Regulation of ClpP:
Role of Substrate Gating and Activation by ClpX

by

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ABSTRACT

AAA+ self-compartmentalized proteases are an important class of proteome regulators that operate to selectively degrade protein substrates. All of these enzymes share the architectural theme of a hexameric ring unfoldase stacked axially onto a barrel-like peptidase, with six- or seven-fold symmetry and sequestered active sites. ClpXP is a model self-compartmentalized protease composed of the regulator ClpX and the serine protease ClpP. Proteolysis occurs by ClpX-dependent substrate selection, unfolding, and translocation into the degradation lumen of ClpP, where rapid and relatively non-specific peptide hydrolysis generates small peptide products.

Prior work had shown that ClpP is unable to degrade polypeptides in the absence of ClpX, suggesting the existence of a mechanism that inhibits the activity of free ClpP. Structures of free ClpP show active sites geometrically competent to perform peptide-hydrolysis chemistry. However, some biochemical results suggested that N-terminal ClpP residues, which line the axial entrance pores, allosterically regulate these active sites. Through measurements of ClpP active-site reactivity, degradation of size-varied peptides, and mutagenesis of the N-termini, I found that peptide degradation is inhibited by steric occlusion, maintained by the N-terminal β-stem loop and α-helix A of ClpP. The N-termini also participate in specifying substrate choice, as mutations within the axial channel prevent degradation of peptides containing stretches of charged amino acids. These data support a model in which ClpX binding opens the axial pore of ClpP to facilitate polypeptide translocation.

Additional residues in ClpP that are important for its function were identified by a selection for dominant-negative mutants impaired in ClpXP-dependent proteolysis. Biochemical studies and mapping of these mutations onto the structure of ClpP suggest that these variants are defective in tetradecamer assembly, peptide binding at the active sites, and ClpX binding. This work provides a foundation for further investigations of the mechanisms of ClpP assembly, degradation, and interactions with ClpX.

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CHAPTER ONE:

Introduction

REGULATED PROTEOLYSIS: FUNCTIONAL SIGNIFICANCE AND REGULATORY MOTIFS

Protein degradation is a key biological process. Initially considered of low biological significance because of the metabolically costly nature of protein synthesis (Wolf 2004), proteolysis is now recognized for its importance in many biological functions, including controlling intracellular protein quality, facilitating rapid responses to external stress factors, and advancing through the cell cycle. The enzymes that catalyze protein degradation – proteases – are exquisitely controlled at multiple levels: gene expression, intracellular localization, cofactor binding, and substrate specificity. In aggregate, these mechanisms for regulating protease activity allow fine-tuning of the proteome, promoting survival in many growth extremes.

Proteases control intracellular protein quality by removing damaged, misfolded, or aggregated proteins. In collaboration with chaperones that encourage protein refolding, the action of proteases limits the intracellular concentrations of damaged proteins that could be deleterious for the cell (Wickner et al. 1999). As an example, the endoplasmic reticulum (ER) has evolved an intricate surveillance mechanism to check the quality of the proteins that need to fold within this organelle (Vembar et al. 2008). This mechanism, termed ER-associated degradation, ensures that proteins which cannot be folded by ER chaperones are retro-translocated to the cytoplasm, where they are degraded.

Unfolded or misfolded polypeptides are typically highly sensitive to degradation, either because the polypeptide backbone is exposed to non-specific proteases or because specific sequences in the denatured protein target it to a specific protease (see below). In addition,

almost all cellular compartments contain proteases, and thus proteolysis competes kinetically with protein folding. Because of mistakes during translation on the ribosome, some proteins cannot fold stably and appear to be rapidly proteolyzed immediately after synthesis. Some estimates indicate that proteolysis targets about 20% of newly synthesized proteins (Wickner et al. 1999). In other cases, oxidation or other types of chemical modification may lead to irreversible protein unfolding. Degradation of proteins that cannot fold or that fold too slowly allows recycling of their amino acids and prevents formation of aggregates, which under some circumstances can be toxic (Turner et al. 2000).

