Mechanistic Studies of the AAA+ Molecular Motor ClpXP

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Abstract

ClpX is an archetypical representative of the AAA+ superfamily of enzymes that serves as the regulatory domain and motor for the ClpXP protease system. ClpX binds protein substrates via an amino acid sequence known as a tag, denatures them, and translocates them into the associated peptidase, ClpP. ClpX utilizes the energy from ATP hydrolysis to pull on bound substrates, destabilizing folded substrates and denaturing them, before they are translocated through the pore of ClpX into ClpP. As a representative of the AAA+ superfamily, mechanistic understanding of the ClpXP protease cycle may elucidate the mechanisms of related enzymes.

I used synthetic peptide substrates to probe what features of a polypeptide chain are recognized during translocation. Surprisingly, side-chain properties including size and charge, and the spacing between peptide bonds had relatively small effects on the rates of translocation by ClpXP. Pulling on tracts of glycine, lysine, or proline also allowed efficient ClpXP degradation of the stable protein GFP, for which unfolding is rate limiting. These results suggest that minimal chemical or structural features may be sufficient for translocation and protein unfolding by ClpX and lead to a new model for translocation based on multiple van der Waal's interactions.

ClpX interacts with its substrates via highly conserved pore loops including the GYVG pore-1 loop. I identify that the hydroxyl group of the tyrosine, Y153, is directly involved in translocation. In addition, I show Y153 is involved in binding substrates and is essential for coupling ATPase activity and translocation.

I investigate substrate tag recognition by ClpX and the role of Y153 in substrate binding via a library of synthetic peptides. In the ClpX recognition tag, ssrA (AANDENYALAA), the last residue plays the largest role in recognition and the penultimate residue plays a smaller role. The incorporation of charges significantly disrupts binding and chirality is essential.

ClpX is a versatile enzyme, able to translocate substrates via the highly permissive and novel mechanism of using van der Waal's interactions. Selectivity and control are exerted on the level of substrate specificity, where ClpX displays high specificity for certain substrate tag sequences that are not tolerant of modification.

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Chapter 1

Protein Degradation and the ClpXP System

Introduction

If asked, "How do you climb a ladder?" one thinks about the physical actions involved- grasping the rungs of the ladder and climbing, pulling yourself up. This type of motion, pulling on a rope or exerting force against a framework, forms the basis of how active translocation of proteins and nucleic acids work in the cell. Yet these motions, and more generally the mechanism of how force is transferred, are not understood on the molecular level.

Molecular motors conduct these driving interactions- hydrolyzing NTPs (nucleotide triphosphates) and converting the energy released into mechanical work (Bustamante et al., 2004; Tomkiewicz et al., 2007). Molecular motors are of interest because of their essential and diverse roles and because gaining the ability to control molecular motors has the potential to start an *in vivo* industrial revolution, wherein they are harnessed for derived rather than evolved roles (Browne and Feringa, 2006; Griffith and Grossman, 2008; Kinbara and Aida, 2005; Moore et al., 2008).

There are several classes of molecular motors, including the AAA+ family of enzymes (Gottesman, 1996; Iyer et al., 2004; Neuwald et al., 1999). AAA+ enzymes can be found in all kingdoms of life and participate in diverse roles including protein unfolding and degradation, membrane dynamics, as a chaperone or in disaggregation, and in vesicle dynamics amongst others (Erzberger et al., 2002).

These diverse functions are carried out using related structures, typically including a hexameric ring shape and containing conserved sequences involved in ATP hydrolysis and sensor regions (Ogura and Wilkinson, 2001; Patel and Latterich, 1998). Interestingly, the hexameric structure plays a role in the ATPase activity, as the site of ATP hydrolysis is located between two subunits in the ring. However, whether or not the conserved architecture transfers to a conserved mechanisms of action, and if so, how this action works, is not known (Mogk et al., 2008). To elucidate this action, I explore the mechanism of an archetypical AAA+ enzyme, ClpXP. The relative simplicity of its structure and the availability of tools to probe it, make ClpXP an ideal system to examine.

Protein Degradation and the ClpXP System

One of the roles played by molecular motors is to regulate protein degradation systems. Protein degradation is essential for many cellular processes as it enables the cell to eliminate abnormal proteins and acts as a control mechanism by changing the lifespan of regulatory proteins. Without protein degradation damaged and non-essential proteins would build up, crippling the cell (Glickman and Ciechanover, 2002).

It is important to note that protein degradation falls into two classes- extracellular protein degradation, where the goal is to degrade proteins into their substituent amino acids for material and energetic purposes, and intracellular protein degradation, which serves many housekeeping functions including stress-response and the removal of damaged proteins. Intracellular degradation requires not just control but dynamic control, which is sensitive

to the current and changing conditions of the cell. Without control intracellular proteolysis would be akin to having a wood-chipper running at all times in your living room- a bad idea. The energy dependent proteases are a subset of the AAA+ family of enzymes and use the AAA+ motor domain to provide the essential dynamic control of degradation (Sauer et al., 2004; Smith et al., 2006). There are five AAA+ enzymes involved in protein degradation in *E.coli*, ClpXP, ClpAP, HslUV, FtsH, and Lon (Gottesman, 1996). In eukaryotes this role is primarily, though not exclusively, filled by the AAA+ 26S proteasome (Coux et al., 1996; Hershko and Ciechanover, 1998).

ClpXP is an AAA+ protein involved in regulated protein degradation in stress response mechanisms and in removal of toxic protein. The functional form of ClpXP consists of two components, the ClpP peptidase chamber, and the ATP dependent regulatory component ClpX (Baker and Sauer, 2006; Sauer et al., 2004). The active form of ClpX is a homohexameric ring that either singly or doubly caps the tetradecameric double ClpP ring. Interestingly, the ring formed by ClpX is skewed and ATP soaking experiments have demonstrated different levels of ATP affinity at different positions (Figure 1, Glynn et al., 2009). This result corresponds with previous experiments that showed saturation of ATP binding at around four ATP per hexamer (Hersch et al., 2005). Thus, as would be expected since ATP hydrolysis is correlated with translocation, the conformation of a ClpX subunit appears coupled with ATP hydrolysis.

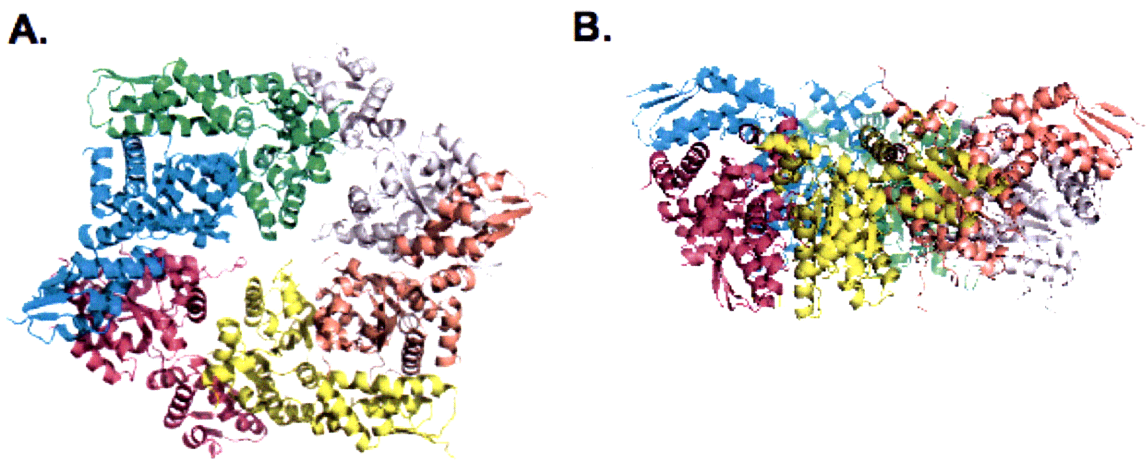

Figure 1: Crystal structure of $ClpX_6$ structure showing top (A) and side-down view (B) $ClpX_6$ forms an assymetric hexamer that is clearly skewed in both the horizontal and vertical planes. ClpX subunits are colored differently to highlight the hexameric nature of the protein. The lack of density in the central pore region is due to the flexible nature of the pore loops. (Glynn et al. 2009)

Control of degradation by ClpXP is important for cell survival in many strains of bacteria. The importance of the ClpXP system can be taken advantage of in multiple ways, for instance, the use of a small molecule that causes pore opening in ClpP leading to unregulated degradation functions as an antibiotic, and an antibody against the highly conserved ClpP sequence in pneomococcas has shown some promise as a human penumococcal vaccine (Brotz-Oesterhelt et al., 2005; Cao et al., 2007). Targeting substrates to ClpX by addition of a tag, has been used as a tool in biochemical studies. ClpXP can degrade a component of a macromolecular assembly and coupled with modified tags can be used in controlled degradation systems (Moore et al., 2008).

Mechanism and Specificity of ClpP

ClpP is a tetradecameric serine protease, consisting of two heptameric rings, which combine to form a barrel shaped degradation chamber. An important property of this degradation chamber structure is that all of the proteolytically active sites are sequestered inside the degradation chamber, which is only accessible via a small axial pore of about 10 Å in diameter (Bewley et al., 2009; Wang et al., 1997). Once a substrate enters the degradation chamber the high concentration of peptidase sites leads to rapid cleavage (Jennings et al., 2008; Kim and Kim, 2008).

Cleavage of a peptide bond is energetically favorable and commonly used proteases such as trypsin and chymotrypsin have no ATP requirement. The energetics of hydrolysis make it unusual that ClpXP and other AAA proteases such as the proteasome are energy dependent. The energy requirement for AAA proteases can be explained by separation of the steps of unfolding and translocation from proteolysis- as in the case of ClpXP. Preparing a substrate for degradation via unfolding and translocation to the ClpP protease active sites is energetically dependent and degradation once the substrate is in position has no energetic requirement.

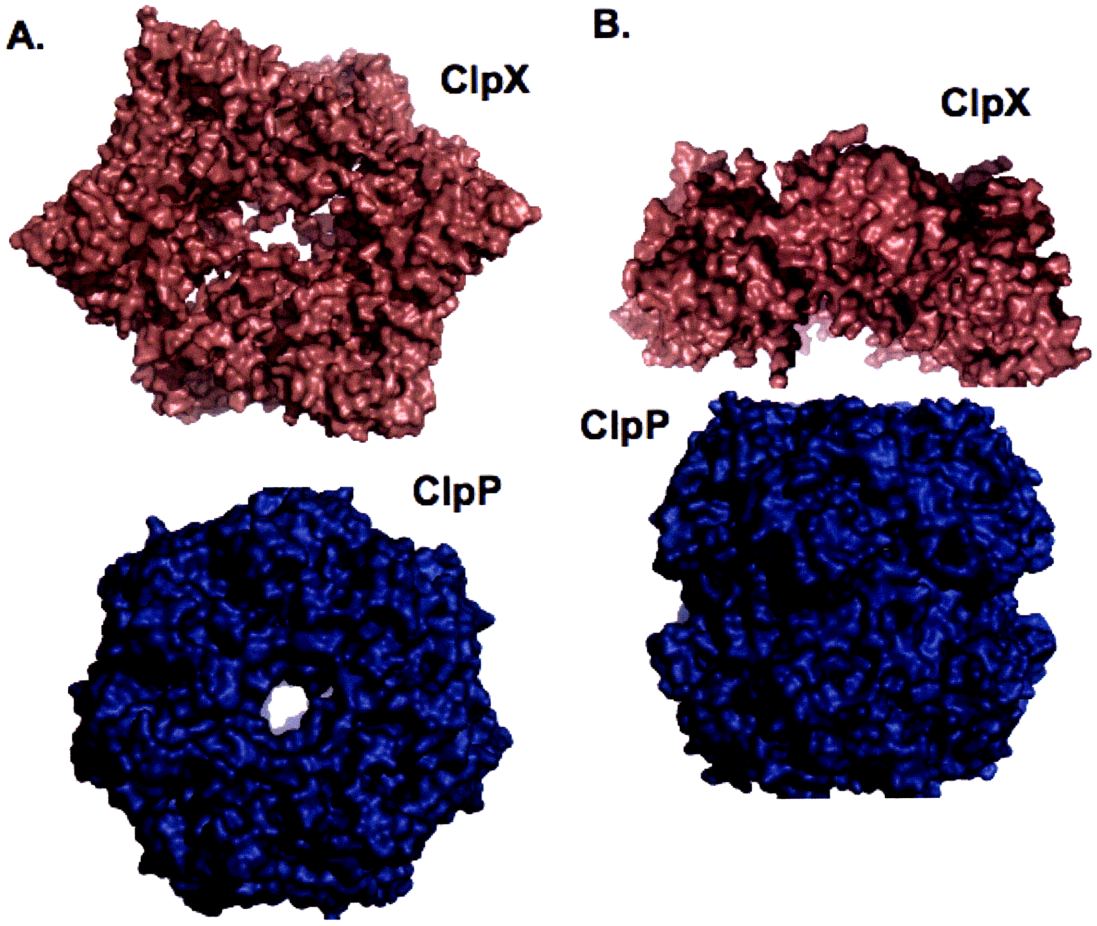

Figure 2: ClpX and ClpP models surfaces from crystal structures (ClpP, Szyk and Maurizi, 2006, PDB 1TYF. ClpX, Glynn et al. 2009). **A**. Pore down views of ClpX and ClpP. ClpX is a skewed hexamer and ClpP is a regular heptamer. **B.** Axial view showing ClpX and ClpP.

ClpP has been previously observed to independently cleave peptides of ~10 amino acids in length, generally at a low rate, though very rapidly in the case of the optimized ClpP propeptide (Figure 3) (Thompson and Maurizi, 1994). Peptide degradation, in the absence of a regulatory domain, probably depends on substrate diffusion through the axial pore into the ClpP degradation chamber. Alternatively, it has been proposed that peptides are able to diffuse into and out of the chamber via gaps at the ring-ring interface of the ClpP barrel (Sprangers et al., 2005). The model for peptide diffusion into ClpP through the

axial pore of ClpP, the same pathway that active translocation uses, is supported by an increase in peptide degradation rate upon deletion of 10-20 amino acids from the N-terminal of the mature form of ClpP (Bewley et al., 2009). This deletion increases the accessibility of the ClpP pore to substrates and even allows degradation of some folded polypeptides.

When ClpP is translated in vivo, it starts with a 14 amino acid sequence that is self-hydrolyzed to form the mature protein. It is believed that this cleavage is necessary to allow access to the degradation chamber and as such is essential to activity. A synthetic version of this 14 amino acid sequence, referred to as the propeptide, has been observed to be cleaved by ClpP at a rate on the order of 10 000 per $ClpP_{14}$, per minute (Thompson and Maurizi, 1994). The rate remains mostly unchanged when the propeptide is shortened to the final 10 amino acids, with a high affinity cleavage site between Met and Ala.

MS|Y|SGERDN<u>FAPHM|ALVP</u>V

ClpP propeptide

Figure 3: ClpP propeptide as initially identified by Thompson and Maurizi. Cleavage sites are indicated in red and shortened propeptide is underlined in bold. The sequence cleaved to form mature ClpP consists of the C-terminal 14 amino acids of this sequence (cleavage between the Met and the Ala).

Protein Degradation Cycle

Protein degradation by ClpXP or another AAA+ protease consists of multiple steps (Figure 4). First, the protein or polypeptide substrate binds to ClpX (this may in turn be subdivided into two steps, a binding step, where ClpX recognizes the substrate and an engagement step where the substrate is actively engaged by the active pore loop residues involved in translocation); second, the substrate (if structured) is denatured; third the substrate is translocated through the ClpX pore into the ClpP proteolytic chamber; fourth the substrate is cleaved and fifth, the fragments are released (Figure 4) (Kenniston et al., 2005, Martin et al., 2008b, Singh et al., 2000). Binding, unfolding, translocation, and peptide release can be rate-limiting, depending on the substrate and the system. Substrate binding is determined by the presence of a "tag" on the substrate.

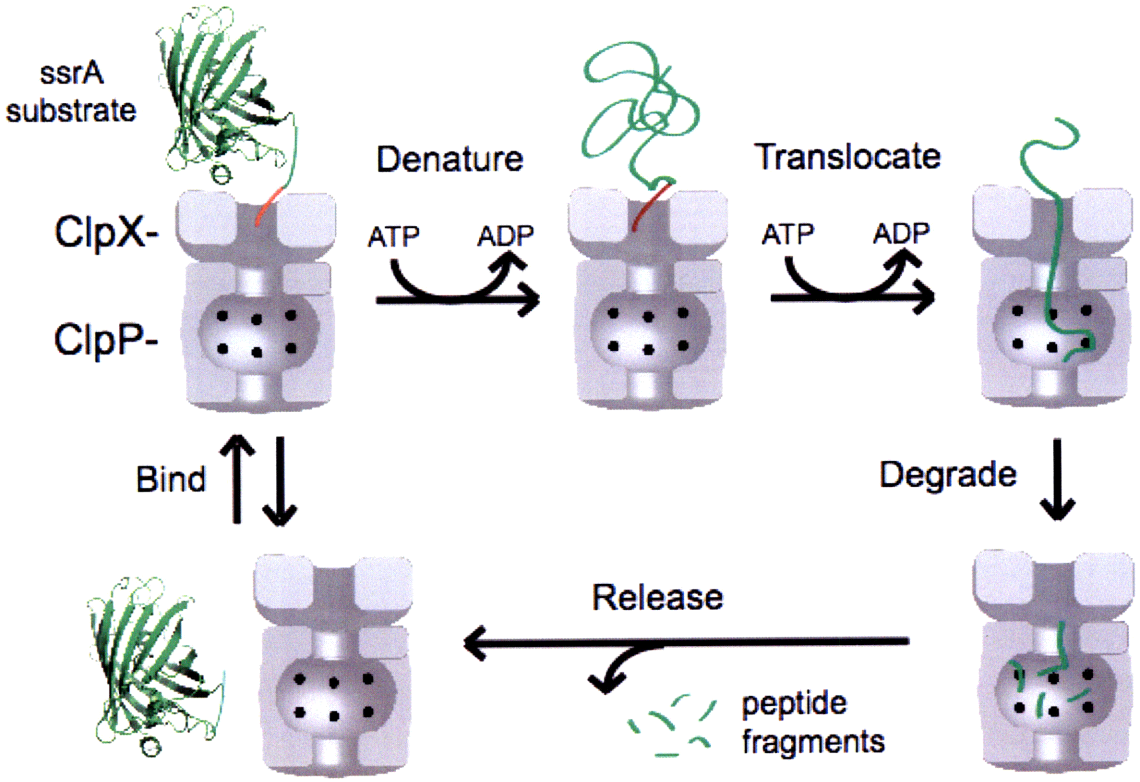

Figure 4: Protein degradation cycle showing binding, denaturation, translocation, degradation and release. Binding could potentially be broken into two steps- recognition and initiation. The black dots in ClpP represent the sequestered peptidase sites. GFPssrA is modeled as the substrate with GFP in green and the ssrA tag (AANDENYALAA) in red.

