolysis in the presence and absence of ADEP1. Reactions contained 0.14 μM of $ClpP_{14}$, 10 μM of ADEP1 and 2 mg/ml of FITC α-casein. B) Rate

of 11mer peptide hydrolysis in the presence and absence of ADEP1. Reactions contained 0.14 µM of $ClpP_{14}$, 10 µM of ADEP1 and 1000 µM of 11mer peptide. C) Wild type ClpP, 9-15G and I19A were incubated at 37°C containing 0.28 µM of $ClpP_{14}$ and 20 µM of ADEP1. Aliquots were removed at the indicated time points and boiled with hot SDS. Samples were resolved on a 15% polyacrylamide gel and stained with Coomassie for visualization. Bars represent a mean ± standard deviation of three replicates.

3.5 The hairpin stabilizing contacts are required for degradation of physiological substrates

While FITC α-casein was utilized as a substrate to test for translocation defects of ADEP1 activated ClpP mutants, it did not represent a physiological polypeptide that is hydrolyzed *in vivo*. Recently FtsZ has been identified as a substrate for ADEP1 activated ClpP and has also been determined as a target for ClpXP during cell division (Sass et al, 2011). *E. coli* FtsZ was purified and utilized as a physiological substrate to determine if the structural determinants of the axial channel were required to translocate physiological substrates.

In the presence of ADEP1 ClpP was observed to degrade *E. coli* FtsZ over a period of 5 hours at 37°C (Figure 7). In the same experimental conditions FtsZ remained intact in the presence of wild type ClpP but without activation by ADEP1 (Figure 7). When the β-hairpin targeting mutants I7P, 9-15G , E14G+R15G+K25G or I19A were tested in the same conditions, limited to no degradation of FtsZ was observed (Figure 7). This indicated that the structural elements required to stabilize the open conformation of the axial channel were required to efficiently translocate physiological substrates such as FtsZ.

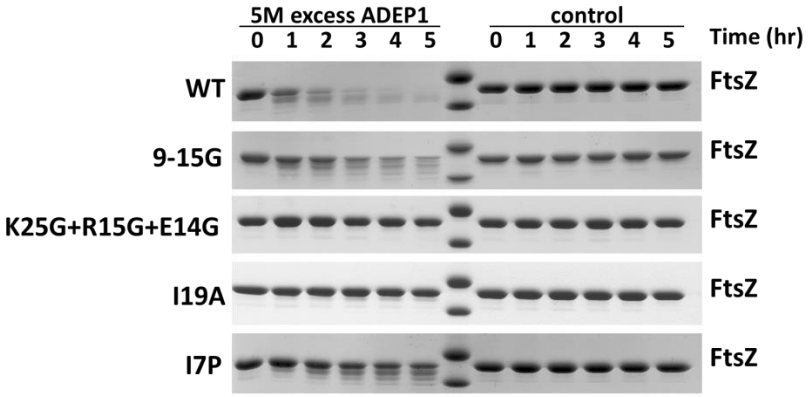

Figure 7. *E. coli* FtsZ degradation by ClpP amino terminal variants.
Wild type ClpP and amino terminal variants were incubated at 37^{o}C containing 0.28 µM of $ClpP_{14}$,20 µM of ADEP1 and 4 µM of FtsZ. Aliquots were removed at the indicated time points and boiled with hot SDS. Samples were resolved on a 15% polyacrylamide gel and stained with Coomassie blue for visualization.

3.6 Translocation deficiency of amino terminal mutants is not an artifact of reduced ADEP1 binding

Formation of an amino terminal β-hairpin appeared to be an important structural element in mediating ADEP1 dependent stabilization of the ClpP open conformation. Targeted disruption of this element by mutation appeared to significantly reduce the function of ClpP to translocate substrates such as FITC α-casein and FtsZ. However it was unclear whether the observed impairment of α-casein degradation was dependent directly on the structural stability of the amino terminal mutants to form an open conformation of the axial channel or a consequence of reduced ADEP1 binding affinity.