In bacteria, rapid cellular responses to external stresses such as heat or osmotic shock are required for survival and are frequently regulated by proteolysis. In general, these responses involve transcription factors that upregulate the expression of gene products that are needed to combat the negative effects of environmental stress. There are multiple strategies by which proteolysis can control transcription-factor activity. In *E. coli*, for example, the expression of heat-shock proteins is controlled by the σ^{32} transcription factor (Guisbert et al. 2008). This factor is constitutively synthesized but is rapidly degraded with a half life of ~1 min under normal growth conditions. Following heat shock, degradation of σ^{32} slows (perhaps because other substrates produced by thermal denaturation compete for proteolysis), resulting in an increase in steady-state levels and enhanced expression of heat-shock genes (Herman et al. 1995). In another example, the σ^{E} transcription factor controls expression of genes required to respond to stress in the periplasm or cell envelope (Ades 2004). In this case, σ^{E} is normally inhibited by binding to a membrane-bound anti-sigma

factor. When protein folding in the periplasm is compromised, a series of proteolytic cleavages destroy this anti-sigma factor, freeing σ^E to activate gene expression.

Proteolysis is also important for temporal coordination of cellular events. For example, degradation of multiple proteins is required for eukaryotic cells to progress through the cell cycle. In this process, degradation of different cyclin proteins controls the activity window of associated cyclin-dependent kinases, allowing precisely-timed phosphorylation of target proteins crucial for cell-cycle progression (Nakayama et al. 2006). Additionally, the transition from metaphase to anaphase requires segregation of sister chromatids, a process made possible by the degradation of cohesin-ring proteins (Nasmyth 2005). Proteolysis also regulates the cell cycle in some prokaryotes. In *Caulobacter crescentus*, for example, degradation of CtrA, a master transcription factor and cell-cycle regulator, allows initiation of DNA replication and triggers transcription of genes required for cell-cycle progression (Biondi et al. 2006; Brown et al. 2009).

As illustrated by the examples discussed so far, proteolysis is important for the elimination of misfolded or damaged proteins and for the regulation of many cellular processes. However, these destructive proteolytic events must take place in a cellular environment that contains thousands of other types of proteins, which need to be spared from degradation. Given the injurious consequences of rogue degradation, numerous mechanisms exist to regulate intracellular proteolysis (Holzer et al. 1980). Some proteases are sequestered in specialized organelles, such as vacuoles or lysosomes, or are only expressed under specific circumstances (Brown et al. 2000; Wolf 2004; Pop et al. 2009). Other proteases are

synthesized as inactive proenzymes that require activation. For example, the apoptotic executioner pro-caspases are activated via endoproteoyolic cleavage by initator caspases (Pop et al. 2009). Cofactor binding is required for certain proteolytic events. For example, the SOS LexA repressor is an autoprotease but only cleaves itself when bound in a complex with RecA and single-stranded DNA (Little 1991). Similarly, the periplasmic DegS protease is only activated when specific peptides bind to its PDZ domain (Walsh et al. 2003). One of the most prevalent forms of proteolytic regulation occurs at the level of substrate recognition (Baker et al. 2006; Schrader et al. 2009). For example, the eukaryotic proteasome recognizes substrates that have been modified by addition of poly-ubiquitin chains. In bacteria, proteasome-like enzymes generally recognize specific peptide sequences in substrate proteins. Many intracellular proteases are regulated via several of these mechanisms, emphasizing the importance of controlling degradation activity.