ClpX recognition tags

Five different classes, or motifs, of tags have been identified to date, three C-terminal tags, and interestingly, two N-terminal tags (Table 1) (Flynn et al., 2003). The existence of multiple classes of recognition tag, and the fact that these tags can occur at either the N or C-terminus, demonstrates the broad range of substrates that ClpX has. It also implies the existence of multiple modes of recognition and raises the question of whether binding and initiation are separate steps. The potential separation of binding and initiation is

highlighted by the fact that ClpX is known to translocate substrates sequentially, starting from the tag, in the case of C-motif 1 substrates (Reid et al., 2001).

Motif	Consensus	Model Substrates	Sequence
N-motif 1	Polar-T/ϕ-ϕ-+-ϕ	λO DpS	NTAKI STAKL
N-motif 2	Met-+-ϕ-ϕ-ϕ-X_5-ϕ	OmpA IscS	NH_2-MKKTAX_5V NH_2-MKLPIX_5A
N-motif 3	ϕ-X-Polar-X-Polar-X-+-Polar	DksA	NH_2-MQEGQNRK
C-motif 1	ϕ-ϕ-ϕ-COOH	SsrA N-RseA	LAA-COOH VAA-COOH
C-motif 2	+-+-+-+-ϕ-ϕ	MuA YbaQ	RRKKAI-COOH RAKKVA-COOH

+ = basic amino acid
ϕ = hydrophobic amino acid
X = any amino acid

Table 1: Five classes of ClpX recognition tag (from J. Flynn, thesis)

Five classes of tags have been identified as interacting with ClpX, but very little is known about exactly what the determinants in these tags ClpX recognizes are and how they bind to ClpX. In addition, some ClpXP substrates that do not have a clear recognition motif have been identified. ClpX has been shown generally to recognize substrates with tags at the N- and C-terminus. In some circumstances these tags are recognized when placed

internally via crosslinking or when placed at the opposite terminus (Flynn et al. 2003, Hoskins et al., 2002).

ClpX has at least two different mechanisms for how it recognizes tagged substrates. C-motif 1 tags are actively bound in the pore of ClpX, however, C-motif 2 tags are bound elsewhere on ClpX, possibly to the N-domain (Wojtyra et al., 2003). For cases where the substrate does not bind in the pore of ClpX, the tag serves as a tethering sequence increasing local concentration of the peptide around the pore. In the case of a tethering tag, translocation could be initiated at a separate point in the substrate from the tag.

The ssrA tag- a mechanism for rescuing stalled ribosomes

The most widely studied of the ClpX tags is the C-motif 1 tag, ssrA. The ssrA-rescue system comes into play if translation stalls, leaving an incomplete protein lodged in the ribosome (Gottesman et al., 1998; Keiler et al., 1996). This system uses trans-translation to append the ssrA tag to the stalled protein, freeing the ribosome, and is highly conserved amongst all bacteria (Ahlawat and Morrison, 2009). Addition of an ssrA tag makes a protein a substrate for degradation by ClpXP. The nature of this system requires that the ssrA tag be sufficient for degradation of a wide-variety of substrates (potentially all proteins).

The ssrA tag serves not just to target proteins for degradation by ClpXP but also by other protein degradation machines in the cell including both ClpAP and FtsH. ClpX and ClpA recognize different sequences of the ssrA tag from each other. How FtsH recognizes the

ssrA tag is not known. ClpX is known to bind the ssrA tag in the pore region, however, for ClpA and FtsH the tag could bind either to the pore or act as a tethering sequence, allowing the initiation of translocation from a separate site. The existence of redundant mechanisms for removal of ssrA tagged substrates highlight the importance of this rescue system (Figure 5) (Flynn et al., 2001; Lies and Maurizi, 2008).

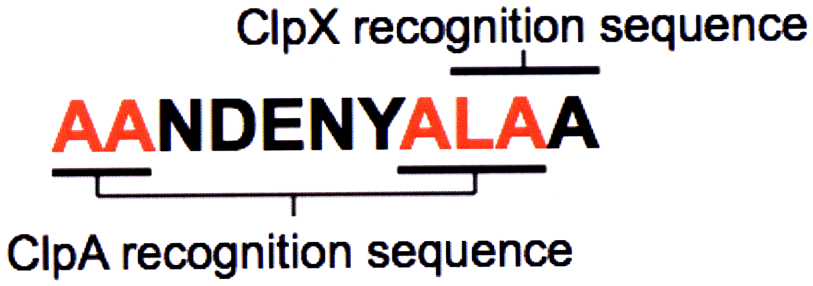

Figure 5: SsrA tag showing sequence bound by ClpX and ClpA. ClpA recognizes the residues in red, ClpX recognizes the final three C-terminal amino acids. (Flynn et al. 2001)

SspB Adaptor Protein

Adaptor proteins modify the specificity of AAA enzymes by tethering substrates to the protein (Dougan et al., 2002). SspB (stringent starvation protein B) is a dimeric adaptor protein that tethers the ssrA tag to ClpX and keeps it away from ClpAP, which also recognizes the ssrA tag. SspB binds part of the ssrA tag (a separate sequence from that recognized by ClpX) and has a high-affinity binding site for binding to ClpX (Figure 6) (Levchenko et al., 2000, Park et al., 2007). Because SspB is a dimer it binds to two ClpX subunits, potentially spanning the pore of ClpX, and holds the ssrA-tagged substrate close to the pore of $ClpX_6$.

It has been hypothesized that hand-off of the tagged substrate from SspB to ClpX is facilitated by the steric clash that occurs when SspB and ClpX are both engaged with the ssrA tag (Flynn et al., 2001, Hersch et al., 2004 Bolon et al., 2004). In this model SspB binds the substrate and tethers it to ClpX, then the substrate binds in the pore region of ClpX, straining the SspB-substrate interaction. In experiments with extended ssrA tags, where additional amino acids are added to provide additional space between SspB and ClpX, Hersch et al. showed an increase in the affinity of SspB for the extended ssrA tags in the presence of ClpX.

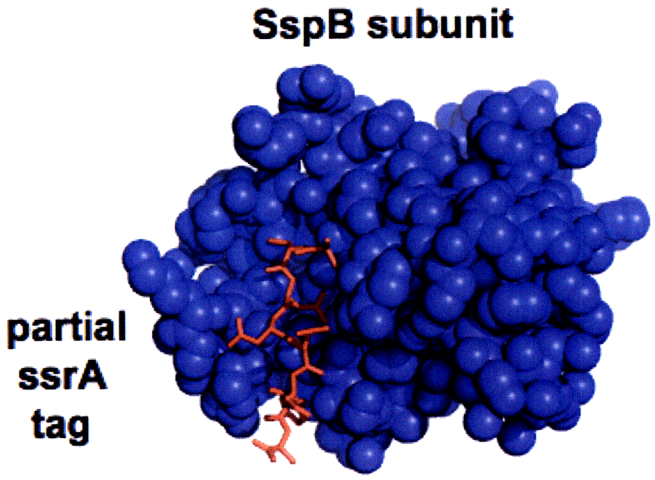

Figure 6: Crystal structure of SspB cross-linked to a partial ssrA tag with the underlined sequence, ACNDENYALAA, the second Ala was mutated to a Cys to allow binding (Bolon et al. 2004, PDB 1twb). Both SspB subunits are capable of binding a substrate but only one is shown here. The ClpX binding region is not shown here but consists of the final three residues, LAA of the tag, which would appear below the image.

Unfolding

Unfolding is the rate-limiting step in ClpXP degradation for most native substrates. It was previously proposed that ClpX unfolds its substrates via a Brownian ratchet motion,

wherein the enzyme traps the protein into a spontaneously occurring unfolded state. However, the Brownian ratchet model is highly unlikely because it fails to explain the rates of degradation observed for highly stable substrates that would seldom sample unfolded states on their own. Instead we believe that ClpX unfolds its substrates by attempting to translocate them. In this model, ClpX engages with the substrate's binding tag and attempts to translocate it through the pore, repeatedly tugging the folded substrate against the ring. This action destabilizes the protein, increasing the likelihood of it denaturing and being successfully translocated through the pore of ClpX. One important aspect of this model is that it does not guarantee success, at least on the first (through nth) try. Instead, successive rounds of ATP-hydrolysis and attempted translocation ultimately result in ClpX unfolding the substrate. While this mechanism seems wasteful, most of the energy exerted in degradation can go into failed unfolding attempts, it has the potential for substrate release. In case of a very stable protein, the ability to release the substrate would prevent ClpXP from becoming jammed, allowing another substrate to be degraded instead (Kim et al., 2000).

Translocation

The heart of ClpXP protein degradation system is translocation by ClpX. Translocation by ClpX allows substrates to be denatured and fed into the peptidase chamber of ClpP. Several models have been previously proposed for the mechanics of how ClpX translocates its substrates. These models include the concerted mechanism, where all of the subunits fire at once dragging the substrate along, and facilitated diffusion, where ClpX passively creates a gradient in the energy landscape making directional travel more

likely, much like how a tire shredder will only damage your car in one direction but allows unimpeded travel in the other. Another possible model involves ClpX using the inherent dipole of a polypeptide backbone, like a bicycle chain, to translocate the substrate.

None of the previously suggested models serves to fully explain the behavior of ClpX. Translocation has been shown not to be the result of a regular interaction between the six subunits of ClpX and does not require all of the subunits to be active (Martin et al., 2005). Only two of the subunits need to be active for translocation to occur, implying some form of stochastic mechanism where each ClpX subunit can bind the peptide and exert force on it. Even with only one active subunit some translocation is observed, however in this system "slipping" or substrate release, easily dominates over successful translocation events. ClpX can translocate proteins sequentially starting at the N-terminus or the C-terminus, implying a lack of dependence upon the polarity of the peptide backbone and ruling out this model (Sauer et al., 2004).

Through cross-linking, it has been demonstrated that ClpXP is not limited to degrading one polypeptide chain at a time. ClpXP was observed to translocate and degrade cross-linked substrates, even though only one of the two was ssrA tagged. After degradation of the crosslinked substrates the cystine bond remained intact (Kenniston et al., 2004). The complete degradation of the crosslinked substrates implies that the central pore of the ClpX hexamer can open to accommodate two or even three strands of polypeptide at a time, and that this expansion does not cripple translocation.

The rate of protein translocation by ClpX appears to have a direct correlation with protein length. In the case of sequential unfolded titan proteins, (1, 2, or 3 titan proteins expressed with linkers and a C-terminal ssrA tag), protein translocation gave a linear relationship between length and number of proteins translocated per minute, with a rate of 387 ± 25 residues per min (Kenniston et al., 2005). I show later in this thesis that this rate holds in the case of peptide substrates where translocation is the rate limiting step, with a maximum observed rate corresponding to approximately 500 residues per min.

These results imply that translocation is sequential but not directional, that ClpX monomers interact with the substrate in a non-concerted manner, and that the pore region is conformationally dynamic. Leaving the question, how is a polypeptide translocated?

Mechanism of translocation: ClpX GYVG pore loop

ClpX translocates its substrates through the pore of the $ClpX_6$ hexamer. This interaction, and substrate binding interactions for some classes of ClpX recognition tags, are mediated by structurally flexible sequences in ClpX that line the pore, referred to as pore loops (Farrell et al., 2007; Martin et al., 2008b). The pore-1 loop is highly conserved across all of the AAA+ proteases, and the pore-2 loop is highly conserved amongst ClpX orthologs. The pore-1 loop consists of the residues GYVG, and the pore-2 loop, consists of the sequence RKH, both occur in the center of a flexible loop region. The RKH loop is directly at the mouth of the pore of ClpX and has been shown to have a role in recognizing and binding substrates. The pore-1 loop is in the center of the pore of ClpX

and can assume a dynamic range of conformations that position the GYVG sequence throughout the length of the pore. The pore-1 loop is involved in substrate recognition, in translocation, and in regulating ATP hydrolysis by the enzyme (Martin et al., 2005; Martin et al., 2008b; Martin et al., 2008c; Siddiqui et al., 2004). However, exactly how these loops convert the energy released by ATP hydrolysis into mechanical work remains unknown.

Relevance to other systems

The AAA+ enzymes are an ancient class of enzymes, as show by the wide range of kingdoms they occur in. Over time different enzymes have evolved to play vastly different roles, however, the core structure has remained conserved. This structural conservation, specifically the high level of conservation of the GYVG pore loop and of the Walker A and Walker B domains, appears to pair with conserved mechanistic elements. As such, the interaction of ClpX with its substrates can be hypothesized to serve as a model for the mechanism of some of the other AAA+ enzymes. I propose later in this thesis that the mechanism is dynamic enough to allow it to be applied to polypeptides, DNA, or RNA with modifications primarily to the substrate binding and recognition properties of the enzyme, but a conserved mode of action.

AAA+ proteases: ClpAP and the 19S Proteasome

ClpP can pair with the ClpX translocation machine or with the related AAA+ enzyme, ClpA to form ClpAP. ClpAP is another example of an AAA+ protease with the

functional unit composed of the $ClpP_{14}$ barrel as in ClpXP, and a homo-hexameric ring of ClpA. ClpA functions in much the same manner as ClpX, denaturing and translocating proteins, and even recognizes the ssrA degradation tag (Gottesman et al., 1998; Wickner et al., 1994). Unlike ClpX, ClpA has two AAA+ domains of which the N-terminal domain is required for hexamer formation, which also requires ATP binding, and the C-terminal domain, which is required for active translocation (Singh and Maurizi, 1994). ClpA has been shown to translocate substrates directionally from the tag for either N or C-terminally tagged proteins (Lee et al., 2001; Reid et al., 2001).

The existence of two proteins that can combine with ClpP to perform the same function presents a possible area of control. One way this control is achieved is via adaptor proteins such as SspB, which does not bind to ClpA, or ClpS that specifically targets N-end rule substrates to ClpA and blocks ssrA tagged substrates.

One of the reasons for interest in ClpAP and ClpXP is their functional and structural homology to the 26S proteasome. The 19S proteasome forms the regulatory domain of the 26S proteasome, making it roughly homologous to ClpX or ClpA. 19S consists of 19 different proteins, of which 9 form the ubiquitin binding "cap" and 10 that form the base, including six different AAA+ proteins that form a hexameric ring (Coux et al., 1996; Rosenzweig et al., 2008). Interestingly, two of the non-AAA+ subunits of the base appear to span the pore of the associated 20S proteasome, most likely being positioned in the center of the AAA+ hexamer. While having the pore occupied by another component of this system seems highly unlikely based on ClpX, the proteasome is significantly

larger, providing room for these extra proteins. If the functional enzyme does have these proteins in the pore region, then it suggests that part of the mechanism involves moving these basal proteins so the AAA+ domains can interact with the substrate directly, or these proteins mediate between the AAA+ domains and the substrates, exerting the driving force on the substrate polypeptide.

E1 Helicase

The papillomavirus E1 helicase is a member of the helicase superfamily III, a subclass of AAA+ enzymes. The active form of E1 is a hexameric ring as with ClpX. The E1 helicase participates in DNA replication by unwinding dsDNA (Tucker and Sallai, 2007). A dimer of E1 helicase rings binds at the origin sequence to two opposing strands of DNA and each translocate one strand through their pore (Enemark and Joshua-Tor, 2006). This model is contrary to how most other helicases work, where a single hexameric ring binds both strands of DNA- one in the pore as with the E1 helicase, and one strand in a cleft on the exterior of the hexamer (Buttner et al., 2007; Enemark and Joshua-Tor, 2008).The back-to-back nature of the rings allows both hexamers to translocate their substrate in the 3' to 5' direction.

Crystallization of the E1 helicase, both in complex with and without substrate bound, has allowed characterization of the interaction between the helicase and the substrate ssDNA and of the conformational changes in the E1 helicase on substrate binding. In the unbound state the E1 helicase pore is very narrow, and even when substrate is bound, the pore only opens sufficiently to allow a single strand of DNA to bind. The structural data

could indicate a lack of flexibility in the E1 helicase pore size, or it could mean that like ClpX the pore size adjusts to the substrate bound. Flexibility in the pore size of E1 seems unnecessary, as the purpose of the enzyme is to separate multiple strands of DNA, so tolerance of multiple strands in the pore region is counter-intuitive. Another distinct structural element is that the double-hexameric complex is off-center, allowing the ssDNA to pass through either hexamer without clash or passing close enough to the other strand to rebind.

Interaction between the E1 helicase and DNA is via a flexible hairpin region. The E1 helicase appears to follow a cyclic translocation model, with subunits cycling between ATP-bound states, and the type of nucleotide bound correlating with the position of the hairpin. The hairpins form what resembles a right-handed spiral staircase, and each hairpin interacts with a unique nucleotide in the substrate ssDNA (Enemark and Joshua-Tor, 2006).