In order to elucidate which of the two processes was responsible competition assays were performed with the most severely attenuated mutants and ADEP1.

Chemically inactivated amino terminal mutants were prepared by treatment with carbobenzoxy-Leu-Tyr-chloromethylketone, a compound which irreversibly inhibits serine proteases. Chemical inactivation was satisfactory resulting in removal of free inhibitory compound from solution and minimal to undetectable peptide cleavage activity as measured with 200 µM of a dipeptide substrate that can freely diffuse into the catalytic chamber (Figure 8A). The chemically inactivated mutants were then assayed for their function to bind and compete for ADEP1 in a reaction containing 0.14 µM $ClpP_{14}$ and 10 µM ADEP1. Increasing the concentration of chemically inactivated wild type up to an excess of 16:1 $ClpPin_{14}$:$ClpP_{14}$ resulted in a significant reduction of α-casein cleavage indicative of competition for ADEP1 between wild type ClpP and the inactivated wild type variant (Figure 8B). Assay of the chemically inactivated β-hairpin formation targeting mutants I7P and 9-15G revealed that these mutants functioned to associate with ADEP1 and thus compete with wild type for free compound (Figure 8B). It was observed that the hairpin anchoring mutant I19A was also able to compete for ADEP1 against wild type ClpP. Competition with wild type ClpP for ADEP1 suggested that the limited degradation of FITC α-casein observed with the mutants was a direct consequence of the amino terminals to adopt an open conformation and not an artifact of reduced binding by ADEP1.