SELF-COMPARTMENTALIZED PROTEASES

A common mechanism of controlling the activity of intracellular proteases is to place the active sites for peptide-bond cleavage in a sequestered chamber and then to regulate the access of substrates to this compartment (Baker et al. 2006; Striebel et al. 2009). Self-compartmentalized proteases are found in the cytoplasm of cells from all kingdoms of life, as well as in mitochodria and chloroplasts. For example, *E. coli* contains five such proteases: ClpXP, ClpAP, HslUV, Lon, and FtsH. Typically, the proteolytic active sites of these enzymes reside within cylindrical or barrel-shaped structures, which are built from rings containing six or seven subunits (Figure 1). For the double-ring $ClpP_{14}$ (part of ClpXP) and $HslV_{12}$ (part of HslUV) enzymes, the "core peptidase" unit is formed from a

single type of polypeptide. For the 26S mammalian proteasome, the core 20S peptidase component consists of multiple types of subunits and active sites (Murata et al. 2009).

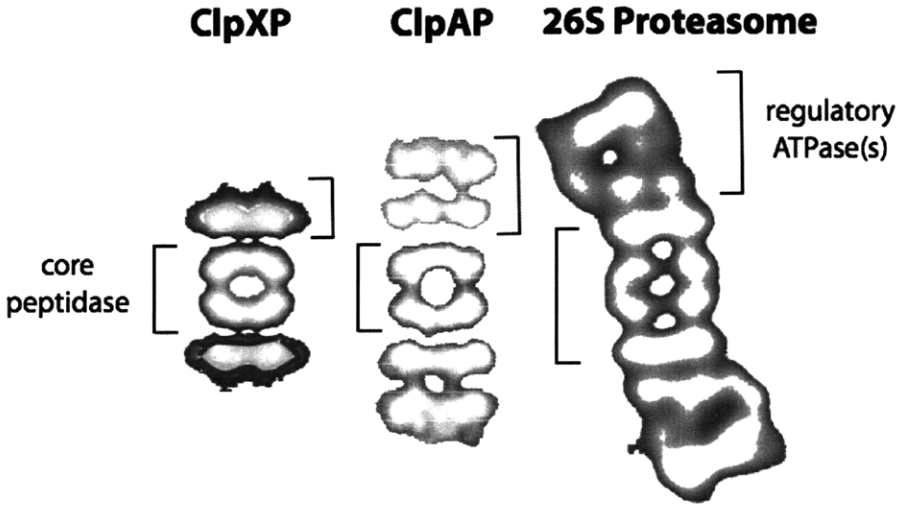

Figure 1. Averaged electron micrograph-derived images of three ATP-dependent self-compartmentalized proteases, *E. coli* ClpXP, *E. coli* ClpAP, and the yeast 26S proteasome (Grimaud et al. 1998; Wickner et al. 1999). The regulatory AAA+ ATPases stack on one or both ends of the core peptidase to form elongated barrel-like structures.

By themselves, $ClpP_{14}$, $HslV_{14}$, and the 20S core of the proteasome cannot cleave protein substrates and show only limited abilities to degrade small peptides. This restriction appears to have a structural basis, as the proteolytic chambers of these enzymes are only accessible through narrow axial channels, which have dimensions smaller than those of native proteins (Sousa et al. 2001; Bajorek et al. 2004; Bewley et al. 2006). In complete self-compartmentalized proteases (e.g., ClpXP, ClpAP, HslUV, or the 26S proteasome), the core peptidase associates with one or two ATPases, which regulate access to the degradation

chamber by recognizing specific substrates, unfolding them, and translocating the unfolded substrate into the lumen of the peptidase (Figures 1 & 2).