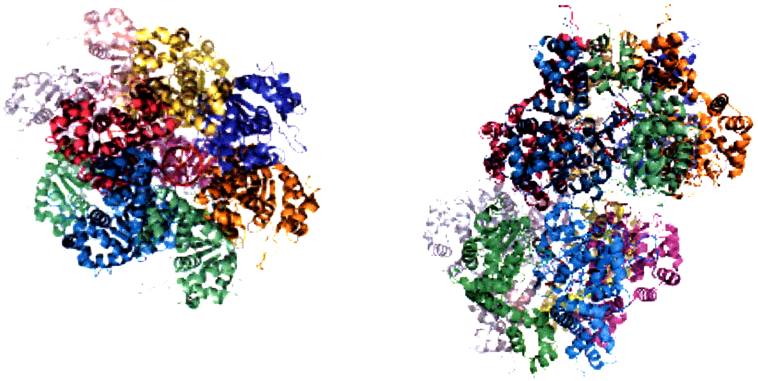

Figure 7: Crystal structure of the E1 helicase with DNA bound (Enemark et al. 2006, PDB 2gxa). The subunits are differently colored to distinguish them. The substrate DNA is shown in red. The off-center nature of the association between the two hexamers is visible in both the top-down and side views of the complex.

Dynein

Dyneins are a subclass of the Helix 2 AAA+ enzymes. Dynein itself consists of at least two "heavy" chains, as well as potentially multiple regulatory proteins referred to as "light" and "intermediate" chains (Oda et al., 2007; Serohijos et al., 2006). The head portion of the heavy chain is also the motor domain of dynein, and consists of 6 AAA+ subunits and a C-terminal domain believed to be involved in regulating the hydrolysis cycle (Figure 8) (Hook and Vallee, 2006; Tucker and Sallai, 2007). Surprisingly, all seven domains are on a single polypeptide chain. The six AAA domains all have unique sequences. An additional idiosyncrasy in the structure of dynein is the presence of a coiled-coiled domain connecting the third and sixth AAA subunits.

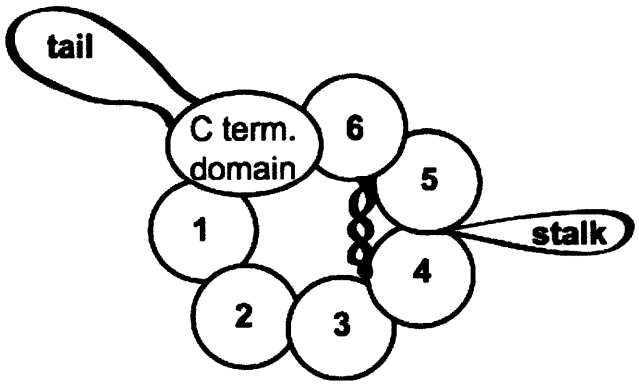

Figure 8: Cartoon of the dynein heavy chain. AAA domains are numbered 1 through 6 and form a ring along with the C-terminal domain. The proposed coiled-coiled is shown between AAA subunits 3 and 6. ATP hydrolysis is proposed to cause a movement in either the stalk or the tail. The active form of dynein consists of two heavy chains. Dimerization occurs via the dimerization domain at the end of the "tail" and the microtubule is contacted via the "stalk."

How does dynein translate ATP hydrolysis into molecular motion? For dynein to "walk" along a microtubule motion needs to be coordinated between the tails of the two heavy chains and the energy for the motion needs to come from ATP hydrolysis in the head region of the heavy chains (Gennerich and Vale, 2009, Numata et al., 2008). The interaction of dynein with its substrates is very different from the AAA proteases or the E1 helicase, as the microtubule is not translocated through the pore or even in direct contact with the AAA domains. Instead, the microtubule is contacted via a stalk that extends from the ring between the fourth and fifth subunits (Figure 8). Not all of the subunits have equivalent roles or flexibility, the first four AAA subunits are believed to be fairly rigid with motion being driven by the fifth and sixth subunits (Kon et al., 2004). This driving force results in either (or potentially both) movement of the stalk or of the tail domain of dynein relative to the central motor unit (Burgess et al., 2003, Roberts et al., 2009).

Understanding of dynein is hindered by the lack of detailed structural data. The dynein complex, which is around 1.2MD, has not been crystallized. However, structural studies have been conducted via EM and some of the subunits of dynein have been crystallized individually. This data has been used to create models of the dynein complex and in attempts to identify the structural elements observed via EM. Large questions remain even in light of these studies, such as whether it is the tail, the stalk, or potentially both moved by ATP hydrolysis. The overall rigidity of four of the AAA domains and the fact that the vast majority of ATP hydrolysis occurs in the remaining two domains, suggests that the presence of six AAA domains owe more to heredity than necessity. The motion

of dynein, while larger, is more constrained in possible contacts than that of AAA enzymes that utilize their central pore through which to translocate their substrates. When a substrate is being translocated through the pore there are at least six possible points of contact and all of the subunits have the potential to drive the interaction. In the case of dynein, none of the subunits are in direct contact with the substrate, and not all of the subunits are even adjacent to the portions of the enzyme that define motion.

F_1-ATPase

F_1-ATPase functions as the soluble portion of the F_0F_1-ATP synthase, by coupling ATP-synthesis with proton transfer across a membrane. On its own, F_1-ATPase instead performs the reverse reaction (Capaldi and Aggeler, 2002). The total enzyme is constructed as two rotary motors that can function and move independently, one in the F_1 part that links catalytic site events with movements and one in the F_0 part, linking movement to proton translocation.

F_1 is a classical molecular motor and is the smallest rotary motor that has been identified to date. It is not a AAA+ enzyme but contains multiple similar motifs, including the Walker A and Walker B motifs, along with an arginine finger that bears similarity to the AAA+ sensor 2 motif based on sequence and structural alignments (Enemark and Joshua-Tor, 2008). In addition, F_1 contains a hexamer consisting of alternating α and β subunits surrounding a central stalk. ATP hydrolysis by the β subunits drives rotation around the stalk leading to changes to conformation of the subunits and associated changes in ATP-binding (Noji et al., 1997). ATP hydrolysis is directly and reversibly linked to mechanical

motion- by forcing the rotor to turn in the opposite direction F_1 synthesizes ATP (Itoh et al., 2004).

Interpretations and Conclusions

There are broad areas of similarity between the molecular motors described here, both in their sequences and structures and potentially in the mechanism of work. The different AAA+ enzymes examined here use apparently dissimilar mechanisms, with the AAA proteases and the E1 helicase translocating their substrates through their pores, while dynein coordinates movement between two heavy chains via stalk regions and the F_1-ATPase rotates around a stalk domain. However, these motions may all be driven by pore region dynamics. The role of pore loops in translocation is apparent in the case of the AAA proteases and the E1 helicase. For the F_1-ATPase it appears that pore loop interactions are directly correlated with ATP hydrolysis due to the fact that reversing mechanical motion, rotation of the pore region around a stalk, also reverses ATP hydrolysis. For dynein, it is more likely that rather than the pore being essential mechanistically, the ring-shape must be held to allow the tail and the stalk a pivot point. Whether or not this requires any form of mediation from the pore is uncertain. However, a better understanding of the driving mechanism that couples ATP hydrolysis and mechanical work, in any of these systems, is likely to expand understanding of the other systems.

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Chapter 2

Polypeptide translocation by the AAA+ ClpXP protease machine

Accepted for publication: Sarah R. Barkow, Igor Levchenko, Tania A. Baker, and Robert T. Sauer (*Chemistry and Biology*, 2009). Polypeptide translocation by the AAA+ ClpXP protease machine.

Abstract

In the AAA+ ClpXP protease, ClpX uses repeated cycles of ATP hydrolysis to pull native proteins apart and to translocate the denatured polypeptide into ClpP for degradation. Here, we probe polypeptide features important for translocation. ClpXP degrades diverse synthetic peptide substrates despite major differences in side-chain chirality, size, and polarity. Moreover, translocation occurs without a peptide –NH and with 10 methylenes between successive peptide bonds. Pulling on homopolymeric tracts of glycine, proline, and lysine also allows efficient ClpXP degradation of a stably folded protein. Thus, minimal chemical features of a polypeptide chain are sufficient for translocation and protein unfolding by the ClpX machine. These results suggest that the translocation pore of ClpX is highly elastic, allowing interactions with a wide-range of chemical groups, a feature likely to be shared by many AAA+ unfoldases.

Introduction

Molecular machines of the AAA+ family (ATPases associated with various cellular activities) use ATP hydrolysis to drive repetitive conformational changes that perform mechanical work within cells (for review, see Hanson and Whiteheart, 2005). Many AAA+ enzymes function by translocating polypeptide or nucleic-acid polymers. Examples include ATP-dependent proteases, protein-secretion translocons, and DNA/RNA helicases, pumps, and viral packaging motors. For AAA+ proteases, ATP hydrolysis is coupled to conformational changes that are used to force unfolding of native protein substrates and then to drive polypeptide translocation into the degradation chambers of enzymes such as ClpXP, ClpAP, HslUV, Lon, FtsH, and the proteasome (for review, see Sauer et al., 2004).

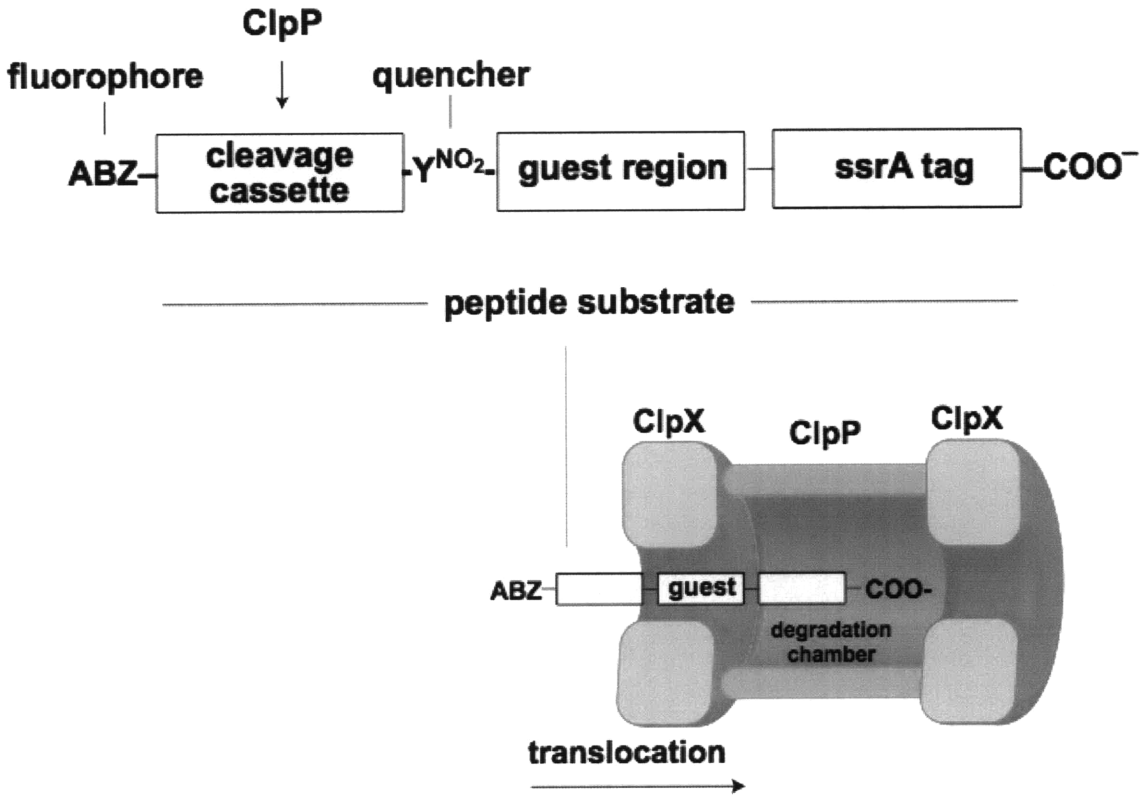

Figure 1: Top — peptide substrates contained an N-terminal sequence (FAPHMALVP) that is cleaved efficiently by a ClpP, a central guest region of variable composition, and a C-terminal ssrA tag (AANDENYALAA). The cleavage cassette had an amino-benzoic acid fluorophore (ABZ) on the N-terminal side and a nitro-tyrosine quencher (Y^{NO2}) on the C-terminal side to allow detection of ClpP proteolysis. Bottom — ClpP cleavage between the ABZ and Y^{NO2} groups of peptide substrates requires prior translocation of the guest region through the axial pore of ClpX.

The ClpXP protease of *Escherichia coli*, which consists of the hexameric ClpX ATPase and the tetradecameric ClpP peptidase, is an archetypal AAA+ protease. ClpP is formed by back-to-back stacking of two $ClpP_7$ rings, placing the proteolytic active sites in an interior chamber accessible through a narrow axial portal in each ring (Wang et al., 1997). Six identical ClpX subunits, each containing a single AAA+ ATPase module, interact to form a hexameric ring with an axial pore. In ClpXP, the pores of one or two hexamers of ClpX align with the ClpP portals, creating channels for polypeptide

translocation into the degradation chamber (Fig. 1; Grimaud et al., 1998; Ortega et al., 2000; Martin et al., 2007). Protein substrates are targeted to ClpXP by short peptide sequences (Flynn et al., 2003). For example, any protein bearing a C-terminal ssrA tag (AANDENYALAA) is a substrate for ClpXP degradation (Gottesman et al., 1998; Kim et al., 2000; Singh et al., 2000; Lee et al., 2001; Kenniston et al., 2003; 2004). The ssrA tag initially binds in the axial pore of ClpX (Siddiqui et al., 2004; Martin et al., 2008b; 2008c).

Polypeptide translocation by ClpX is required for protein unfolding and for transporting denatured substrates into ClpP for degradation. Translocation of the ssrA tag of a native substrate appears to pull the attached protein structure against the entrance to the axial pore, thereby generating a denaturation force because the pore is smaller than the folded protein (for review, see Sauer et al., 2004). For a very stable native substrate, hundreds of cycles of ATP hydrolysis by ClpXP can be required before denaturation occurs, suggesting that enzymatic unfolding is a stochastic process with only a small probability of success per pulling event (Kenniston et al., 2003). A translocation-induced unfolding model is supported by the finding that mutations in the GYVG loops, which line the axial pore of ClpX, slow translocation of unfolded substrates, reduce the rate of unfolding of native substrates, and increase the ATP-hydrolysis cost of both processes (Martin et al., 2008c).

AAA+ enzymes use two kinds of "active" sites for ATP-dependent polypeptide translocation. One traditional "chemical" site mediates the binding and hydrolysis of ATP

to generate conformational changes in the enzyme. The other "mechanical" site transmits force generated by these conformational movements to the substrate. What features of a polypeptide chain are recognized by the mechanical site of ClpX to allow the pulling events that lead to translocation and unfolding? The answer is unclear. There appears to be no obligatory directionality to translocation, as ClpXP can degrade substrates starting either from the N-terminus or from the C-terminus (Gottesman et al., 1998; Gonciarz-Swiatek et al., 1999; Lee et al., 2001; Flynn et al., 2003; Hoskins et al., 2002; Kenniston et al., 2005; Farrell et al., 2007). In principle, the pore loops of ClpX could bind to the peptide bonds, interact with certain types of side chains, or recognize the chiral branching of side chains in the unfolded polypeptide. Based on mutant studies in the related HslUV protease and the conservation of a critical aromatic side chain in the pore loops of all AAA+ unfoldases, it has been postulated that π-cation and π–π interactions between the unfoldase and aromatic side chains in a substrate may be important for translocation and unfolding (Park et al., 2005). Glycine/alanine-rich stretches and other low-complexity sequences appear to prevent unfolding of very stable domains by the 26S proteasome, suggesting that side-chain variety may be an important component in translocation-dependent denaturation of hyper-stable structures (Tian et al., 2005; Hoyt et al., 2006).

Here, we probe the chemical and structural features of a polypeptide chain that allow it to be translocated by ClpX and find that this process is remarkably promiscuous. Peptides can be translocated even when they contain homopolymeric tracts of amino acids that are chemically and structurally diverse, including D-amino acids, residues that lack a peptide –NH group, or amino acids bearing insertions of as many as nine methylene groups

between successive peptide bonds. These results, which run counter to traditional "lock and key" notions of enzymatic specificity, have important implications for the mechanism of ClpX translocation and unfolding and may be a common feature of other ATP-dependent unfoldases.

Results

Design of substrates

All peptide substrates were prepared by solid-phase synthesis and contained three segments: an N-terminal module containing a ClpP cleavage site, a variable central guest region, and a C-terminal ssrA tag (Fig. 1). To detect peptide-bond cleavage, we used a peptide sequence (FAPHMALVP) that ClpP cleaves at a rate $\geq 10^4$ min^{-1} $ClpP_{14}^{-1}$ (Thompson and Maurizi, 1994), flanked on one side by an aminobenzoic acid fluorophore (ABZ) and on the other side by a nitro-tyrosine quencher (Y^{NO2}). Cleavage within this segment results in an increase in fluorescence. The guest region was typically 10 residues in length, which exceeds the ClpX translocation step-size (Kenniston et al., 2005; Martin et al., 2008b). In an extended conformation, 10 residues are also sufficient to span the entire ClpX pore (S. Sundar, A. Martin, R.T.S., unpublished). Consequently, active translocation of the guest region of a peptide substrate is required for the cleavage module to enter ClpP for proteolysis (Fig. 1). To improve solubility, most substrates also had two lysines (KK) between the guest region and the ssrA tag. Table 1 lists the sequences of the peptides used for this study. We refer to peptides using the one letter code for the sequence of the guest region. For example, the [Q_{10}] peptide contains ten

glutamines in the guest region, and the $[VG]_5$ peptide has a guest region with the sequence VGVGVGVGVG.