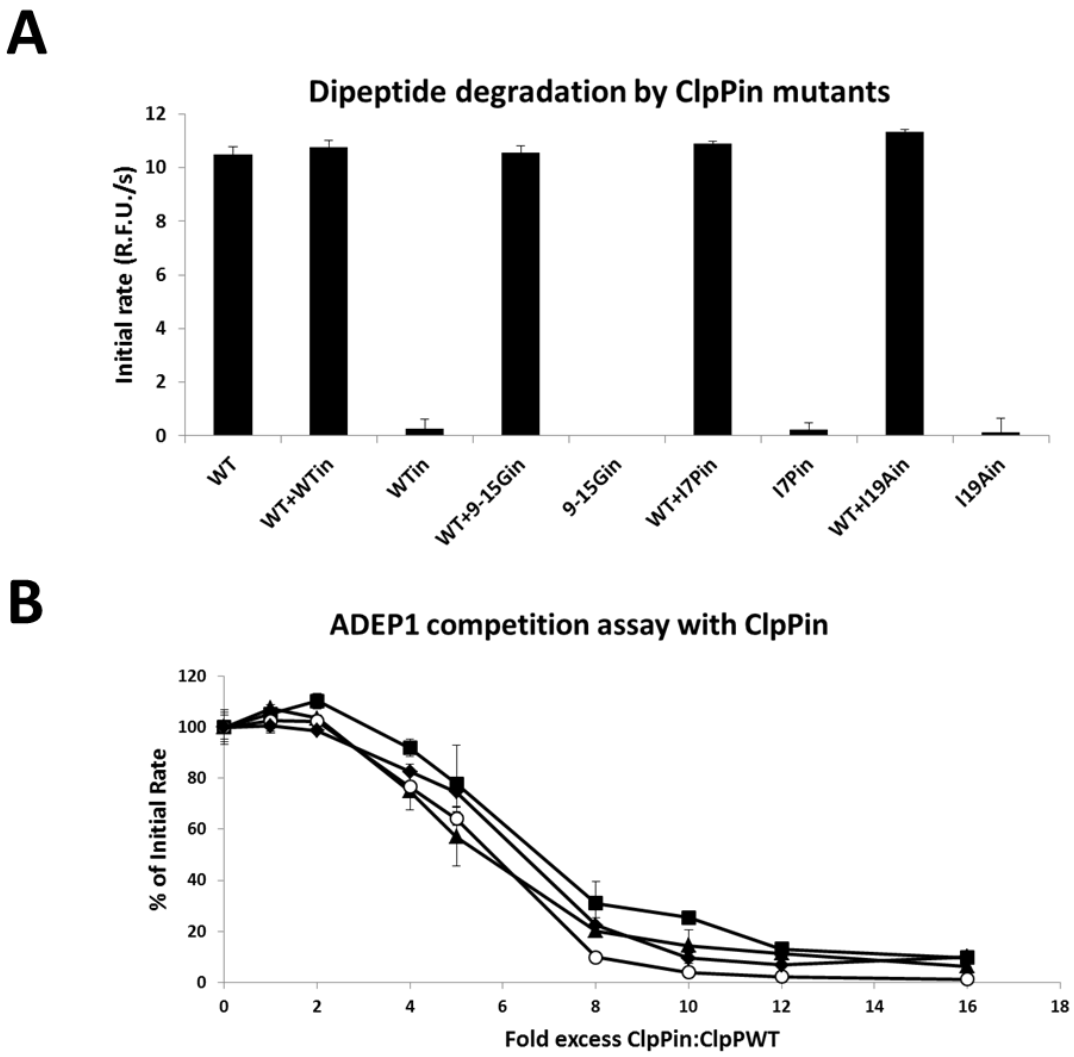

Figure 8. ADEP1 binds to amino terminal variants of ClpP

A) Hydrolysis rates of the dipeptide Suc-LY-AMC for wild type ClpP and amino terminal variants after chemical inactivation. Reactions contained 0.14 µM of wild type $ClpP_{14}$ or the indicated amino terminal variant and when displayed a mixture of 0.14 µM of wild type ($ClpP_{14}$) and 0.14 µM of the amino terminal variant ($ClpP_{14}$), 200 µM of Suc-LY-AMC. Rates were calculated as the initial rate and were plotted relative to wild type as 100%. B) Chemically inactivated wild type and amino terminal variants of ClpP were titrated into a reaction containing 0.14 µM of wild type $ClpP_{14}$ and *10 µM of*

ADEP1. The initial rate of FITC α-casein hydrolysis was measured and plotted relative to the hydrolysis rate measured with 0.14 μM of wild type $ClpP_{14}$ and 10 *μM of ADEP1before addition of chemically inactivated ClpP. wild type: shaded triangle, I19A: shaded square, I7P: shaded diamond, 9-15G: open circle. Bar/values represent a mean ± standard deviation of three replicates.*

3.7 Amino terminal mutation does not disrupt the function of ClpP to hydrolyze peptide bonds

While a variety of substrates had been utilized to assay the role of the targeted residues in mediating β-hairpin formation and stabilization of the open conformation it was unclear whether intrinsic substrate hydrolysis was significantly different for all of these mutants. In the literature various mutations of ClpP can affect assembly and effectively the function of the enzyme to cleave peptide bonds (Geiger et al, 2011, Lee et al, 2010b). It was possible that the reduced substrate cleavage rates observed with FITC α-casein and FtsZ were in effect a product of reduced function to cleave peptide bonds.

In order to ensure that the low activity of these mutants was not a consequence of reduced peptide hydrolysis a dipeptide substrate that can freely diffuse into the catalytic chamber was utilized to assay for hydrolysis defective mutants. Wild type was observed to hydrolyse the dipeptide with a Km of $2.8x10^2$ μM (Table 1). This value was in agreement with previously published values obtained for ClpP mediated degradation of the dipeptide at $5.8x10^2$ μM (Geiger et al, 2011). The β-hairpin targeting mutants 9-15G, I7P and I19A were observed to hydrolyse the dipeptide with a Km of $2.2x10^2$ μM, $4.9x10^2$ μM and $3.1x10^2$ μM respectively (Table 1). Similarly the Km of each mutant did

not differ by more than a log indicating that substrate affinity was similar between wild type and each mutant. Accordingly Vmax was measureable and found to be within a log difference to wild type (Table 1). This result indicated that disruption of the hairpin element by mutation did not significantly alter the ClpP mutants to function as enzymes and cleave peptide bonds.