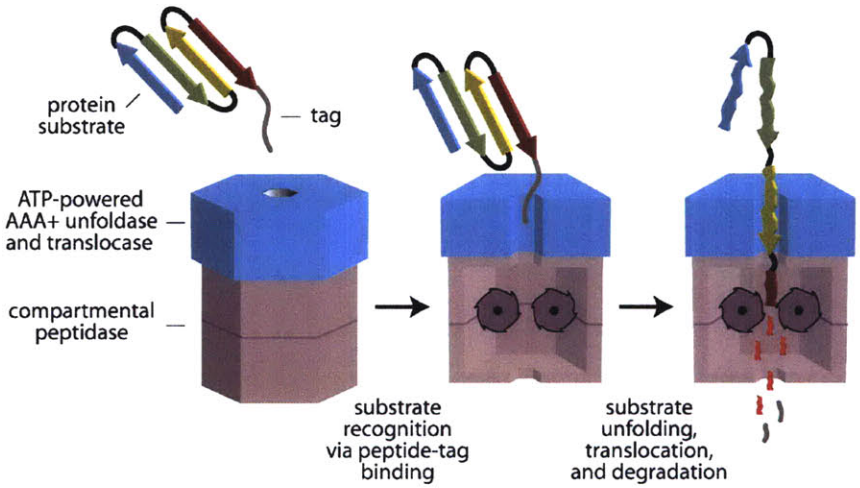

Figure 2. Schematic of the ClpXP degradation cycle (Sauer et al. 2004), depicting one ClpX hexamer (blue) bound to $ClpP_{14}$ (purple). A native protein substrate containing a ClpX recognition tag binds ClpX and is then mechanically unfolded and translocated into the chamber of ClpP for proteolysis.

Degradation by self-compartmentalized proteases is processive and requires ATP hydrolysis (Thompson et al. 1994b; Kim et al. 2000; Sauer et al. 2004; Martin et al. 2008b). Figure 2 shows a cartoon view of the ClpXP degradation reaction. Recognition is mediated by the binding of a peptide tag at the terminus of a native substrate in the axial pore of the ClpX hexamer. In a process that requires numerous cycles of ATP hydrolysis and appears to be mediated by mechanical pulling, ClpX then denatures the native protein and translocates it into ClpP for degradation (Ortega et al. 2000; Kenniston et al. 2003). Degradation is processive because once protein unfolding occurs, there are no significant barriers that would lead to release of the substrate. Moreover, within the ClpP chamber the concentration of active sites is very high and thus most sites in the polypeptide that can be cleaved are

proteolyzed. The research described in this thesis focuses on the ClpXP system, with specific attention to ClpP determinants that limit the degradation activity of the free peptidase but allow it to cooperate effectively with ClpX in protein degradation.

ClpP AND ClpX

E. coli ClpXP is one of the best characterized compartmental proteases. Crystal structures of $ClpP_{14}$ and $ClpX_6$ (but not of the complex) are known (Wang et al. 1997; Glynn et al. 2009). Numerous peptide signals that target substrates to ClpX, either directly or via adaptor proteins, have been identified (Gottesman 1996; Levchenko et al. 2000; Flynn et al. 2003). Studies of ClpXP degradation of model substrates have probed the energetic costs of protein denaturation and translocation, and the processivity of degradation (Kenniston et al. 2003; Kenniston et al. 2004; Kenniston et al. 2005). Below, I summarize some of the relevant information about these enzymes.

The crystal structure of *E. coli* $ClpP_{14}$ was solved more than a decade ago (Wang et al. 1997). Face-to-face stacking of two homoheptameric rings results in a roughly spherical degradation chamber with an internal diameter of ~50 Å (Figure 3). Each ClpP subunit contains a Ser-His-Asp catalytic triad, which is the hallmark of serine proteases like chymotrypsin. In the tetradecamer, 14 active sites occupy positions near the ring-ring interface (Figure 3C). Modification of the ClpP active-site serine with diisopropylfluorophosphate (DFP) inactivates the peptidase activity of ClpP and the protease activity of ClpXP (Thompson et al. 1994b). Based on the structure, the only entrance to the degradation chamber is through two narrow channels located at the axis of each heptameric

ring. These channels appear to have a diameter of ~10 Å, but this value could be lower if unstructured portions of ClpP fill some of the space. Nevertheless, in the absence of substantial conformational changes, the dimensions of the ClpP axial channel are far too small to allow passage of native proteins. This simple structural argument was one of the first indications that polypeptides enter the degradation lumen as elongated and unfolded structures.