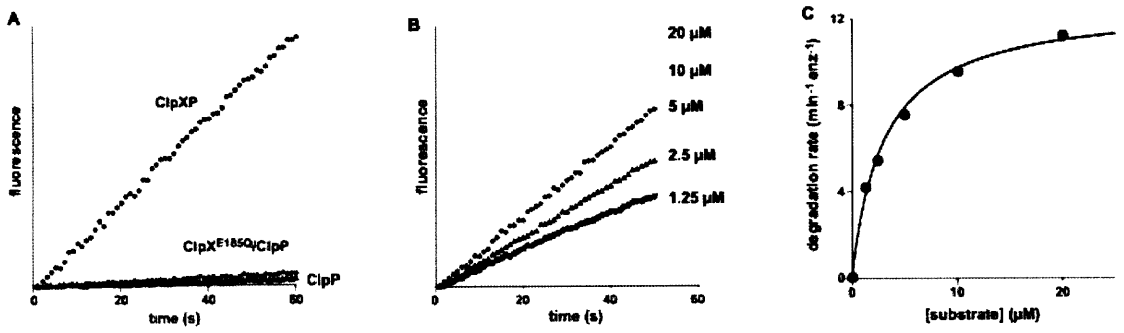

Figure 2: (**A**) Efficient cleavage of the $[G_{10}]$ peptide by ClpP was observed in the presence of wild-type ClpX but not in the absence of ClpX or with $ClpX^{E185Q}$, which cannot hydrolyze ATP. All reactions contained 10 μM of the $[G_{10}]$ substrate and 300 nM $ClpP_{14}$. When present, the concentration of $ClpX_6$ or the ATPase-defective mutant was 800 nM. (**B**) Degradation of different concentrations of the $[VG]_5$ peptide by 800 nM $ClpX_6$ and 300 nM $ClpP_{14}$. (**C**) Steady-state rates of $[VG]_5$ peptide degradation by ClpXP were calculated from the data in panel C and fit to the Michaelis-Menten equation (K_M = 3.1 μM; V_{max} = 12.7 min^{-1} $ClpP^{-1}$).

Degradation requires ATP-dependent translocation

Using the fluorescence assay to monitor substrate cleavage, we assayed the rate of degradation of 10 μM $[G_{10}]$ peptide by ClpXP (Fig. 2A). Control experiments established that cleavage was almost entirely dependent on ATP-dependent translocation by ClpX. First, cleavage by ClpP alone occurred at a 40-fold slower rate than cleavage by ClpXP (Fig. 2A). Second, the ATPase and translocation defective $ClpX^{E185Q}$ mutant, which still binds ClpP and ssrA-tagged substrates in an ATP-dependent fashion (Hersch et al., 2005), did not markedly stimulate ClpP cleavage of this peptide substrate (Fig. 2A). Similar results were observed for all peptide substrates; peptide cleavage by ClpP alone was always at least 20-fold slower than that by ClpXP (not shown). We conclude that the

vast majority of ClpXP peptide degradation in our assays occurs via active ATP-dependent translocation.

For each peptide, we measured steady-state rates of ClpXP degradation at different substrate concentrations and fit the data to obtain K_M and V_{max} values (Table 1). Figs. 2B and 2C show these experiments for the $[VG]_5$ peptide. In all cases, we report maximal degradation rates normalized by the total concentration of ClpP. For example, the steady-state kinetic parameters obtained by fitting one set of $[VG]_5$ degradation reactions were $K_M = 3.1\ \mu M$ and $V_{max} = 12.7\ min^{-1}\ ClpP^{-1}$. Although both K_M and V_{max} varied for different peptides, the latter parameter is more important for understanding effects on ClpX translocation. Indeed for different peptides, average V_{max} values obtained from 2-3 experiments ranged from 2.7 to 14.5 $min^{-1}\ ClpP^{-1}$ (Fig. 3; Table 1). These results show that the identity of residues in the peptide guest region influences the overall rate of ClpXP degradation.

Polyglycine translocation

Glycine is the smallest amino acid, with only a hydrogen atom for a side chain. ClpXP degraded peptides with seven or ten glycines in the guest region with V_{max} values of approximately 14 $min^{-1}\ ClpP^{-1}$ (Table 1; Fig. 3), demonstrating successful translocation of polyglycine sequences. When we permuted the sequence of the $[G_{10}]$ peptide by moving the KK solubility sequence to the middle of the guest region in the $[G_5KKG_5]$ substrate, V_{max} for ClpXP degradation was unchanged (Table 1; Fig. 3).

Table 1: Steady-state kinetic parameters for ClpXP degradation of peptide substrates

name	V_{max} min^{-1} $ClpP^{-1}$	K_M μM	V_{max} with SspB min^{-1} $ClpP^{-1}$	cost ATP/peptide with SspB	sequence	length
$[G_7]$	13.7 ± 1.3	5.4 ± 1.9	13.5 ± 0.7	30	ABZ-FAPHMALVPY^{NO2}G_7KKAANDENYALAA	30
$[G_{10}]$	14.3 ± 0.8	6.1 ± 0.2	11.5 ± 0.4	30	ABZ-FAPHMALVPY^{NO2}G_{10}KKAANDENYALAA	33
$[G_5KKG_5]$	14.5 ± 1.9	4.5 ± 2.2	12.1 ± 0.1	36	ABZ-FAPHMALVPY^{NO2}G_5KKG_5AANDENYALAA	33
$[\beta]_{10}$	9.8 ± 0.9	4.1 ± 0.3	8.8 ± 1.1	43	ABZ-FAPHMALVPY^{NO2}β_{10}KKAANDENYALAA	33
$[\gamma]_{10}$	6.4 ± 0.3	3.7 ± 0.6	6.5 ± 0.4	57	ABZ-FAPHMALVPY^{NO2}γ_{10}KKAANDENYALAA	33
$[\varepsilon]_{10}$	6.1 ± 0.6	4.7 ± 0.4	5.9 ± 0.3	61	ABZ-FAPHMALVPY^{NO2}ε_{10}KKAANDENYALAA	33
$[O]_5$	7.0 ± 0.2	4.3 ± 0.4	6.8 ± 1.3	53	ABZ-FAPHMALVPY^{NO2}O_5KKAANDENYALAA	28
$[U]_4$	6.2 ± 0.6	9.0 ± 2.0	7.8 ± 0.5	47	ABZ-FAPHMALVPY^{NO2}U_4KKAANDENYALAA	27
$[P_5]$	11.4 ± 0.2	n.d.	12.9 ± 1.1	45	ABZ-FAPHMALVPY^{NO2}P_5KKAANDENYALAA	28
$[P_{10}]$	6.4 ± 0.2	2.8 ± 0.2	6.5 ± 0.2	65	ABZ-FAPHMALVPY^{NO2}P_{10}KKAANDENYALAA	33
$[P_{15}]$	3.4 ± 0.8	3.6 ± 0.7	3.3 ± 0.3	125	ABZ-FAPHMALVPY^{NO2}P_{15}KKAANDENYALAA	38
$[VG]_5$	14.4 ± 1.7	3.4 ± 0.3	13.6 ± 0.4	30	ABZ-FAPHMALVPY^{NO2}$[VG]_5$KKAANDENYALAA	33
$[DVG]_5$	14.0 ± 0.4	6.7 ± 1.6	14.2 ± 0.2	28	ABZ-FAPHMALVPY^{NO2}$[DVG]_5$KKAANDENYALAA	33
$[FG]_5$	12.1 ± 1.7	4.2 ± 1.0	14.1 ± 0.8	22	ABZ-FAPHMALVPY^{NO2}$[FG]_5$KKAANDENYALAA	33
$[Q_{10}]$	8.7 ± 0.5	9.1 ± 1.7	8.8 ± 0.8	48	ABZ-FAPHMALVPY^{NO2}Q_{10}KKAANDENYALAA	33
$[E_{10}]$	6.0 ± 0.1	2.0 ± 0.1	6.3 ± 0.2	62	ABZ-FAPHMALVPY^{NO2}E_{10}KKAANDENYALAA	33
$[K_{10}]$	2.7 ± 0.1	≤ 0.2	2.8 ± 0.1	160	ABZ-FAPHMALVPY^{NO2}K_{10}KKAANDENYALAA	33
$[R_{10}]$	8.0 ± 0.4	0.2 ± 0.1	8.4 ± 0.1	53	ABZ-FAPHMALVPY^{NO2}R_{10}KKAANDENYALAA	33

β (β-alanine); γ (γ-aminobutyric acid), ε (ε-amino caproic acid); O (8-aminooctanoic acid), U (11-aminoundecanoic acid).

V_{max} and K_M values are means of 2-3 independent determinations (n) with errors calculated as $\sqrt{\frac{1}{(n-1)}\sum_{1}^{n}(value-mean)^2}$

Altered peptide-bond spacings

Successive peptide bonds in all natural proteins are separated by a single carbon atom and are related by two dihedral angles (Φ, Ψ), resulting in restrictions in possible backbone conformations. To probe the importance of this geometry, we synthesized peptides in which the guest region contained unnatural amino acids with additional carbon atoms between successive peptide bonds by inserting β-alanine (2-carbon spacing), γ-aminobutyric acid (3-carbon spacing), ε-aminocaproic acid (5-carbon spacing), 8-aminooctanoic acid (7-carbon spacing), or 11-aminoundecanoic acid (10-carbon spacing) in the guest region. Strikingly, peptides with 4-10 residues of these "stretched" amino acids in the guest region were degraded at 40-70% of the [G_{10}] peptide degradation rate (Fig. 3). Because substrates with guest-region spacings of 2-10 methylene groups between successive peptide bonds were translocated and degraded at substantial rates compared to peptides with the normal single-carbon spacing, we conclude that the spacing of peptide bonds along the polypeptide backbone is not a major determinant of recognition during translocation of substrates by ClpXP.

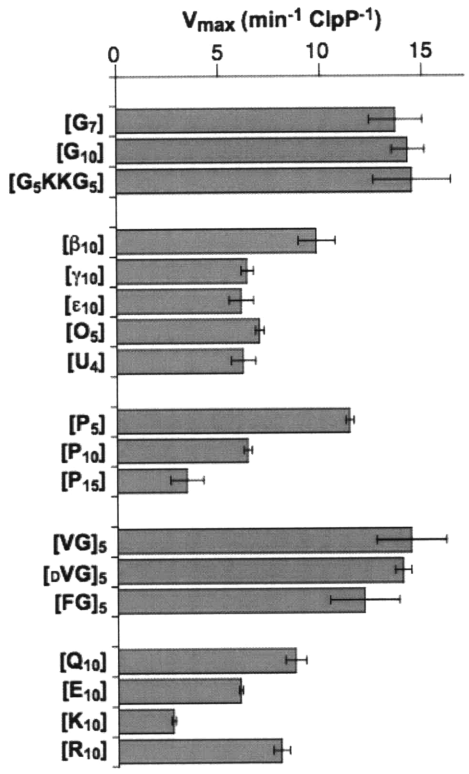

Figure 3: Maximum rates of ClpXP degradation of peptide substrates with different guest regions were determined from multiple experiments like those shown in Fig. 2B and 2C. See Table 1 for sequences of individual peptides and definition of error bars.

Polyproline translocation

Proline lacks a peptide –NH group, and successive prolines severely constrain the polypeptide backbone and often adopt a left-handed polyproline-II helix (Φ = -75°, Ψ = 150°; Schulz and Schirmer, 1979; Adzhubei and Sternberg, 1993). The maximal rates of ClpXP degradation of the [P_5], [P_{10}], and [P_{15}] peptides were 11.4, 6.4, and 3.4 min^{-1} $ClpP^{-1}$, respectively (Table 1; Fig. 3). Hence, a peptide –NH group is not required for translocation nor is the ability to assume an α-helix, a β-strand, or other conformations incompatible with polyproline sequences. Because ClpX degradation slowed in

proportion to the length of the polyproline segment, however, a polyproline helix might be difficult to translocate or may need to be disrupted to allow translocation.

Side-chain chirality and size

Natural amino acids, with the exception of glycine, are L-isomers. To assess the effect of side-chain chirality on ClpXP translocation, we measured degradation rates for substrates with five successive L-Val-Gly repeats in the guest region (V_{max} = 14.4 min^{-1} $ClpP^{-1}$) or five successive D-Val-Gly repeats (V_{max} = 14.0 min^{-1} $ClpP^{-1}$). Because these rates are essentially the same, we conclude that peptides containing D-isomers can be translocated as well as those containing L-isomers. Thus, ClpX appears to be indifferent to side-chain chirality.

The maximum degradation rates for the $[G_{10}]$, $[VG]_5$, and $[FG]_5$ peptides were 14.3 min^{-1} $ClpP^{-1}$, 14.4 min^{-1} $ClpP^{-1}$, and 12.1 min^{-1} $ClpP^{-1}$, respectively. The similarities in these rates suggest that the presence of larger residues in a substrate, including β-branched and aromatic side chains, plays little role in ClpXP translocation.

Side-chain polarity and charge

Does the charge or polarity of amino-acid side chains affect ClpX translocation? To address this question, we determined V_{max} values for ClpXP degradation of peptides with guest regions containing 10 lysines (V_{max} = 2.7 min^{-1} $ClpP^{-1}$), 10 arginines (V_{max} = 8.0 min^{-1} $ClpP^{-1}$), 10 glutamic acids (V_{max} = 6.0 min^{-1} $ClpP^{-1}$), or 10 glutamines (V_{max} = 8.7

min^{-1} $ClpP^{-1}$). These results show that ClpXP can translocate homopolymeric stretches of charged and polar side chains. Degradation of the $[K_{10}]$ peptide was slower than any of the other peptides tested in this study. However, the $[R_{10}]$ peptide was degraded about three-fold faster, showing that positive charge *per se* is not the sole cause of slow $[K_{10}]$ peptide degradation. The $[Q_{10}]$ peptide was degraded about 50% faster than the $[E_{10}]$ peptide. Thus, negatively charged glutamic-acid side chains are modestly more difficult for ClpX to translocate than uncharged but isosteric glutamine side chains.

ATP cost of translocation

Rates of substrate degradation by ClpXP need not be correlated with energetic efficiency, because the ATPase activity of ClpX can vary substantially for different substrates (Kenniston et al., 2003; 2004; Martin et al., 2008c). To assess energetic costs, we measured the rate of ATP hydrolysis and the maximum rate of peptide degradation during ClpXP proteolysis under the same conditions. To ensure saturation of the enzyme by substrate in these studies, we used 10-20 μM substrate in the presence of equimolar SspB adaptor, which reduces K_M for ClpXP degradation of ssrA-tagged substrates to a value of 200 nM or less (Levchenko et al., 2000; Wah et al., 2003; Bolon et al., 2004). We then divided the ATPase rate by the degradation rate for the SspB-bound peptide to provide an estimate of the number of ATPs hydrolyzed during degradation of a single molecule of each peptide substrate. This value is an average. It includes energy consumed during productive and non-productive work (for example, substrate slipping or ATP hydrolyzed during engagement), much as the fuel economy of a vehicle traveling over

rough muddy terrain might be reduced by occasional spinning of the wheels without net movement.

As shown in Table 1, the cost of degradation ranged from approximately 20 to 160 ATPs per substrate, with the highest costs associated with the slowest V_{max} values. Peptides with non-polar amino acids in the guest region were degraded with the lowest costs, whereas peptides with "stretched" amino acids, prolines, or polar residues had higher costs. For the most efficient substrates, an average of about one ATP was hydrolyzed per amino acid translocated and degraded. For the least efficient substrate, an average of roughly five ATPs were hydrolyzed per amino acid translocated and degraded. For several substrates, we also performed experiments to calculate the ATP cost of peptide degradation in the absence of SspB and obtained values within 20% of those measured with the adaptor (not shown).

Translocation under load

It might be argued that homopolymeric stretches of glycines, prolines, or other residues are easy to translocate in the absence of an opposing force, but may not allow ClpX to grasp a substrate firmly enough to allow it to unfold a stable native protein. To test this idea, we fused degradation tags containing stretches of glycine, proline, or lysine to green fluorescent protein (GFP). Denaturation is known to be the rate-limiting step in ClpXP degradation of ssrA-tagged variants of GFP (Kim et al., 2000). Michaelis-Menten experiments, like the one shown in Fig. 4A, revealed that ClpXP degraded all of these GFP substrates at comparable maximal rates (Fig. 4B). For polyglycine substrates, the

maximal rate of degradation was similar regardless of whether the homopolymeric stretch was immediately adjacent to GFP, and thus would occupy the pore during unfolding, or was separated from GFP by several residues. Similar results for tags containing polyglycine have been obtained independently (P. Chien and T.A. Baker, unpublished). Hence, ClpX must grip polyglycine, polyproline, or polylysine sequences tightly enough to allow translocation-mediated unfolding of GFP.