Mutant	Km (x10²µM)	Vmax (RFU s^{-1})
WT	2.8 ± 0.3	15.7 ± 0.7
E8K	1.7 ± 0.2	10.7 ± 0.4
K25E	4.8 ± 1.6	4.3 ± 0.6
E14A R15A	2.2 ± 0.3	10.1 ± 0.4
K25A R15A E14A	3.2 ± 0.8	28.2 ± 2.4
E8G	3.7 ± 0.9	9.7 ± 0.9
K25G	2.8 ± 0.7	22.6 ± 1.9
E14G R15G	3.4 ± 0.7	18.0 ± 1.4
E14G R15G K25G	3.0 ± 0.7	11.4 ± 0.9
9 GGGGGGG 15	2.2 ± 0.4	16.7 ± 0.9
I7P	4.9 ± 2.0	9.7 ± 1.8
I19A	3.1 ± 1.2	3.8 ± 0.6

Table 1. Kinetic parameters of amino terminal mutants studied with the dipeptide N-succinyl-Leu-Tyr-7-amido-4-methylcoumarin. *Bars represent a mean ± standard error of three replicates.*

3.8 ClpA recovers structural defects at the amino terminus of ClpP that disrupt translocation of protein substrates

ADEP1 represents a novel chemical activator of the ClpP system, however ClpA and ClpX are the natural binding partners that associate with ClpP and regulate activation *in vivo*. Both of these ATPases have been described to directly interact with the amino

terminal residues of ClpP by a pore-2-loop element and with a hydrophobic pocket near the axial surface by a flexible IGF motif (Martins et al, 2010). In order to determine the contributions of ClpA binding during stabilization of the ClpP open conformation the hydrolysis rate of FITC α-casein was measured for the significantly disrupted mutants. Mutants affected in hairpin formation such as the E14G+R15G, I7P and 9-15G mutants were observed to recover to wild type activity when activated by ClpA (Figure 9A). Conversely this was not the case for I19A or K25G+R15G+E14G which only displayed partial activity in the presence of ClpA (Figure 9B).