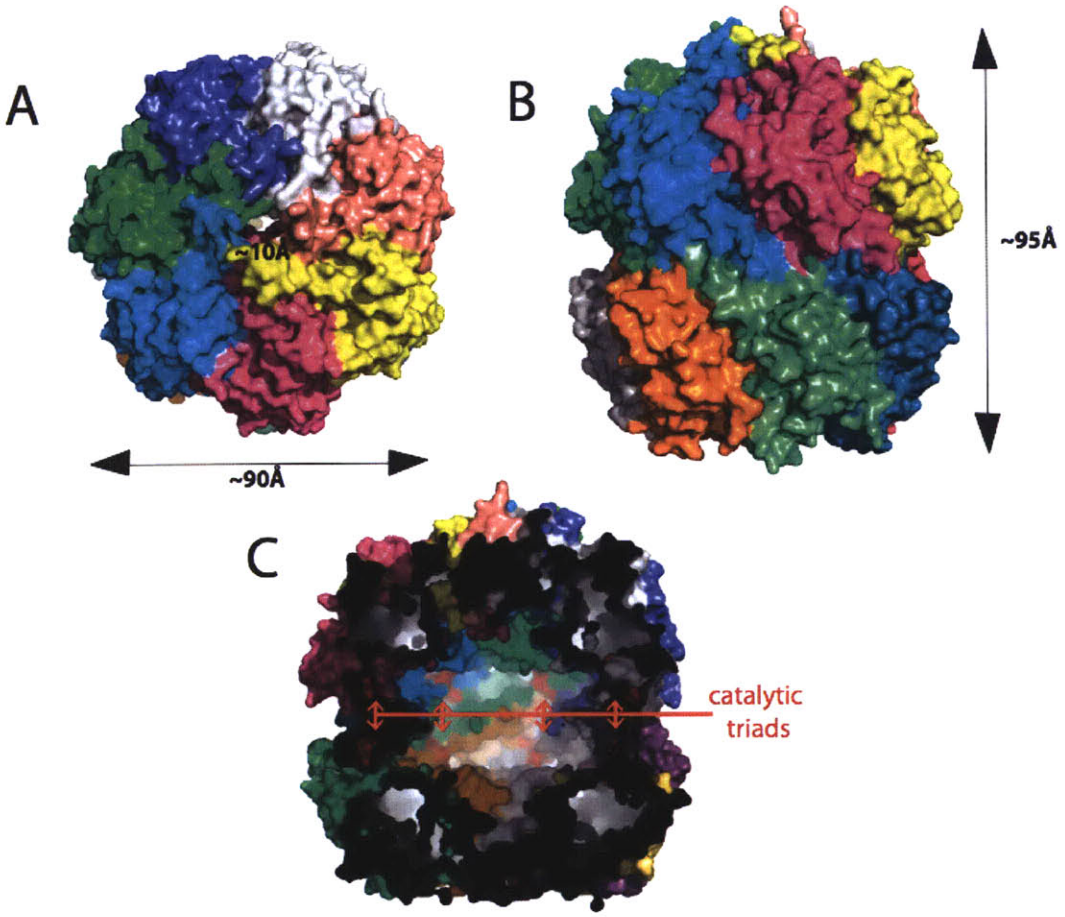

Figure 3. Crystal structure of homotetradecameric *E. coli* ClpP (PDB 1YG6) with each monomer shown in a different color. (A) Top view. (B) Side view. (C) Cutaway of the side view shows the degradation lumen. The catalytic triads (colored in red and indicated by red arrows) are near the ring-ring interface.