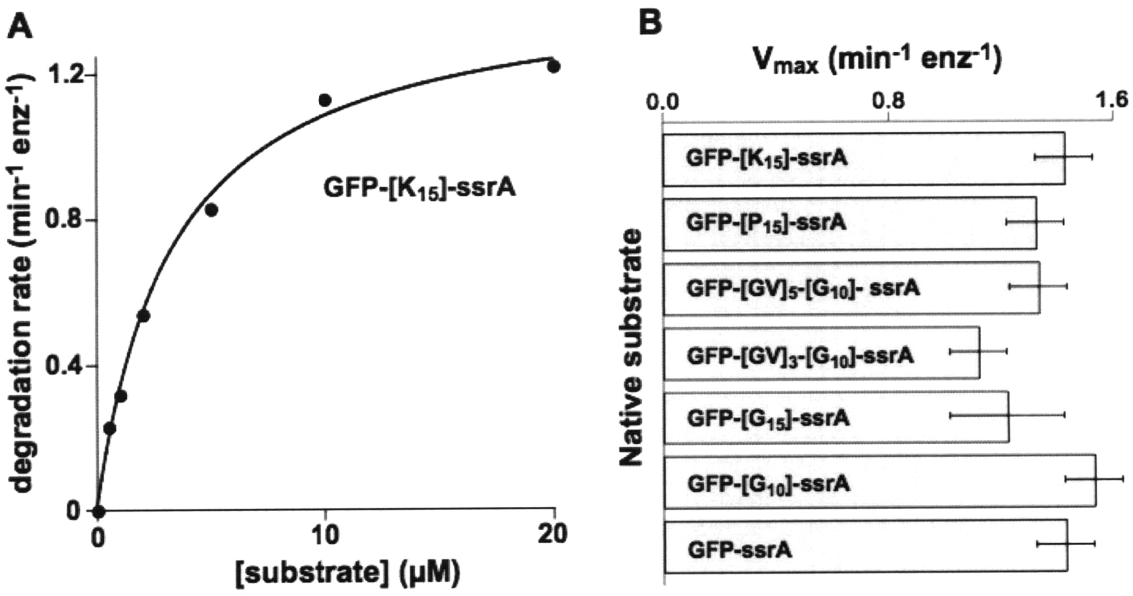

Figure 4: (**A**) Michaelis-Menten analysis of ClpXP degradation of GFP-[K15]-ssrA. The solid line is a non-linear least-squares fit (*K*M = 3.3 ± 0.3 μM; Vmax = 1.44 ± 0.05 min-1 ClpP-1). (**B**) Maximum rates of ClpXP degradation of native GFP substrates with homopolymeric sequences of lysine, proline, or glycine between the folded body of GFP and the ssrA degradation tag. Error bars represent the uncertainty of a non-linear least-squares fit of experimental data to the Michaelis-Menten equation. *K*M's for the fits not shown in panel A were GFP-ssrA (3.3 ± 0.4 μM); GFP-[P15]-ssrA (7 ± 2 μM); GFP-[GV]5-[G10]-ssrA (2.4 ± 0.4 μM); GFP-[GV]3-[G10]-ssrA (2.0 ± 0.2 μM); GFP-[G15]-ssrA (2.1 ± 1 μM); GFP-[G10]-ssrA (4.1 ± 0.5 μM).

Discussion

Molecular translocation can be viewed as moving a biological polymer through a stationary machine or alternatively as tracking of a dynamic machine along a fixed polymer. For example, many DNA and RNA helicases track in a 3′ to 5′ direction along one strand of a nucleic-acid duplex, and simultaneously disrupt interactions with the complementary strand (Patel and Picha, 2000; Singleton et al., 2007; Pyle, 2008; Enemark and Joshua-Tor, 2008). For some helicases, including those belonging to the AAA+ family, the enzyme interacts with the sugar-phosphate backbone of the DNA/RNA strand and has a step size of one nucleotide per ATP hydrolyzed. This type of fixed step-size drive mechanism is analogous to the relationship between the teeth on a sprocket and the roller links on a bike chain, which allows forward pedal movement to be tightly coupled to the rotation of the bike wheel.

Although ClpX conceptually tracks along an unstructured polypeptide chain, our results suggest that this polypeptide-translocation machine operates by a rather different mechanism than related hexameric helicases. For example, there is no obligatory directionality to ClpX translocation in the sense that degradation can start near a degradation tag at either terminus of a protein substrate (Lee et al., 2001; Hoskins et al., 2002; Kenniston et al., 2005). Moreover, we find no evidence that ClpX translocation requires a fixed spacing between successive peptide bonds or side chains. These results appear to rule out drive-train mechanisms that rely on strict geometric coupling between the movement of ClpX machine parts and the properties of the polypeptide chain. Indeed, there may not be an invariant ClpX translocation step size. For unfolded proteins, the

average step size of ClpXP translocation has been estimated to range from one to five amino acids per ATP hydrolyzed (Kenniston et al., 2005; Martin et al., 2008a), and we observed significant variations in the energetic cost of ClpXP degradation of the different peptides studied here, suggesting that they are also translocated with variable average step sizes. Another difference between ClpX and many hexameric helicases involves the order in which subunits around the hexameric ring hydrolyze ATP. A strictly sequential firing mechanism has been proposed for the T7 gp4 helicase, the Φ12 RNA packaging ATPase P4, and the pappilomavirus E1 helicase (Singleton et al., 2000; Mancini et al., 2004; Enemark and Joshua-Tor, 2006), whereas ClpX appears to employ a probabilistic mechanism in which the order of ATP hydrolysis in different subunits is not predetermined (Martin et al., 2005).

Our results show that ClpXP translocation is relatively indifferent to the chirality, size, polarity, or charge of protein side chains. The ClpXP enzyme from *E. coli* has hundreds of natural substrates (Flynn et al., 2003; Neher et al., 2006), and attaching an ssrA tag to numerous proteins makes them substrates for ClpXP degradation (Gottesman et al., 1998; Kim et al., 2000; Singh et al., 2000; Lee et al., 2001; Kenniston et al., 2003; 2004). During translocation of these substrates, the sequence of the polypeptide segment being actively moved through the ClpX pore changes continually. Thus, ClpX must be able to translocate an enormous number of different polypeptide sequences, each with distinct chemical properties and conformational preferences. Viewed from this perspective, our results make both functional and biological sense.

How has nature evolved a protein-degradation machine that is exquisitely specific in terms of substrate choice but cares little about the detailed chemical and structural properties of these substrates? The answer is that degradation, like many key cellular processes, is controlled at the level of initiation. Only proteins bearing degradation tags that bind specifically to the protease are engaged, translocated, and then degraded. For example, the ssrA tag of a substrate initially binds in the axial pore of ClpX, where it makes specific interactions with pore loops whose ATP-fueled movements subsequently drive translocation (Siddiqui et al., 2004; Martin et al., 2008b; 2008c). However, once translocation of the ssrA-tagged substrate commences, the chemical properties of the rest of the polypeptide chain seem to have only small influences on the rate of degradation. An analogy with a macroscopic machine is apt. Conveyor belts can move objects of vastly different sizes and shapes, but these objects must first be placed on the belt.

How does ClpX translocate polypeptide substrates without strict recognition of chemical or geometric features? One possibility is that the ClpX pore is relatively elastic and collapses around a polypeptide, allowing flexible pore loops to maintain atomic contact with the substrate. Then, during the power stroke of the ATPase cycle, conformational changes in ClpX could drag the substrate along by van der Waals forces that create friction between the enzyme and the unfolded polypeptide. Because van der Waals interactions occur between all types of atoms, they would be ideally suited for interactions with substrates like unfolded polypeptides, which have highly variable atomic compositions. Pore elasticity could also explain how ClpXP can simultaneously

translocate multiple polypeptide chains during degradation of disulfide-bonded proteins (Burton et al., 2001; Bolon et al., 2004).

Variation in the average step size for ClpXP translocation (from one to five residues per ATP hydrolyzed Kenniston et al., 2005; Martin et al., 2008a) can be explained in several ways. First, different polypeptide sequences may adopt different conformations during translocation, and a power stroke of a fixed length might move more substrate residues in a compact conformation than in an extended conformation. Second, some substrates might not move during each power stroke, or might slip back afterwards, in a manner that depends upon the precise sequence and the elasticity of the pore. There is precedent for substrate slipping. For example, during attempts to unfold some native ssrA-tagged proteins, engaged substrates slip from of the grasp of ClpXP and are released without being translocated through the pore (Kenniston et al., 2005). In addition, mutating the GYVG pore loops of ClpX results in a smaller average translocation step size per power stroke. This result is expected if such mutations weaken substrate contacts and result in an increased number of mechanical cycles that fail to move the polypeptide substrate (Martin et al., 2008c).

Regardless of the exact mechanism, our results show that ClpXP can grip and translocate homopolymeric stretches of glycine, proline, and lysine forcefully enough to denature an attached GFP protein. Because spontaneous solution denaturation of GFP occurs with a half-life of years, enzymatic unfolding of this protein by ClpXP represents a major challenge (Kim et al., 2000). Taken together, these results indicate that minimal features

of a polypeptide chain are adequate for ClpXP translocation, even when acting against a substantial resisting force. These findings are also consistent with experiments demonstrating that ClpXP can completely degrade some substrates containing several folded protein domains (Lee et al., 2001; Kenniston et al., 2005; Martin et al., 2008b; 2008c). In these instances, the primary degradation tag attached to the first domain is proteolyzed before the second domain is encountered, and translocation through the ClpX pore of a segment of the first domain or a linker drives unfolding of the second domain. Thus, there appears to be no requirement for translocation of specialized sequences to allow denaturation of attached native domains. It is possible, of course, that some polypeptide sequences are somewhat "slippery" during normal ClpX translocation and thus do not allow efficient force transfer and subsequent ClpXP denaturation of hyper-stable substrates, as has been demonstrated for the eukaryotic proteasome (Tian et al., 2005; Hoyt et al., 2006).

Given that our studies show that minimal sequence determinants are required for substrate translocation by ClpXP, it is reasonable to ask if this ability to translocate radically different natural and unnatural polypeptide sequences is a specialized adaptation or represents a general property of other ATP-dependent proteases as well. Because all AAA+ proteases share the ability to degrade a wide variety of protein substrates, we anticipate that translocation tolerance may be a common feature of this entire enzyme family.

Significance

Prior to this work, it would have been reasonable to assume that translocation by ClpX involves recognition either of regular chemical features of the polypeptide backbone or of specific side chains in a substrate. Strikingly, our experimental results fail to support either model. Instead, we find that translocation and subsequent degradation by ClpXP is remarkably tolerant to a wide range of natural and non-natural amino acids. We find no evidence that sequence diversity is necessary for normal translocation. For example, homopolymeric stretches of charged amino acids (Lys, Arg, Glu), polar residues (Gln), and small non-polar residues (Pro, Ala, Gly) all appeared to be translocated. Similarly, the presence of D-amino acids or residues with 2-10 methylenes between successive peptide bonds failed to halt translocation by ClpX. Moreover, previous studies had shown that there is no requisite directionality to ClpXP degradation and that more than one polypeptide chain can be translocated at the same time (Burton et al., 2001; Lee et al., 2001; Hoskins et al., 2002; Bolon et al., 2004; Kenniston et al., 2005). A new model is required to account for this collective information. The translocation pore of the ClpX hexamer must be elastic and highly adaptable, and general chemical features, such as van der Waals interactions, must allow ClpX to grip substrates tightly enough to couple nucleotide-dependent changes in hexamer structure to vectorial movement of the translocating polypeptide. It will be important to decipher the structural basis of this translocation mechanism and to test whether it also applies to other families of AAA+ proteases.

Experimental Procedures

Peptides and proteins

Peptides were synthesized by standard solid-phase techniques and were purified by reverse-phase HPLC chromatography on an LC-10AD-VP column (Shimadzu), using a gradient from 0 to 80% acetonitrile in 0.06% TFA. The expected masses of purified peptides were confirmed by MALDI-TOF mass spectrometry. Peptide concentrations were determined by nitro-tyrosine absorption at 381 nm (ε = 2200 M^{-1} cm^{-1}; Means and Feeny, 1971).

Variants of *E. coli* ClpX with an N-terminal His_6 tag and *E. coli* ClpP with a C-terminal His_6 tag were purified as described (Kim et al., 2000; Hersch et al., 2004). PCR-mediated mutagenesis was used to construct GFP variants with an N-terminal His_6 tag, a variable sequence, and a C-terminal ssrA tag after the GFP-coding sequence. These variants were expressed, under IPTG control, from a pACYC vector in *E. coli* BLR/λDE3 *ΔclpX* cells and were purified by Ni^{2+}-NTA affinity after lysis under non-denaturing conditions. GFP concentrations were determined by absorbance at 280 nm (ε = 19770 M^{-1} cm^{-1}). Purified GFP-ssrA and $ClpX^{E185Q}$ were gifts from Greg Hersch (MIT).

Assays

Degradation assays were performed at 30 °C in PD buffer (25 mM HEPES [pH 7.6], 200 mM KCl, 5 mM $MgCl_2$, 0.032% NP-40, and 10% glycerol). For ClpXP degradation, assays included 300 nM Clp_{14}, 800 nM wild-type or mutant $ClpX_6$, and an ATP

regeneration system consisting of 4 mM ATP, 16 mM creatine phosphate, and 0.32 mg/mL creatine phosphokinase. For ClpP degradation, ClpX and the ATP regeneration system were omitted. All reaction components except substrate were preincubated at 30 °C, and reactions were initiated by the addition of substrate and monitored by changes in fluorescence using a QM-2000-4SE spectrofluorimeter (Photon Technology International). For peptide degradation assays, samples were excited at 320 nm and fluorescence at 420 nm was monitored. In a control experiment, we found that complete ClpXP degradation of the $[VG]_5$ peptide resulted in the same final fluorescence as degradation of this substrate by chymotrypsin, and that peptide-degradation rates calculated from changes in fluorescence corresponded well to rates determined by loss of the substrate peak following HPLC separation. For GFP degradation assays, samples were excited at 467 nm and fluorescence emission was monitored at 511 nm; assays monitored by SDS-PAGE showed similar rates of GFP degradation by ClpXP.

For measurement of rates of ATP hydrolysis during degradation, we used the SspB adaptor protein (a gift from Natalia Ivanova, MIT) to ensure that ClpX was saturated with the ssrA-tagged peptide substrate. For these experiments, reactions contained 800 nM $ClpX_6$, alone or with 300 nM $ClpP_{14}$, and equimolar SspB and peptide substrate (10-20 μM) in PD buffer. Rates of ATP hydrolysis at 30 °C were measured by changes in absorbance at 340 nm using a coupled assay system with 5 mM ATP, 1 mM NADH, 2 mM phosphoenolpyruvate, 3 U/ml lactate dehydrogenase, and 3 U/ml pyruvate kinase (Nørby, 1988). For the experiments containing $ClpX_6$ and $ClpP_{14}$, the ATPase activity of ClpX hexamers in the doubly capped $ClpX_6$•$ClpP_{14}$•$ClpX_6$ complex was calculated by

correcting for the activity of free ClpX hexamers (25% of total). In control experiments, we found that SspB enhanced the rate of ClpXP degradation of sub-saturating concentrations of peptide substrates but suppressed the very slow rate of ATP-independent proteolysis of these substrates by $ClpX^{E185Q}$/ClpP and ClpP (not shown).

Acknowledgements

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Chapter 3

ClpX GYVG Pore Loop Interactions

Summary

Flexible loops in the pore of ClpX are believed to drive translocation and to mediate substrate-binding interactions. The GYVG pore-1 loop is essential for ClpX activity and is highly conserved among AAA+ proteases. In this chapter, I explore the role of Tyr153 in the GYVG loop. Mutation of this Tyr to Phe, Ile, or Leu causes changes in ATP-hydrolysis rates, activation of ATP hydrolysis by substrates, and substrate binding and degradation.

Introduction

ClpXP consists of the AAA+ unfoldase, ClpX, and the associated peptidase, ClpP. ClpX recognizes protein substrates that contain specific degradation tags (see Chapter 1, Table 1), unfolds these proteins if necessary, and then translocates the unstructured polypeptide into ClpP for proteolysis. Flexible loops that line the pore of ClpX are involved in substrate recognition, ATPase activation, and substrate unfolding and translocation (Figure 1). There are three sets of ClpX pore loops. The pore-2 loops are near the bottom of the ClpX pore and mediate interactions with ClpP, participate in recognition of the ssrA tag, and play roles in substrate unfolding (Martin et al., 2007). The RKH loops are positioned around the entry to the pore. Mutations of the RKH loops alter substrate specificity for different degradation tags, reducing affinity for the C-motif 1 ssrA tag, but increasing affinity for C-motif 2 and N-motif substrates (Farrell et al., 2007). This result suggests that ClpX has evolved moderate affinity for multiple classes of tag instead of high affinity for any single type of tag.

The GYVG or pore-1 loop is located roughly midway through the pore and is important for substrate recognition, unfolding, translocation, and the coupling of mechanical processes to ATP hydrolysis (Siddiqui et al., 2004; Martin et al., 2008a; Martin et al., 2008b). Mutation of Val154 in the GYVG loop causes poor recognition of C-motif 1 substrates but has little effects on recognition of other classes of substrates (Siddiqui et al., 2004). In FtsH and ClpAP, mutations at corresponding positions also affect substrate specificity but do not prevent ATP- hydrolysis-dependent translocation (Okuno et al., 2006; Yamada-Inagawa et al., 2003, Farbman et al., 2008). These results suggest that

different tags are recognized by different binding sites in the pore. The pore-1 loop is also involved in translocation and unfolding. A Tyr→Ala mutation in the GYVG loops of all subunits in $ClpX_6$ kills activity (Siddiqui et al., 2004), and even mutations in just two subunits decreases the speed and energetic efficiency of translocation and unfolding (Martin et al., 2008a). Diminished protein degradation activity was observed when the aromatic residue in the pore-1 loop was mutated to an alanine in either FtsH or ClpAP (Okuno et al., 2006; Siddiqui et al., 2004; Yamada-Inagawa et al., 2003). Non-aromatic hydrophobic side chains at this position in FtsH provided improved performance relative to alanine but were not as good as the wild-type phenylalanine. In this chapter, I explore the effects of substituting Tyr153 in ClpX with Ile, Leu, and Phe.

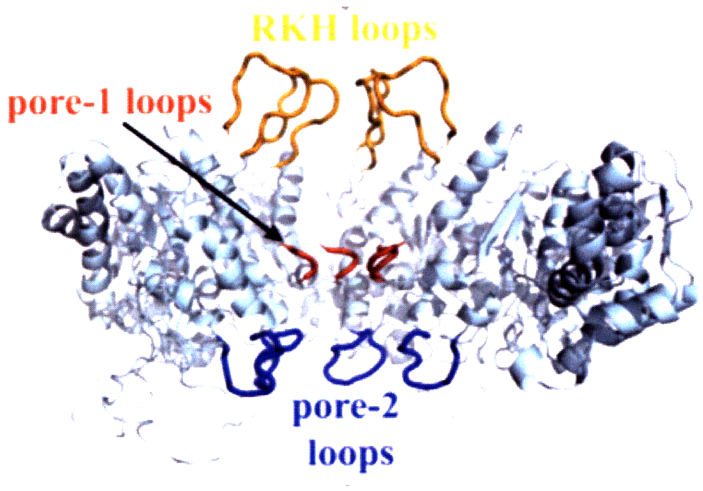

Figure 1: The RKH (gold), pore-1 or GYVG loops (red), and pore-2 loops (blue) are highlighted in a model of the ClpX hexamer based (Kim and Kim, 2003, Bochtler et al., 2000). Three subunits of the hexamer were removed to allow visualization of the pore loops. Figure taken from (Martin et al., 2007).