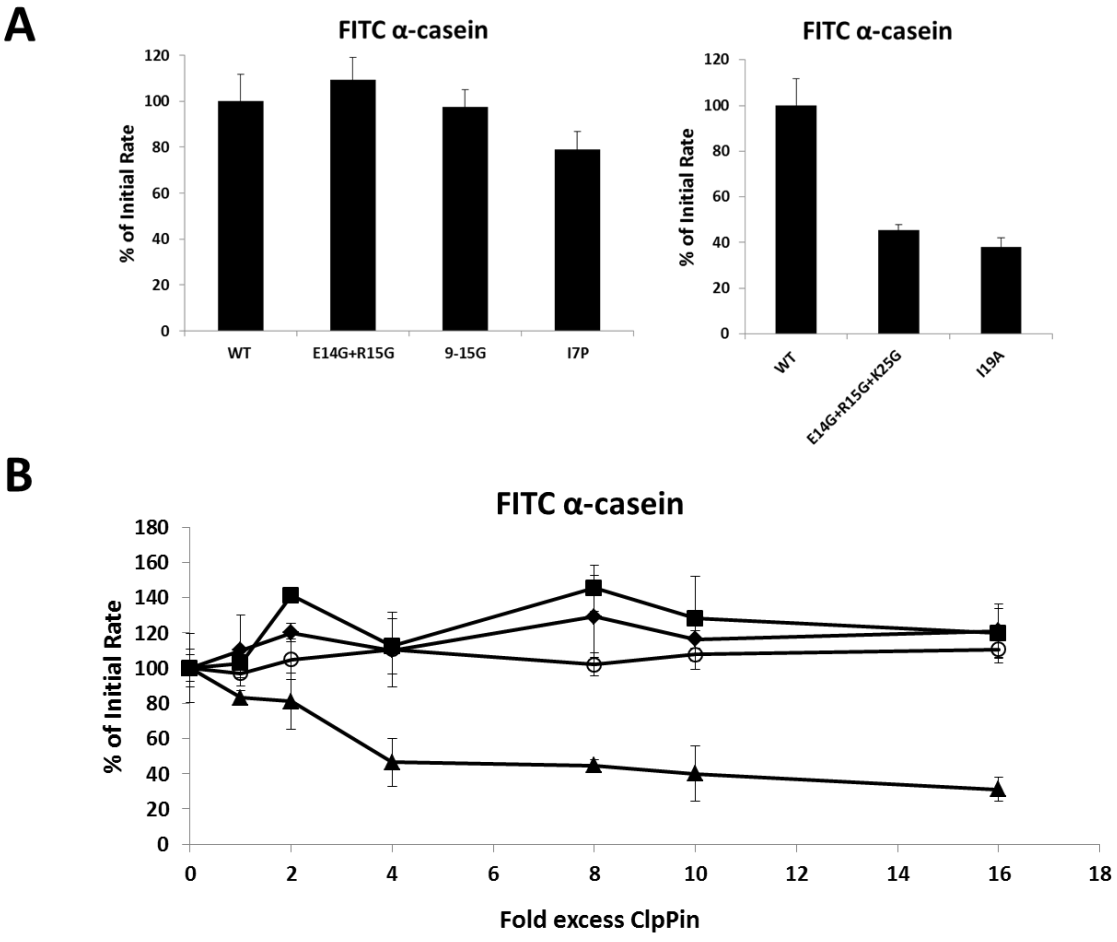

Figure 9. ClpA mediates recovery of structural defects at the amino terminus of ClpP for unfolded substrates

A) Initial rates of FITC α-casein hydrolysis for wild type and ClpP amino terminal variants. Reactions contained 0.14 μM of $ClpP_{14}$ and 0.56 μM of $ClpA_6$ along with an ATP regeneration system. B) Initial rates of FITC α-casein hydrolysis for wild type and ClpP amino terminal variants. Reactions contained 0.14 μM of $ClpP_{14}$ and 0.56 μM of $ClpA_6$ along with an ATP regeneration system. C) Competition assay between wild type ClpAP and chemically inactivated wild type of amino terminal variants of ClpP. Reactions contained 0.14 μM of $ClpP_{14}$ and 0.56 μM of $ClpA_6$ with an ATP regeneration system. Initial rates of FITC α-casein hydrolysis were measured and plotted relative to wild type ClpAP (0.14 μM of $ClpP_{14}$:0.56 μM of $ClpA_6$) as 100% of initial rate. Wild type (triangle), I19A (square), I7P (diamond), 9-15G (open circle). Bars/values represent a mean ± standard deviation of three replicates.

Interestingly these mutants were observed to poorly displace wild type ClpP in complex with ClpA indicating that their ability to form stable complexes with ClpA was disrupted (Figure 9C).

In order to determine the additional effects of these amino terminal mutations in a situation where substrate unfolding is required an SsrA tagged EGFP was utilized as a substrate. In this assay ClpA recognizes the SsrA tag and subsequently unfolds EGFP resulting in degradation by associated ClpP complexes. Regardless of whether hairpin formation or hairpin anchor was targeted, only partial recovery by ClpA was observed for hydrolysis of EGFPSsrA (Figure 10A). EGFP is a folded substrate and requires a committed interaction between ClpA and ClpP to mediate unfolding. It is likely that for folded substrates such as EGFP ClpA can only partially compensate for structural abnormalities at the ClpP amino terminus. These results indicate that while the amino

terminals are required to structurally organize to form an open axial gate, association with ClpA provides additional stabilization of the open conformation of the axial channel.