Mapping of the sites of cleavage in model substrates revealed that ClpP preferentially cleaves after hydrophobic and aliphatic residues (Thompson et al. 1994a). As a consequence of the high local concentration of active sites within the degradation chamber and the broad cleavage specificity of ClpP, the products of ClpXP or ClpAP degradation are typically peptides of 3-7 amino acids in length (Choi et al. 2005). Small peptides can enter the degradation chamber of ClpP in the absence of a ClpX or ClpA partner, and cleavage rates as high as ~10,000 min^{-1} $ClpP_{14}^{-1}$ have been reported (Thompson et al. 1994b; Kenniston et al. 2003). By contrast, the maximal rates of protein degradation by ClpXP or ClpAP are typically at least 1000-fold slower (Kim et al. 2000; Kenniston et al. 2003), indicating that peptide-bond hydrolysis is not the rate limiting factor in degradation. In some cases, the maximal rate of protein degradation is limited by the rate of protein unfolding. In other cases, translocation appears to be the slow step in overall protein degradation. Because ClpP degradation occurs in a enclosed chamber, cleavage products must eventually exit the chamber to allow new substrates to enter. Two models have been proposed for product egress: (*i*) cleaved peptides may exit via the same axial channels used for substrate entry; or (*ii*) peptide exit may occur through small pores near the ring-ring interface, which exist only transiently and are not observed in the wild-type crystal structure. Although indirect evidence for the latter model has been presented (Gribun et al. 2005), this issue remains unresolved.

ClpX is a member of the Clp/Hsp100 subfamily of AAA+ (ATPases associated with various cellular activities) ATPases (Schirmer et al. 1996; Neuwald et al. 1999). All members of this subfamily assemble as ring homohexamers and function to unfold, disassemble, or

disaggregate proteins. These activities do not depend on association with a peptidase. For example, Hsp104 and ClpB do not have peptidase partners, but function by themselves to solubilize protein aggregates (Doyle et al. 2009). In addition, ClpX and ClpA can unfold substrates or disassemble macromolecular complexes without assistance from ClpP (Levchenko et al. 1995; Weber-Ban et al. 1999; Kim et al. 2000). The unfolding/disassembly activities of ClpX, ClpA, and other Clp/Hsp100 enzymes require ATP hydrolysis, which is thought to drive conformational changes that allow these enzymes to mechanically unfold native substrates by attempting to pull these molecules through the narrow axial channel of the ring hexamer. For both ClpX and ClpA, these axial pores have been shown by mutagenesis and cross-linking experiments to serve as binding sites for the peptide tags of specific substrates (Hinnerwisch et al. 2005; Martin et al. 2008a).

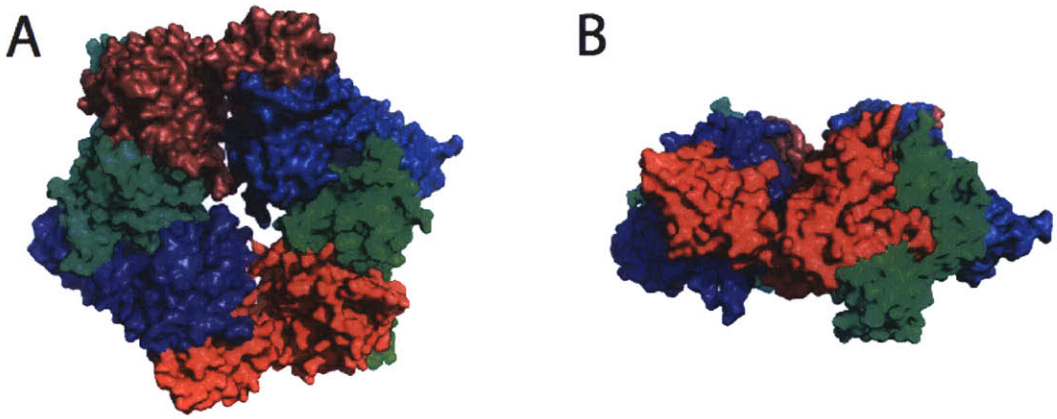

Figure 4. Top (A) and side (B) views of the nucleotide-bound ClpX homohexamer (PDB 3HWS), with each subunit depicted in a different color (Glynn et al. 2009).