Materials and Methods

Protein Mutagenesis, Expression, and Purification

Quick-change mutagenesis was used to modify the GYVG loop of *E. coli* ClpX. Variants of *E. coli* ClpX with an N-terminal His_6-tag and *E. coli* ClpP with a C-terminal His_6-tag were purified as described (Hersch et al., 2004; Kim et al., 2000). Three ClpX Tyr153 variants ($ClpX^{YF}$, $ClpX^{YL}$ and $ClpX^{YI}$) were expressed, under IPTG control, from a pET20b vector in *E. coli* BLR/λDE3 cells. After lysis, cell extracts were loaded onto Ni^{2+}-NTA column in 50 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole and the ClpX mutants were eluted with 250 mM imidazole and were approximately 95% pure as estimated by SDS-PAGE. ClpX and variants were stored in Clp buffer containing 50 mM HEPES-KOH [pH 7.5], 200 mM KCl, 25 mM $MgCl_2$, 0.1 mM EDTA and 10% glycerol. GFP-ssrA was a gift from G. Hersch (MIT); SspB adaptor protein was a gift from N. Ivanova (MIT).

Peptides were synthesized by standard Fmoc chemistry and purified by HPLC on a C-18 column in 0.06% TFA using a gradient from 0 to 80% acetonitrile. Two peptides were used for degradation studies. The $[G]_{10}$ peptide had the sequence ABZ-*FAPHMALVPY*^{NO2}G_{10}KKAANDENYALAA, where ABZ is an aminobenzoic acid quencher, the sequence in italics contains a highly efficient ClpP cleavage site, Y^{NO2} is a nitrotyrosine fluorophore, and the underlined sequence is the ssrA tag. In the $[FG]_5$ peptide, the G_{10} sequence was replaced by FGFGFGFGFG.

Assays

ClpXP degradation assays were performed at 30 °C in PD buffer (25 mM HEPES [pH 7.6], 200 mM KCl, 5 mM $MgCl_2$, 0.032% NP-40, and 10% glycerol), with 300 nM $ClpP_{14}$, 800 nM wild-type or mutant $ClpX_6$, and an ATP-regeneration system consisting of 4 mM ATP, 16 mM creatine phosphate, and 0.32 mg/mL creatine phosphokinase. For lambdaO-Arc degradation reactions contained 800 nM wild-type or mutant $ClpX_6$ and either 300 nM $ClpP_{14}$ or 800 $ClpP_{14}$.

For GFP degradation assays, samples were excited at 467 nm and fluorescence emission was monitored at 511 nm. Degradation of lambdaO-Arc was assayed by SDS-PAGE. For peptide degradation assays, samples were excited at 320 nm and fluorescence emission was monitored at 420 nm.

ATPase assays were carried out using either 300 or 800 nM $ClpX_6$ with or without 300 nM $ClpP_{14}$. Activation of the ATPase activity of ClpX was assayed in the presence of peptide substrates (10-100 μM) or with peptide substrates and equimolar quantities of the SspB adaptor protein (up to 20 μM). Rates of ATP hydrolysis at 30 °C were measured by changes in absorbance at 340 nm using a coupled assay system with 5 mM ATP, 1 mM NADH, 2 mM phosphoenolpyruvate, 3 U/mL lactate dehydrogenase, and 3 U/mL pyruvate kinase (Norby, 1988).

Results

The Tyr153→Phe ($ClpX^{YF}$), Tyr153→Ile ($ClpX^{YI}$), and Tyr153→Leu ($ClpX^{YL}$) mutants were active in ATP hydrolysis, indicating that these enzymes form hexamers. This result is unsurprising as these mutations do not affect the subunit interfaces in ClpX. However, the rate of ATP hydrolysis and the sensitivity to the presence of substrate, differed significantly from wild-type ClpX depending on the identity of the mutation (Figure 2). Only the wild-type enzyme showed a significant increase in ATP hydrolysis in the presence of ssrA-tagged substrates.

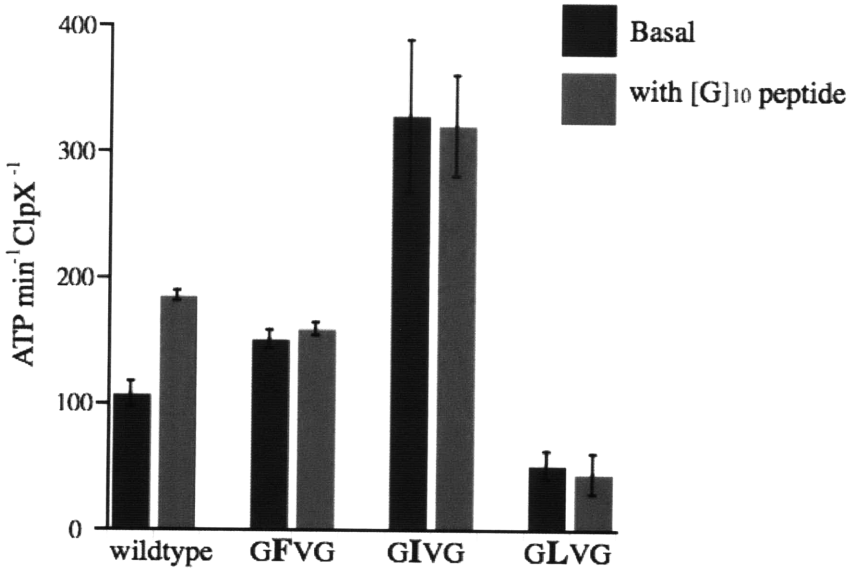

Figure 2: ATP-hydrolysis rates for wild-type and mutant enzymes. Basal ATPase activity (0.3 μM $ClpX_6$) is shown in maroon. Activated rates of ATP hydrolysis (0.3 μM $ClpX_6$) were measured in the presence of 50 μM ssrA-tagged $[G]_{10}$ peptide. GFVG is Tyr153→Phe ($ClpX^{YF}$), GIVG is Tyr153→Ile ($ClpX^{YI}$), and GLVG is Tyr153→Leu ($ClpX^{YL}$).

Large differences in activity between the position-153 mutants were observed in degradation assays. In the presence of ClpP, no degradation of ssrA-tagged GFP or the G_{10} or $[FG]_5$ peptide substrates was observed for either the $ClpX^{YL}$ or $ClpX^{YF}$ mutants (Table 1; Figure 3; data not shown). Notably, substrate degradation by these ClpX mutants was not rescued by the presence of 10 μM SspB adaptor, which increases the affinity of ssrA-tagged substrates for wild-type ClpX to 20-50 nM (Figure 3; Hersch et al., 2004; Bolon et al., 2004). Thus, if the Tyr153→Ile/Leu mutations reduce degradation activity solely by reducing substrate affinity, then this reduction must be substantially greater than 1000-fold.

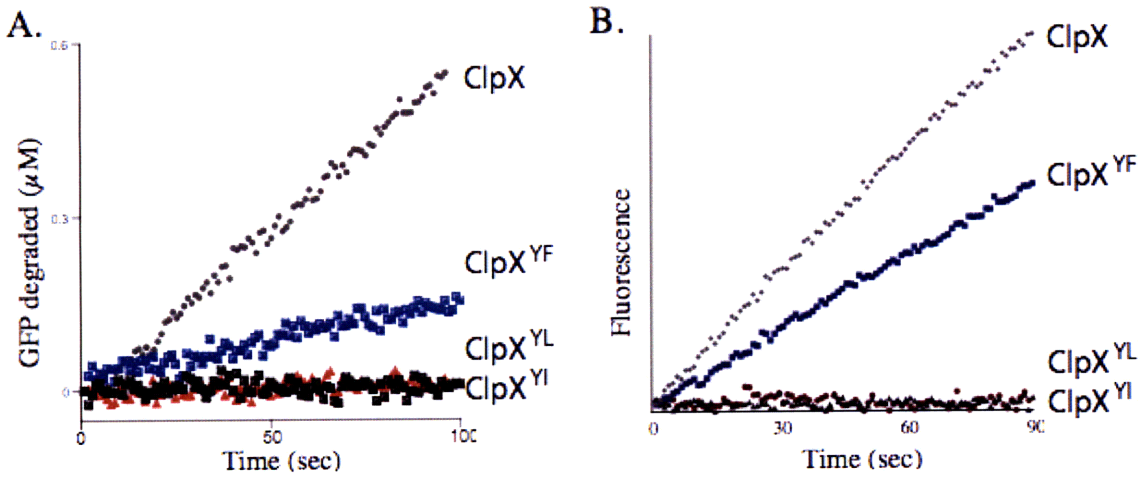

Figure 3: **A**. GFP-ssrA (5 μM) degradation by wild-type ClpXP (gray circles), $ClpX^{YF}$/ClpP (blue squares), $ClpX^{YI}$/ClpP (red triangles), and $ClpX^{YL}$/ClpP (black squares). **B**. Degradation of the ssrA-tagged $[G]_{10}$ peptide (10 μM) with equimolar SspB by wild-type ClpXP (gray circles), $ClpX^{YF}$/ClpP (blue squares), $ClpX^{YI}$/ClpP (red triangles), and $ClpX^{YL}$/ClpP (black squares). All reactions contained 800 nM $ClpX_6$ or variants and 300 nM Clp_{14}.

ClpX enzyme	degradation rate (min^{-1} $[ClpP]^{-1}$)
Wild-type	0.82
$ClpX^{YF}$	0.47
$ClpX^{YI}$	not detected
$ClpX^{YL}$	not detected

Table 1: Rate of degradation of GFP-ssrA by ClpX and position-153 mutants. Reactions contained 2 μM GFP-ssrA preincubated with 2 μM SspB.

The $ClpX^{YF}$ mutant was roughly half as active as the wild-type enzyme in ClpP-mediated degradation of ssrA-tagged GFP and peptide substrates (Table 1; Figure 3). For the ssrA-tagged peptide substrate, Michaelis-Menten analysis revealed that the reduction in the steady-state rate of degradation was caused by a modest increase in K_M combined with a modest decrease in V_{max}. The data in Figure 4 were obtained using the $[G]_{10}$ peptide. Similar values for steady-state kinetic parameters were also obtained using the $[FG]_5$ peptide described in Chapter 2 (data not shown).

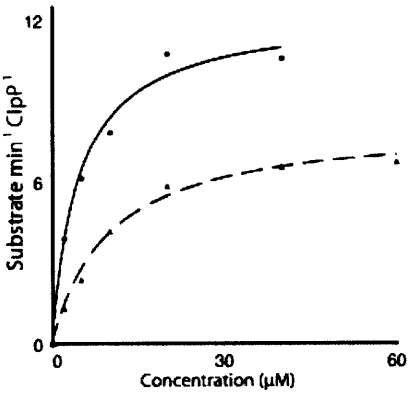

Figure 4: Michaelis-Menten plot for the degradation of the ssrA-tagged $[G]_{10}$ peptide by ClpXP (circles; V_{max} = 12.9 min^{-1} $ClpP_{14}^{-1}$; K_M = 4.7 μM) or $ClpX^{YF}P$ (triangles; V_{max} = 7.8 min^{-1} $ClpP_{14}^{-1}$; K_M = 9.8 μM).

Some mutations in the GYVG loop of ClpX affect degradation of ssrA-tagged substrates but not substrates bearing the lambaO degradation tag (Siddiqui et al., 2004). Thus, I assayed for degradation of lambdaO-tagged Arc repressor by the position-153 mutants and ClpP using SDS-PAGE (Figure 5). $ClpX^{YI}$ and $ClpX^{YL}$ were again inactive in this assay (data not shown), whereas $ClpX^{YF}$ showed an approximate 4-fold reduction in activity compared to wild-type ClpX.

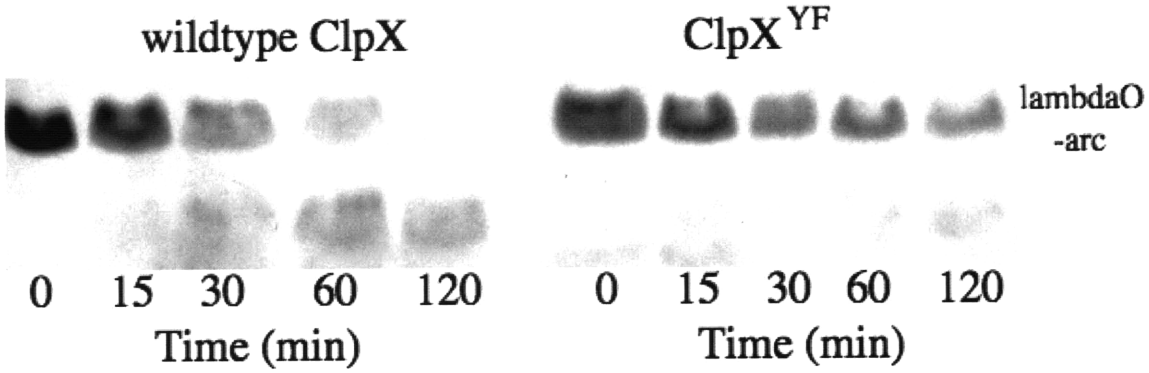

Figure 5: Degradation of lambdaO-Arc (10 μM) by ClpP (800 nM) with wild-type ClpX (800 nM) or with $ClpX^{YF}$ (800 nM) No degradation of the lambdaO-Arc was observed in similar assays using $ClpX^{YI}$ or $ClpX^{YL}$.

Discussion

When I mutated Tyr153 in ClpX to other large hydrophobic residues, the basal ATPase activity was increased modestly for $ClpX^{YF}$, increased substantially for $ClpX^{YI}$, and decreased significantly for $ClpX^{YL}$. Indeed, the altered position of a single methyl group between the Ile153 and Leu153 side chains resulted in a 7-fold difference in the rate at which ClpX hydrolyzes ATP. Clearly, interactions within ClpX mediated by the side chain of residue 153 play a major role in setting the basal rate of ATP hydrolysis.

Moreover, in contrast to wild-type ClpX, none of the position-153 mutants displayed substrate stimulation of ATP-hydrolysis activity. For the Ile153 and Leu153 mutants, this defect could arise from an inability to bind substrate. For the Phe153 mutant, however, no stimulation of ATPase activity was observed at a substrate concentration where binding was approximately 80% saturated. I conclude that the hydroxyl group of the Tyr153 side chain plays an important role in sensing substrate and/or in coupling substrate binding/translocation to changes in the ATP-hydrolysis rate. Previous studies have shown that mutation of the aromatic residue in the pore-1 loops of ClpA (GYVG) and FtsH (MFVG) also alters the basal ATPase rate and activation by substrate (Farbman et al., 2008; Yamada-Inagawa et al., 2003; Okuno et al., 2006). One difference, however, is that FtsH still exhibits substrate-dependent ATPase activation upon mutation of the Phe in its pore-1 loop to Ala/Lys/Glu for some substrates (Yamada-Inagawa et al., 2003). Thus, a Tyr or an aromatic side chain is not needed at this pore-loop position in FtsH to couple substrate binding/degradation with the rate of ATP hydrolysis.

In ClpX, mutation of Tyr153 side chain to Ala in just two subunits of the hexamer compromises substrate recognition, translocation, and unfolding (Martin et al., 2008b; 2008c). I found that mutating Tyr153 to Ile or Leu in the ClpX homohexamer resulted in no detectable degradation activity for peptide or protein substrates bearing either the ssrA degradation tag or the lambdaO degradation tag. Moreover, degradation activity for these mutants was not rescued by addition of the SspB adaptor protein. I suspect that degradation defects observed for $ClpX^{YI}$ and $ClpX^{YL}$ are caused both by poor substrate binding and by poor substrate translocation/unfolding, but none of my experiments rule

out models in which these mutations only affect binding or only affect translocation. Because peptide-degradation activity was not rescued by the addition of SspB, $ClpX^{YI}$ and $ClpX^{YL}$ would have to bind ssrA-tagged substrates more than 5000-fold more weakly than the wild-type enzyme for their relative inactivity to be caused solely by changes in substrate-binding affinity.

I found that mutating Tyr153 to Phe caused a 2-fold increase in K_M and a 40% decrease in V_{max} for degradation of an ssrA-tagged peptide substrate. The change in V_{max} is likely to reflect slower translocation by the mutant. Because wild-type ClpX hydrolyzes ATP more rapidly than the Phe153 mutant in the presence of substrate, slower translocation could reflect slower ATP hydrolysis. Indeed, the cost in ATP consumption for degradation of GFP-ssrA and the ssrA-tagged $[G]_{10}$ peptide substrate were roughly comparable for wild-type ClpX and $ClpX^{YF}$. Martin and colleagues (2008a) reported that the ability of ClpX to unfold GFP-ssrA required a sufficiently fast rate of ATP hydrolysis. In my experiments, GFP-ssrA and the $[G]_{10}$ peptide were both degraded about 40% more slowly when Tyr153 was mutated to Phe. Because unfolding is the rate-limiting step for ClpXP degradation of GFP (Kim et al., 2000), $ClpX^{YF}$ appears to have a modest defect in unfolding this substrate. This reduced activity could easily be caused by the lack of substrate-stimulation of ATP hydrolysis for $ClpX^{YF}$.