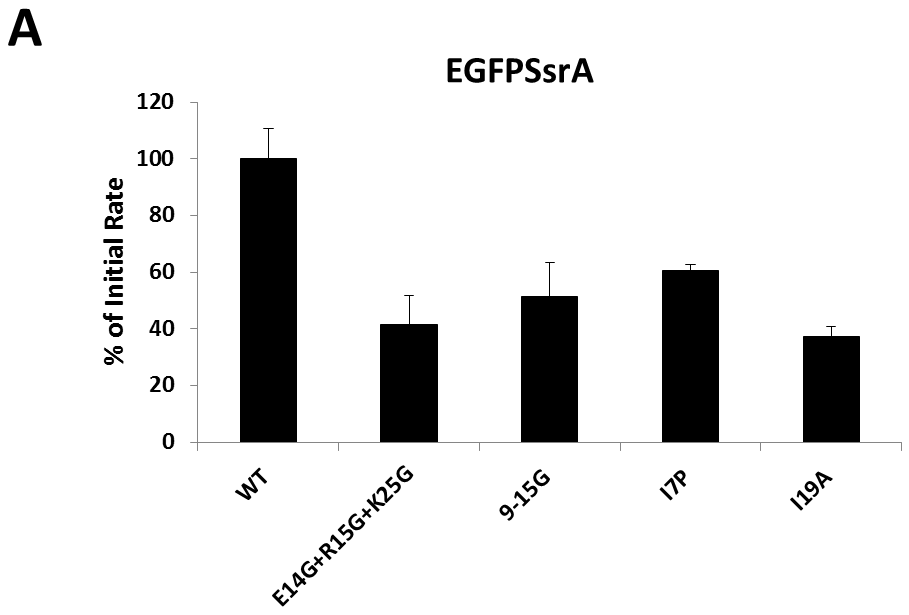

Figure 10. ClpA mediates partial recovery of structural defects at the amino terminus of ClpP for folded substrates

A) Initial rates of EGFPSsrA hydrolysis for wild type and ClpP amino terminal variants. Reactions contained 0.14 µM of $ClpP_{14}$ and 0.56 µM of $ClpA_6$ along with an ATP regeneration system. Initial rates of EGFPSsrA hydrolysis were measured and plotted relative to wild type ClpAP (0.14 µM of $ClpP_{14}$:0.56 µM of $ClpA_6$) as 100% of initial rate. Bars represent a mean ± standard deviation of three replicates.

4.0 Discussion

The amino termini of ClpP are a dynamic region of the protein and are required to structurally transition from a closed to open state in order to mediate restriction and translocation of polypeptides respectively. Cryo-EM reconstructions of the ClpAP complex have failed to reveal insights into the structural requirements of the amino

terminals during substrate translocation (Effantin et al, 2010a, Effantin et al, 2010b), while X-ray crystallography has been unable to observe the gated state as the amino terminals are often disordered and not visualized in published structures (Wang et al, 1997, Bewley et al, 2006, Kang et al, 2004). However the recent crystal structure of ADEP1 bound *E. coli* ClpP revealed that the amino terminals can adopt a structured β-hairpin configuration (Li et al, 2010). Additionally unpublished crystal structures in the Protein Data Bank (PDB) reveal that in some of the ClpP promoters the amino termini can be stabilized as a β-hairpin such as in the case for *Coxiella burnetii* (PDB 3Q7H) ClpP and less so for *Francisella tularensis* ClpP (PDB 3P2L) although not to as a significant extent as in the ADEP1 bound structure. Based on these findings it was predicted that the amino termini of ClpP require the formation of a structured β-hairpin to facilitate efficient translocation of polypeptides.

The work presented in this thesis focused on the investigation of this prediction by generating ClpP amino terminal variants that modified the amino acids observed to stabilize the open conformation during ADEP1 activation (Li et al, 2010). It was observed that replacement of amino acids responsible for the stabilization of the β-hairpin element significantly reduced the function of ClpP to translocate protein substrates. Additionally replacement of residues implicated in anchoring of the hairpin element by a cluster of hydrophobic residues at the base of the hairpin was also observed to significantly reduce substrate translocation. These observations suggest that the open conformation of the ClpP axial channel requires the formation of a structured amino terminal at each protomer consisting of a β-hairpin element that is stabilized at the base

by hydrophobic interactions. While electrostatic interactions were observed to stabilize the hairpin element in the ADEP1 bound crystal structure (Li et al, 2010) it was found that the intramolecular interactions were relatively important while the intermolecular interactions were dispensable for substrate translocation.

In tandem with these observations it was observed that stabilization of the closed conformation of the axial channel also required a structural commitment from the amino terminal residues. Disruption of either the electrostatic interactions or β-hairpin stabilizing contacts significantly increased the basal degradation rate of an 11 amino acid peptide. This observation agreed with results observed in a similar study of residues 8-15 on the amino terminus and the first α-helix adjacent the amino terminus whereby point mutation significantly increased the basal degradation rate of a 10 amino acid peptide (Lee et al, 2010a). Importantly background degradation was not observed for the larger substrates (FITC α-casein and FtsZ) in the study, suggesting that the closed conformation requires a substantial rearrangement of the amino terminus to facilitate efficient substrate translocation.

These observations contrast with an alternate ClpP crystal structure solved with the acyldepsipeptide compounds (ADEP1 and ADEP2) in *B. subtilis* where the amino terminal residues are not observed (Lee et al, 2010b). In a model hypothesized from the work of Lee at al the amino terminals are suggested to adopt a disorganized state and become highly motile upon activation. This model disagrees with the current observations whereby increasing the flexibility of the amino terminal residues in *E. coli* significantly reduces the ability of ClpP to translocate large polypeptide substrates during

activation with ADEP1. Additionally variants of *E. coli* ClpP that increase the flexibility of the amino terminals were also observed to undergo enhanced processing during activation. Combined with the observation that the β-hairpin destabilizing mutants poorly competed with wild type ClpAP complexes indicates a functional requirement for a structured amino terminus during the activation process of ClpP.

In deciding which of the two models have functional relevance in describing the structure of the amino terminals during ADEP1 activation and more importantly during ATPase association it is important to compare the role of the crystal packing environments in the two structures. While the *B. subtilis* structure was crystalized in monocyclic form consisting of one heptameric ring in the asymmetric unit, the *E. coli* ClpP structure was solved with two full tetradecamers in the asymmetric unit. It has previously been observed that when solved as a tetradecamer the amino terminals ClpP can adopt independent structural configurations at each apical surface highlighted by the up and down configurations observed in *E. coli* ClpP (Bewley et al, 2006). A detailed comparison of the crystal packing in both of the acyldepsipeptide activated (*E. coli* and *B. subtilis)* structures revealed that the amino terminal region was involved in crystal contacts in both structures where there was limited space to extend outwards from the axial channel (Figure 11). However it was only in the *E. coli* structure which offered 4 unique heptameric units that the amino terminals were observed to be stabilized in an upright β-hairpin configuration as previously reviewed (Alexopoulos et al, 2012). In agreement, the unpublished X-ray structure of *C. burnetii* ClpP (PDB 3Q7H) 4 out of 7 amino terminals are observed to form full β-hairpin elements with the amino terminals

occupying the groove between two heptamers of an adjacent tetradecameric unit, suggesting that the amino terminals favourably adopt hairpin elements when provided enough space (Figure 11).

This work describes the use of ADEP1 as a molecular probe to study the activation mechanism of the bacterial protease ClpP in order to better understand the functional role of ATPase binding. While ATPase binding facilitates structural rearrangement of the ClpP amino terminals only co-crystalized ADEP:ClpP structures exist to elucidate the structural arrangement of the amino terminals during this process. This thesis provides a biochemical characterization of the structural elements required to stabilize the open conformation of the ClpP axial channel observed during crystallization of ADEP1 bound ClpP (Li et al, 2010). This work provides a direct comparison of the *in vitro* roles mediated by ADEP1 and ClpA in stabilization the open conformation, which is currently not clear in the literature. This work sets the foundation for future studies of the structural requirements of the amino terminal region of the ClpP system. In addition this study has provided a methodology for the analysis of other regulated compartmentalized protease system through the use of molecular probes that mimic functional contacts of activating partners.