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Chapter 4

ClpX Substrate Recognition

Summary

ClpX recognizes protein and polypeptide substrates by binding to sequences known as degradation tags. Although five classes of ClpX tags have been described, relatively little is known about the chemical and structural determinants in these sequences that mediate ClpX recognition. In this chapter, I probe the interaction of ClpX with a library of synthetic peptides containing ssrA tags with modifications of the two C-terminal amino acids. The C-terminal alanine is extremely important for ClpX recognition. D-alanine at the C-terminus is not recognized, conservative replacements with glycine or α-aminobutyric acid decrease binding affinity, and the α-carboxylate plays a role in recognition. The penultimate alanine of the ssrA tag is also an important determinant of ClpX recognition, as is the peptide-bond spacing. Preliminary experiments reveal that comparable experiments will be possible for two different classes of degradation tags, the C-terminal MuA tag and the N-terminal lambdaO tag. The Tyr153→Phe mutation in the GYVG loop of ClpX shows non-additive effects with mutations at the penultimate position of the ssrA tag but does not seem to affect recognition of the MuA or lambdaO tags.

Introduction

Intracellular degradation is vital for protein-quality control and many regulated cellular processes. For example, cells use specific degradation pathways to control transcriptional responses to environmental stress, to remove damaged proteins, and to rebalance the proteome to adapt to changing growth conditions. At the same time, it is obviously important to avoid degradation of the majority of catalytic, regulatory, and structural proteins in the cell. ClpXP is a cytoplasmic AAA+ protease found in most bacteria and mitochondria. In this ATP-fueled proteolytic machine, ClpX serves to recognize specific protein substrates, unfold them as necessary, and then translocate the unfolded polypeptide into the degradation chamber of ClpP, where proteolysis to small peptide fragments occurs (Thompson and Maurizi, 1994; Thompson et al., 1994). By itself, ClpP is unable to degrade proteins or polypeptides of more than 30 amino acids, because these molecules cannot pass through the narrow portals that serve as entryways into the degradation chamber. Interestingly, small molecules that appear to open the portal of ClpP result in unregulated degradation and function as antibiotics (Bottcher and Sieber, 2008; Brotz-Oesterhelt et al., 2005). Thus, the role of ClpX as the regulatory gatekeeper for ClpP is extremely important. In chapter 2, however, I showed that ClpX is able to translocate an enormously diverse set of natural and unnatural peptide sequences. Thus, degradation by ClpXP is controlled by initial recognition, which in turn depends on ClpX binding to degradation tags or sequences that are accessible in the substrate.

ClpX recognizes at least five different classes of tags, which are typically located near the N or C-terminus of substrates but can sometimes function at internal sequence positions

(Flynn et al., 2003; Hoskins et al., 2002). Currently, two classes of C-motif tags (CM) and three classes of N-motif tags (NM) have been identified. If ClpX serves as a locked gate regulating substrate entry to ClpP, then at least five different keys allow entrance. The best characterized degradation signal for ClpX is the ssrA tag (AANDENYALAA; Dougan et al., 2003; Flynn et al., 2001; Gottesman et al., 1998; Keiler et al., 1996), an 11-residue C-motif 1 sequence. Recognition of this tag occurs in two steps. The first involves an electrostatic interaction between the positively charged "RKH" loops of ClpX and the negatively charged α-carboxylate of the ssrA tag (Farrell et al., 2007). The second involves interactions of the two or three C-terminal tag residues with the GYVG (pore-1) and pore-2 loops in the translocation channel of ClpX (Flynn et al., 2001; Gottesman et al., 1998; Farrell et al., 2007; Siddiqui et al., 2004; Martin et al., 2007; 2008b; 2008c). By contrast, the MuA tag (RRKKAI; C-motif 2) and the lambdaO tag (TNTAKILNFGR; N-motif 1) are not believed to bind in the pore but rather to tether substrates to the N-domain of ClpX (Wojtyra et al., 2003). For tethering tags, binding to ClpX and engagement to allow initiation of translocation are likely to be distinct steps, whereas they could easily be concerted for tags that bind directly in the pore itself. To probe the determinants in the ssrA tag that are important for ClpX binding, I created a synthetic substrate library and assayed degradation by wild-type ClpXP and a variant containing the ClpX Tyr153→Phe mutation. I also carried out preliminary studies to probe ClpXP degradation of peptide substrates containing the MuA or lambdaO tags.

Results

Determinants of ClpX recognition in the ssrA tag

Figure 1 shows the basic design of peptides used to probe the importance of residues in the ssrA tag. I focused on sequence changes at the two C-terminal residues of the ssrA tag (Table 1). Although the antepenultimate leucine in the tag plays some role in ClpX recognition (Flynn et al., 2001), the importance of this position is marginal and was not investigated further here. For each variant substrate, I determine the steady-state kinetic parameters (K_M and V_{max}) for degradation by ClpXP and by $ClpX^{YF}$/ClpP (Table 1). Most peptides had similar values of V_{max} but a very large range of K_M values were observed.

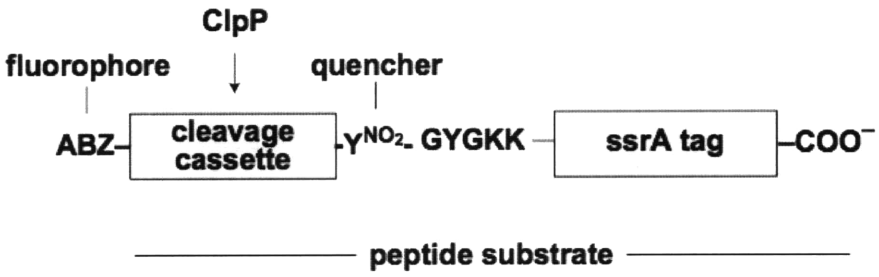

Figure 1: Structure of the wild-type ssrA-tagged substrate used to probe interactions with ClpX. The sequence of the cleavage cassette was FAPHMALVP.

The C-terminal alanine of the ssrA tag is important for ClpX recognition but some other residues at this position allow lower-affinity binding. For example, the most conservative mutations at this position (Ala→Gly or Ala→αAbu) caused approximately three-fold decreases in K_M, whereas larger non-polar substitutions (Ala→Ile and Ala→Phe) reduced K_M approximately 10-fold. Replacing the negatively charged α-carboxyl group with an

uncharged carboxamide group increased K_M about 5-fold. Replacing the C-terminal alanine with the stretched β-alanine analog increased K_M more than 10-fold relative to alanine and about 4-fold relative to glycine, suggesting that the spacing between the α-carboxylate and first peptide bond is not a critical determinant of recognition. By contrast, chirality is exceptionally important. Indeed, the substrate with a C-terminal D-alanine was completely refractory to degradation. Charged residues were also highly deleterious. K_M was increased 30-fold when aspartic acid was the C-terminal residue and degradation was not detectable when lysine was the C-terminal residue.

		wild-type ClpX		$ClpX^{YF}$	
Position Modified	C-terminal sequence	V_{max} (min^{-1} $ClpP^{-1}$)	K_M (μM)	V_{max} (min^{-1} $ClpP^{-1}$)	K_M (μM)
-	A L A A-COOH	11 ± 1	6.7 ± 1	7.8 ± 0.4	10 ± 1
C-term.	A L A A-$CONH_2$	12 ± 1	38 ± 8	7.4 ± 0.7	25 ± 7
ALAx	A L A DA	not detected	-	not detected	-
	A L A G	6.3 ± 0.1	19 ± 1	4.5 ± 0.3	60 ± 9
	A L A αAbu	11.6 ± 0.9	19 ± 3	6.3 ± 0.4	26 ± 3
	A L A βAla	10.7 ± 0.7	81 ± 9	5.6 ± 0.3	95 ± 13
	A L A D	8.9 ± 0.7	183 ± 25	5.1 ± 0.2	150 ± 15
	A L A I	9.8 ± 0.7	63 ± 9	8 ± 1	70 ± 20
	A L A F	8.7 ± 0.9	78 ± 8	6.7 ± 0.8	85 ± 20
	A L A K	not detected	-	not detected	-

AlxA	A L G A	12.7 ± 0.7	39 ± 5	6.4 ± 0.4	82 ± 13
	A L βAla A	10.4 ± 0.7	17 ± 4	4.6 ± 0.3	39 ± 6
	A L D A	11.8 ± 0.9	102 ± 15	5.1 ± 0.4	112 ± 16
	A L I A	10.4 ± 0.7	24 ± 4	6.4 ± 0.2	56 ± 5
	A L F A	10 ± 1	49 ± 12	6.5 ± 0.7	67 ± 16
	A L K A	9.6 ± 0.6	108 ± 16	3.8 ± 0.6	94 ± 4

Table 1: Steady-state kinetic parameters for degradation of peptide substrates by ClpXP or a variant with the Tyr153→Phe ClpX mutation. The wild-type peptide sequence was ABZ-FAPHMALVPY^{NO2}GYGKKAANDENYALAA. The underlined sequence is the ssrA tag.

The penultimate alanine in the ssrA tag is also important for ClpX recognition. Among non-polar substitutions, the Ala→Ile and Ala→βAla mutations increased K_M about 3-fold, whereas the Ala→Gly and Ala→Phe substitutions increased K_M approximately 6-fold to 7-fold. Charged substitutions were again highly deleterious. The penultimate Ala→Asp and the Ala→Lys mutations both increased K_M about 15-fold, showing that substitutions with charged residues were slightly more disruptive than those with large non-polar substitutions.

To test for potential interactions between the GYVG loop and the ssrA tag, I also determined steady-state kinetic parameters for degradation of the mutant ssrA-tagged peptides by ClpX^{YF}/ClpP (Table 1; Table 2). In most cases, the energetic effects of the Tyr→Phe mutation in ClpX and the mutation in the ssrA tag were approximately additive. In two cases, however, there were suggestions of interactions. For example, the COO→CONH$_2$ mutation at the tag C-terminus increased K_M for wild-type ClpXP but

decreased K_M for $ClpX^{YF}$/ClpP, hinting at a potential interaction between the Tyr153 hydroxyl and the α-carboxylate. In another case, the Ala→Gly mutation at the C-terminus caused a larger decrease in binding for the mutant ClpX than the wild-type ClpX. I note, however, that double-mutant cycle analysis gives small interaction energies for these "interactions" (≈ 0.4-0.5 kcal/mol) with large errors.

The MuA and lambdaO tags

When the MuA tag was placed at internal positions in a protein substrate, some recognition by ClpXP was still observed (Hoskins et al., 2002), suggesting that a free α-carboxylate is not essential for recognition. Indeed, a prior experiment suggested that the MuA α-carboxylate might inhibit ClpX binding (Sarah Bissonnette, personal communication). I performed assays similar to those described for the ssrA tag, to probe the importance of the α-carboxylate of the MuA tag (RRKKAI-COOH) and confirmed. this surmise. The base peptide used for the MuA studies is shown in Figure 2.

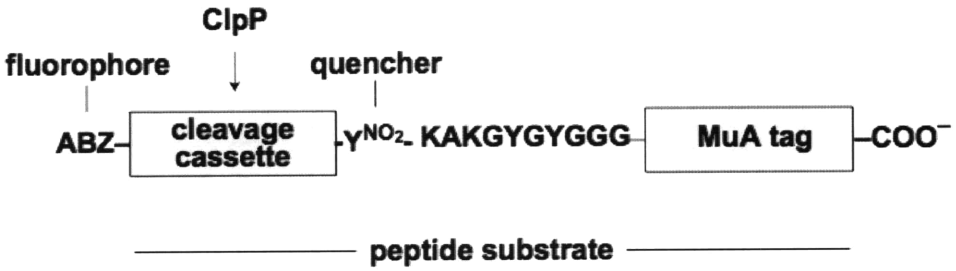

Figure 2: Peptide design for MuA (C-motif 2) tagged substrates. The MuA tag has the sequence RRKKAI. The cleavage cassette is the one described in Fig. 1.

Substitution of the carboxylate with a carboxyamide decreased K_M from approximately 50 μM to 25 μM (Table 2). I also studied ClpXP degradation of a MuA peptide with four

additional residues (GRYD) appended to the C-terminus and found that K_M was increased, but only by a factor of roughly two (Table 2). This result is consistent with the model that ClpX can recognize the MuA tag when it is not positioned at the C-terminus of a substrate. Finally, I found that the Tyr153→Phe mutation in ClpX had no effect on recognition of the MuA tag. Indeed, wild-type ClpX and $ClpX^{YF}$ had indistinguishable K_M's for degradation of MuA peptides with free or amidated C-termini (Table 2).

	wild-type ClpX		**$ClpX^{YF}$**	
MuA tag variant	V_{max} (min^{-1} $ClpP^{-1}$)	K_M (μM)	V_{max} (min^{-1} $ClpP^{-1}$)	K_M (μM)
RRKKAI-COOH	10.8 ± 2.0	48 ± 17	4.6 ± 0.4	49 ± 5
RRKKAI-$CONH_2$	12.6 ± 0.4	25 ± 2	6.6 ± 0.6	23 ± 5
RRKKAIGRYD-COOH	9.4 ± 0.6	82 ± 11	(n.d.)	(n.d.)

Table 2: Steady-state degradation parameters for MuA-tagged peptide variants by ClpXP or by $ClpX^{YF}P$. (n.d.); not determined.

The lambdaO tag appears to consist of five N-terminal amino acids (TNTAK; Flynn et al., 2003). To develop a peptide-degradation system to investigate this tag, I designed the peptide shown in Figure 3, which had an additional methionine at the N-terminus. Wild-type ClpXP degraded this peptide with a K_M of 34 μM and a V_{max} of 5.6 min^{-1} enz^{-1} (Table 3). Thus, the N-terminal methionine does not appear to prevent ClpX recognition of this tag. $ClpX^{YF}$/ClpP degraded the same peptide at less than half the maximal speed, consistent with its slower degradation of the ssrA-tagged and MuA tagged peptide substrates (average V_{max} reduction 1.8 ± 0.4 fold). Thus, the translocation defect of the

Tyr153→Phe mutant does not seem to depend on whether a substrate is recognized by an N-terminal tag or a C-terminal tag. Interestingly, degradation mediated by the $ClpX^{YF}$ mutant had a slightly lower K_M than wild-type (Table 3), but this difference was close to the error of the measurements.

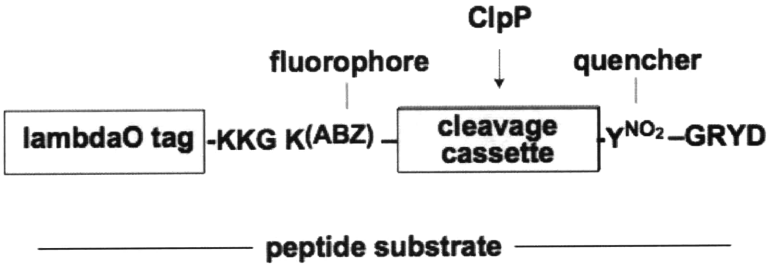

Figure 3: Peptide design for lambdaO (N-motif 1) tagged substrate. The lambdaO tag has the sequence MTNTAKILNFGR. The cleavage cassette is the one described in Fig. 1.

	wild-type ClpX		$ClpX^{YF}$	
lambdaO N-terminus	V_{max} (min^{-1} $ClpP^{-1}$)	K_M (μM)	V_{max} (min^{-1} $ClpP^{-1}$)	K_M (μM)
unmodified	5.6 ± 0.7	34 ± 9	2.1 ± 0.2	19 ± 6
acetylated	4.9 ± 0.2	30 ± 4	(n.d.)	(n.d.)

Table 3: Steady-state degradation parameters for lambdaO-tagged peptide variants by ClpXP or by $ClpX^{YF}P$. (n.d.); not determined.

A previous study demonstrated that ClpXP recognition of an N-motif 2 degradation tag did not require a free α-amino group (Spector et al., 2003). When I acetylated the N-terminus of the lambdaO peptide substrate, ClpXP degraded it with steady-state kinetic parameters within error of the unacetylated peptide (Table 3). I conclude that a free amino group is not an important determinant of ClpX recognition of the lambdaO degradation tag.

Conclusions

For the ssrA tag, the α-carboxyl group contributes to recognition by ClpX but the binding defect was only about 5-fold when this group was replaced with a carboxamide. I did not probe whether addition of extra C-terminal residues would decrease binding even more. For the MuA tag, the negatively charged carboxylate was slightly detrimental for ClpX recognition. This C-motif 2 tag is recognized quite well even at internal positions, as expected from previous studies (Hoskins et al., 2003). Similarly, ClpX recognition of the lambdaO N-motif 1 tag did not require a free N-terminus.

My results clarify recognition determinants for ClpX in the ssrA tag. In addition to the α-carboxylate, the methyl side chain of the C-terminal alanine is important. Removing this methyl group (glycine) or adding one additional methyl group (α-butyric acid) reduced apparent affinity modestly. However, even large non-polar side chains (isoleucine, phenylalanine) at the C-terminal residue only increased K_M by 10-fold. These results suggest significant tolerance in terms of side-chain size at this position. Similarly, non-polar substitutions for the penultimate alanine were detrimental but did not eliminate ClpXP degradation. The view that emerges is of a relatively hydrophobic binding pocket that fits Ala-Ala best but can also accommodate smaller or larger hydrophobic side chains with some loss of binding energy. Charged side chains at either position caused large decreases in apparent affinity, with lysine being more detrimental than aspartic acid at the C-terminal residue. β-alanines were deleterious but tolerated at both C-terminal positions. I interpret this result to mean that the spacing of the peptide amides at these

positions is not critical for ClpX recognition. Because the β-alanine substitutions increase backbone flexibility, reposition the methyl/methylene group, and change the backbone spacing, it is not possible to ascribe the reduced affinity to any single effect.

Interestingly, a D-alanine at the last position prevented any detectable ClpXP degradation even in the presence of the adaptor protein SspB. At present, I do not know if this substitution blocks binding or prevents engagement for translocation. If the latter model is true, then the D-alanine variant should be an excellent competitive inhibitor. It will be interesting to see how D-alanine at the penultimate position of the ssrA tag affects ClpXP degradation. I anticipate that more extensive studies of the tolerance of the ssrA tag and other ClpX recognition motifs to natural and non-natural amino-acid substitutions will improve understanding of the ways in which ClpX chooses substrates for degradation.