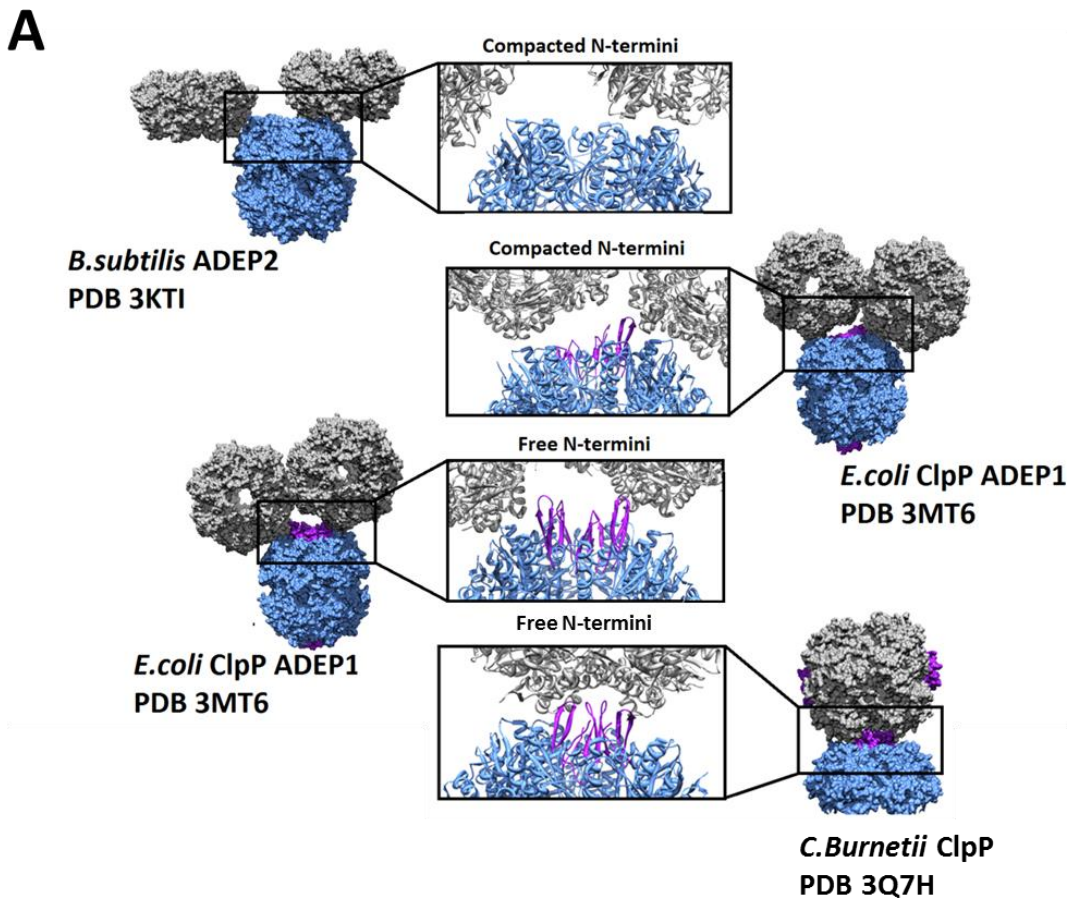

Figure 11. Crystal packing influences the structure adopted by the amino termini of ClpP

A) Crystal packing of adjacent tetradecamers are shown for the E. coli ADEP1 and B. subtilis ADEP2 crystal structures. In the top panel one heptameric face of B. subtilis ClpP is observed to impact part of the axial face of the full tetradecamer leaving no amino terminal residues. A similar packing is observed in the second panel of E. coli ClpP, this time with packing between the side of an adjacent tetradecamer and the axial surface of another tetradecamer. Partial amino terminal residues are observed in this packing but most are disordered. The third panel depicts the packing in the second tetradecamer in the asymmetric unit of the E. coli structure, this time leaving space for the amino terminals which appear to adopt an upright conformation. The fourth panel

shows the amino terminals of C. burnetii ClpP adopting an upright β-hairpin configuration with packing of the axial channel in the groove between two heptameric rings of an adjacent tetradecamer.

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