Materials and methods

Protein Expression and Purification

Variants of *E. coli* ClpX with an N-terminal His_6 tag and *E. coli* ClpP with a C-terminal His_6 tag were purified as described (Hersch et al., 2004; Kim et al., 2000). The ClpX Tyr153→Phe variant ($ClpX^{YF}$) was purified as described in Chapter 3. The lambdaO-Arc protein was a gift from J. Flynn (MIT). SspB was a gift from N. Ivanova (MIT).

Peptide Synthesis, Modification, and Purification

Peptides were synthesized via standard Fmoc chemistry and purified by HPLC on a C-18 column in 0.06% TFA using a gradient from 0 to 80% acetonitrile. The parental ssrA-tagged peptide had the sequence ABZ-*FAPHMALVPY*^{NO2}GYGKKAANDENYALAA, where ABZ is an aminobenzoic acid fluorophore, the sequence in italics contains an efficient ClpP cleavage site, Y^{NO2} is a nitrotyrosine quencher, and the underlined sequence is the ssrA tag. For MuA-tagged peptides, the ssrA tag in this peptide was replaced by the sequence RRKKAI. The MuA and MuA-$CONH_2$ peptides were gifts from S. Bissonnette. The base lambdaO peptide had the sequence MTNTAKILNFGRKKGK(ABZ)-*FAPHMALVPY*^{NO2}GRYD, where K(ABZ) is Lys with an ABZ group attached to the ε-amino group and the underlined sequence is the lambdaO tag.

Assays

ClpXP degradation assays were performed at 30 °C in PD buffer (25 mM HEPES [pH 7.6], 200 mM KCl, 5 mM $MgCl_2$, 0.032% NP-40, and 10% glycerol), with 300 nM $ClpP_{14}$, 800 nM wild-type or mutant $ClpX_6$, and an ATP-regeneration system consisting of 4 mM ATP, 16 mM creatine phosphate, and 0.32 mg/mL creatine phosphokinase. $ClpP_{14}$ concentration was normalized for activity to published values in a GFP-ssrA degradation assay. Assays with SspB contained equimolar SspB to peptide. For peptide-degradation assays, samples were excited at 320 nm and fluorescence emission was monitored at 420 nm.

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Appendix A

Translocation under load: ClpX biotin pull-off assay

Summary

ClpXP is able to exert sufficient force to dissociate an ssrA-tagged biotinylated peptide from avidin. Using this system, I used peptide substrates with different guest regions to probe how the sequence being translocated affects the ability of ClpX to pull the biotinylated peptide away from its binding site in avidin. My results suggest that ClpX can pull more forcibly on sequences with bulky side chains and less forcibly on sequences with small side chains, and also suggest that the effective length of the sequence segment on which ClpX pulls is between 7 and 10 residues.

Introduction

Degradation of a multidomain protein by a AAA+ protease requires sequential unfolding of each independent domain. As a consequence, when the protease encounters the second folded domain, the sequence in the translocation channel is generally a remnant of the undegraded portion of the first domain or an inter-domain linker. For the proteasome, the chemical nature of this "remnant" can determine whether unfolding of a hyper-stable domain is successful. Specifically, if the sequence in the translocation channel is glycine/alanine rich or low-complexity, then the proteasome fails to degrade the N-terminal domain of NFκB, a DHFR•methotrexate complex, or ornithine decarboxylase (Goldberg, 2003; Palombella et al., 1994; Tian et al., 2005; Hoyt et al., 2006; Moorthy et al., 2006). Eventually, the undegraded portion of the substrate is released by the proteasome, resulting in processing rather than complete degradation. Processing of multidomain proteins has also been observed using ClpXP (Lee et al., 2001; Lin and Kobayashi, 2003; Kenniston et al., 2005). In all of these cases, the attempt to unfold the hyper-stable domain results in a large resisting force, which apparently prevents translocation of the sequence in the pore. Because some sequences allow unfolding under these circumstances, whereas others do not, the ability to translocate against a substantial resisting force must be a function of the interaction of the sequence with the translocation machinery. In other words, some sequences appear to be too "slippery" to allow sufficiently forceful pulling to overcome the resisting force.

In Chapter 2, I demonstrated that ClpX is capable of translocating different homomeric peptide sequences, including $[G_{10}]$, $[A_{10}]$, $[P_{10}]$. $[K_{10}]$, $[Q_{10}]$, $[E_{10}]$, etc. Pulling on $[G_{15}]$,

[P_{15}], and [K_{15}] sequences by ClpX also allowed unfolding of GFP, a stable native protein. In the work presented here, I use a different assay to investigate the ability of ClpX to translocate sequences against a substantial resisting force. The binding of biotin to avidin is one of the strongest known biological interactions with a K_D of 10^{-15} (Green, 1963). However, Andreas Martin (unpublished data) has shown that ClpX can forcibly extract a biotinylated ssrA peptide from avidin. I have used this system to probe the limitations on ClpX translocation under extreme load. Specifically, I designed a series of peptides containing a C-terminal ssrA tag, a central "guest" region, and an N-terminal cysteine that was crosslinked to biotin-maleimide (Figure 1). The biotinylated substrates were in turn bound to immobilized avidin, and I then assayed the ability of ClpX to disrupt the complex of avidin with the biotinylated ssrA peptide.

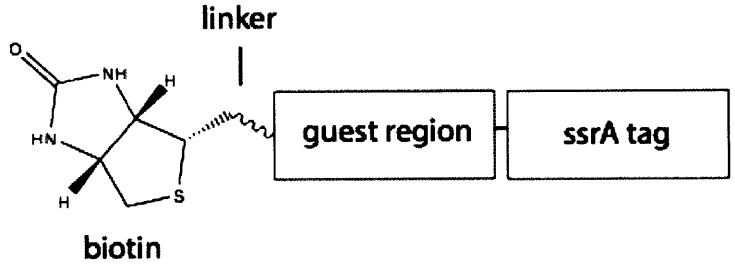

Figure 1: Substrate design for the biotin pull-off assay. The guest regions consisted primarily of homopolymeric amino-acid sequences from 4 to 10 amino acids in length. Biotin-PEO_2-maleimide was crosslinked to the N-terminal cysteine of each peptide.

Experimental Procedures

A biotinylated oligonucleotide (ATGAGTCTTAGCTGCCATATβ-BioTEG) was radiolabeled by 5′ end labeling with [γ-^{32}P]-ATP using polynucleotide kinase. The

radiolabeled product was purified using a BioRad quick spin DNA-binding column and was diluted 1:250 with the unlabeled biotinylated oligonucleotide for pull-off assays. Peptides containing N-terminal cysteines were synthesized by the MIT Biopolymers laboratory, reduced with DTT for 1 hr at room temperature, and purified by HPLC. Peptide identities were confirmed by MALDI mass spectrometry. The purified peptides were biotinylated at pH 7.5 with EZLink biotin-maleimide (Thermo Scientific) overnight at room temperature. Biotinylated peptides were purified on a Superdex peptide column equilibrated in 50 mM HEPES [pH 7.6]. The level of biotin incorporation was assayed using a HABA assay (ε_{500} = 34000 M^{-1} cm^{-1}) (Pemberton and DeJong, 1971). Peptide concentrations were determined by UV absorption at 280 nm using an extinction coefficient of 1400 M^{-1} cm^{-1}.

Each biotinylated peptide was preincubated with equimolar avidin or streptavidin-agarose (purchased from VWR Scientific). Biotin, in at least twofold excess over avidin, was then added to ensure that all binding sites were filled. The peptide-avidin complex was buffer exchanged into 50 mM HEPES [pH 7.6] using a spin column to remove excess biotin. Pull-off assays contained 0.25 μM $ClpX_6$, 0.8 μM $ClpP_{14}$, and 4 μM of the biotinylated peptide, 4 μM avidin, and 4 μM of the radiolabeled oligonucleotide. $ClpX_6$, H_6-$ClpX_6$, and H_6-$ClpP_{14}$ were purified as described in chapter 2, and all reactions were carried out at 30 °C using the standard reaction conditions described in Chapter 2.

In this assay, ClpX-mediated disruption of the complex of avidin with the biotinylated ssrA peptide allows the radiolabeled biotinylated oligonucleotide to bind to the

unoccupied sites in avidin or streptavidin. The concentration of the free biotinylated oligonucleotide (assayed either by PAGE or scintillation counting) is then an inverse measure of the success of the pull-off reaction. Gels contained 10% acrylamide, 2.2 M urea, 5% sucrose, 22 mM Tris-borate [pH 8.3], 25 mM EDTA, 0.03% SDS, 0.012% bromophenol blue, and 0.012% xylene cyanol, and were quantified using a Typhoon Phosphorimager. To quantify the free biotinylated oligonucleotide, aliquots were taken at set time points and applied to a streptavidin-agarose spin column. Any biotinylated oligonucleotide that remained bound to avidin was able to flow through the column, and progress of the reaction could be followed by the decrease in radioactivity in the flow-through fraction. Both avidin and streptavidin were tested to optimize reaction conditions. Avidin was used for experiments presented here.

Results and Discussion

Table 1 summarizes the results of these assays. In the right-hand column, "Translocated" means that ClpXP successfully removed the biotinylated peptide from avidin or streptavidin. "Resistant" means that no significant pull-off of the biotinylated peptide was observed.

Name	Sequence	Result
bPEO_2C_5ssrA	Biotin-PEO_2CKWGRGAANDENYALAA	Translocated
bG_4KKssrA	Biotin-$PEO_2C G_4$KKAANDENYALAA	Translocated
bG_7KKssrA	Biotin-$PEO_2C G_7$KKAANDENYALAA	Translocated
bG_{10}KKssrA	Biotin-$PEO_2C G_{10}$KKAANDENYALAA	Resistant

bKKG$_{10}$ssrA	Biotin-PEO$_2$CKKG$_{10}$AANDENYALAA	Resistant
b(ßA)$_9$KKssrA	Biotin-PEO$_2$C(ßA)$_9$KKAANDENYALAA	Translocated
bPEO$_{11}$C$_5$ssrA	Biotin-PEO$_{11}$CKWGRGAANDENYALAA	Resistant
bDA$_9$KKssrA	Biotin-PEO$_2$CDA$_9$KKAANDENYALAA	Resistant
bA$_{10}$KKssrA	Biotin-PEO$_2$CA$_{10}$KKAANDENYALAA	Resistant
bK$_{10}$KKssrA	Biotin-PEO$_2$CK$_{10}$KKAANDENYALAA	Translocated
bE$_{10}$KKssrA	Biotin-PEO$_2$CE$_{10}$KKAANDENYALAA	Translocated
b(VG)$_5$KKssrA	Biotin-PEO$_2$C(VG)$_5$KKAANDENYALAA	Translocated

Table 1: Summary of results from the biotinylated peptide pull-off assay. For the resistant substrates, no increase, above background noise, was visible in the incorporation of radiolabeled biotinylated oligonucleotide.

Side-chain bulk increases pulling force

Depending on the guest region, some biotinylated peptides were clearly removed from avidin by ClpXP, whereas other peptides were poor substrates in this assay (Figure 2, Table 1). The poor substrates had runs of 10 glycines, 10 alanines or D-alanines, 9 β-alanines, or 11 polyethylene-glycol units. By contrast, a run of 10 lysines, 10 glutamates, or 5 VG repeats allowed ClpXP to forcibly remove the biotinylated peptide from avidin. Thus, under high-load, ClpX appears to interact in a preferential manner with substrates containing bulky side-chains but has difficulty translocating long stretches of very small side chains.

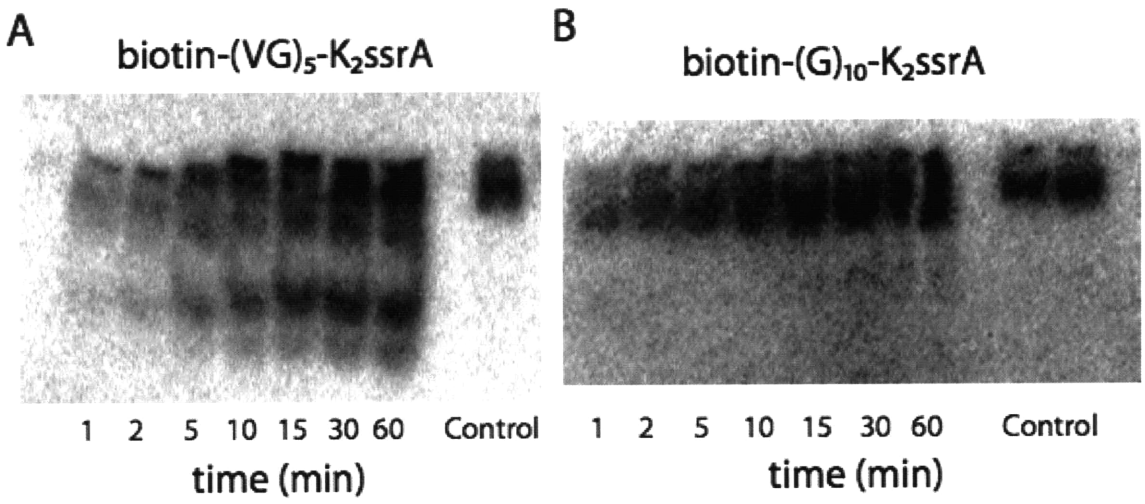

Figure 2: Biotin pull-off assay. A) Successful translocation of biotin-$(VG)_5$-K_2-ssrA. B) Unsuccessful or questionable translocation of biotin-G_{10}-K_2-ssrA where the control (without ClpXP) shows a comparable level of translocation. Avidin is a tetradecamer explaining the four different bands that appear on binding of one to four radiolabeled biotinylated oligonucleotide in panel A.

An unexpected result is that ClpX was able to translocate biotin-$(ß\text{-Ala})_9$-KK-ssrA but not biotin-$(G)_{10}$-KK-ssrA. Due to the extended backbone of ß-Ala, this peptide has a longer guest region in an extended conformation. However, ß-Ala is able to adopt a range of conformations that are forbidden to polyglycine, raising the possibility that one or more of this extended set of conformations allows successful translocation.

The ClpX Power stroke "sees" a span of 7 to 10 residues

The clear cut-off between translocation of a biotinylated substrate with $(G)_7$ versus $(G)_{10}$ showed an unexpectedly sharp limitation in the length of the power stroke executed by ClpX. The successful translocation of bG_7KK-ssrA and failure to translocate bG_{10}KK-ssrA implies that those three additional residues are sufficient to cripple ClpX. This result can be explained if ClpX, during its power stroke, recognizes a stretch of approximately

10 amino acids in the substrate via positioning of different pore loops. This would allow ClpX to be able to "pull" on the residues spanning the seven-residue polyglycine tract, thus being able to pull the substrate off of avidin even if it cannot exert sufficient force on the polyglycine stretch itself. This result is reasonable. Based on structural data and experimental results, the ClpX pore region can be spanned by 7 to 10 residues in an extended conformation (S. Sundar and A. Martin, personal communication; Glynn et al., 2009).

Background Rate

One major complication in using and interpreting this assay was the unexplained presence of a background exchange rate (Figure 3). Considering the high affinity of biotin and avidin, visible background exchange should require days rather than minutes. The background rate is probably because of a contaminant in one of the protein stocks, either in the avidin itself or potentially in the ATP regeneration mixture that was added to all reactions regardless of the presence or absence of ClpX.

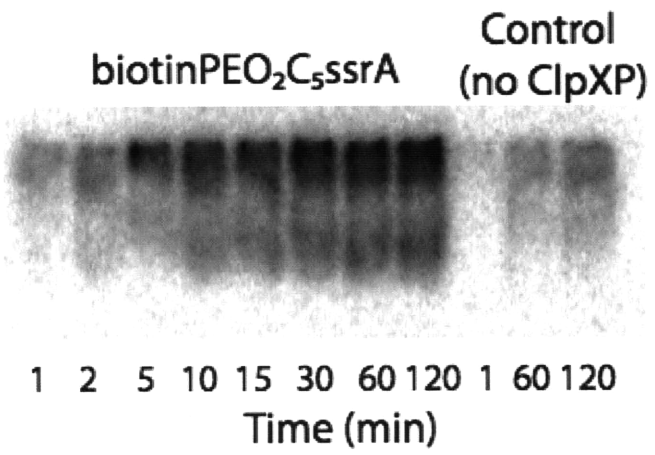

Figure 3: Active translocation by ClpXP versus background rate. The control contains the same reaction mixture but no ClpXP. Over 2 hrs biotin and avidin should not spontaneously dissociate enough to be detected by this assay.

Conclusions

It is clear that ClpX is able to exert a significant amount of force when attempting to translocate a substrate. However, successful separation of biotin and avidin depends upon the polypeptide sequence that ClpX must translocate in attempting to dislocate the biotinylated ssrA-tagged peptide. ClpX appears to form stronger interactions with sequence stretches containing any type of bulky side chain, regardless of charge. This increased interaction with bulky side chains can be explained by stronger van der Waals interactions between ClpX and the substrate, as proposed in chapter 2.

The interaction footprint, over which ClpX is able to exert force on a substrate, appears to be longer than seven amino acids. The successful translocation of bG_7KK-ssrA but not $bG_{10}KK$-ssrA suggests that ClpX can still interact with the two lysine residues that it encounters prior to the guest region when it pulls biotin and avidin apart in the former

substrate. In light of the successful translocation of bG_7KK-ssrA, the failure of ClpX to translocate both $bG_{10}KK$-ssrA and $bKKG_{10}$-ssrA has two possible explanations: either ClpX does not see the residues directly adjacent to the substrate it is attempting to denature (it requires a linker region) or ClpX "stalled" on the polyglycine tract can no longer work efficiently. The first explanation is most likely, because avidin would be pulled against the hexameric face ClpX before biotin enters the pore region of ClpX, and because ClpX was shown, in chapter 2, to successfully translocate polyglycine stretches when it was not under load.

Acknowledgements

Andreas Martin originally developed the biotin pull-off assay.

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Curriculum Vitae

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