Crystal structures of ClpX hexamers in nucleotide-free and ADP-bound forms were determined recently (Glynn et al. 2009). These structures have approximate two-fold

symmetry, but the positions of the large and small AAA+ domains of individual subunits are staggered up and down relative to the axis of the hexamer (Figure 4). As in other AAA+ ATPases, nucleotide binds at an interface formed by the large and small AAA+ domains of one subunit and the large domain of a neighboring subunit (Hanson et al. 2005). The rotation between domains in each subunit varies, however, and as a consequence only four sites in the hexamer bind ADP or ATP. This asymmetry in nucleotide binding is consistent with studies that show that the ClpX hexamer binds a maximum of four nucleotides in solution (Hersch et al. 2005; Martin et al. 2005). Studies using covalently linked single-chain hexamers of ClpX also support an asymmetric mechanism of ATP hydrolysis (Hersch et al. 2005; Martin et al. 2005). In fact, ATP hydrolysis in a single ClpX subunit is sufficient to support ClpXP-mediated degradation of native protein substrates. Two structural features help explain this observation. First, the major subunit-subunit interface of the hexamer consists of a rigid body formed by packing of a small AAA+ domain against the large AAA+ domain of a neighboring subunit. Second, nucleotide binding alters the rotation between the large and small domains of a single subunit. Thus, ATP binding or hydrolysis in a single subunit could change the orientation between domains in this subunit, and these conformational changes would be propagated around the entire hexameric ring via the rigid-body interfaces. These changes, in turn, could drive up-and-down movements of loops in the axial pore, which could be linked to substrate unfolding and/or translocation (Glynn et al. 2009). Indeed, mutations in these pore loops cause defects in translocation and unfolding (Martin et al. 2008a).

The axial channel of ClpX is rather narrow in the crystal structure, and some conformational rearrangements would appear to be necessary to accommodate even a single translocating polypeptide (Glynn et al. 2009). Nevertheless, biochemical studies using disulfide-crosslinked substrates have shown that ClpXP can translocate as many as three polypeptide chains simultaneously (Burton et al. 2001; Bolon et al. 2004). Although it is not yet known how this is accomplished, the crystal structures suggest that unraveling of the domain-domain linkers in the ClpX subunits that do not bind nucleotide might allow the pore to open in an elastic fashion.

INTERACTIONS BETWEEN ClpX AND ClpP

ClpX rings are hexameric whereas ClpP rings are heptameric. Thus, the interactions that stabilize the ClpXP complex and allow alignment of their pores to form a continuous translocation channel must be inherently asymmetric. Because ATP-powered changes in ClpX conformation can occur hundreds of times during degradation of a single protein substrate (Kenniston et al. 2003), interactions between ClpX and ClpP must also be flexible enough to avoid dissociation, which would lead to incomplete degradation.

Bioinformatic comparisons initially revealed the presence of surface loops containing a hydrophobic-glycine-hydrophobic motif in all AAA+ family members that interacted with ClpP (Kim et al. 2001; Martin et al. 2007). In *E. coli* ClpX, these loops are roughly 30 amino acids in length, contain an IGF tripeptide motif, and appear to be flexible, as most residues are disordered in the crystal structure. Modeling shows that these "IGF" loops would extend away from the base of the ClpX hexamer in a fashion that would allow at least

some of them to dock into hydrophobic depressions on the periphery of the top surface of a ClpP ring (Figure 5). Point mutations in the IGF sequence of ClpX severely compromised ClpP binding but did not affect ATP hydrolysis or ClpP-independent disassembly of macromolecular complexes (Kim et al. 2001; Martin et al. 2007). Similarly, deletion of a substantial portion of the IGF loop of ClpX eliminated detectable interactions with ClpP but did not prevent ATP-dependent protein unfolding by the mutant enzyme (Joshi et al. 2004). Using single-chain ClpX variants, Martin et al. (2007) showed that ClpX hexamers lacking a single IGF loop bound ClpP ~50-fold less well than wild-type ClpX but still functioned in ClpXP degradation, whereas variants lacking two IGF loops failed to bind ClpP or support degradation, irrespective of the geometric positions of the missing loops. Thus, a minimium of five "peripheral" contacts between the IGF loops of a ClpX hexamer and ClpP are required for formation of a functional proteolytic complex.

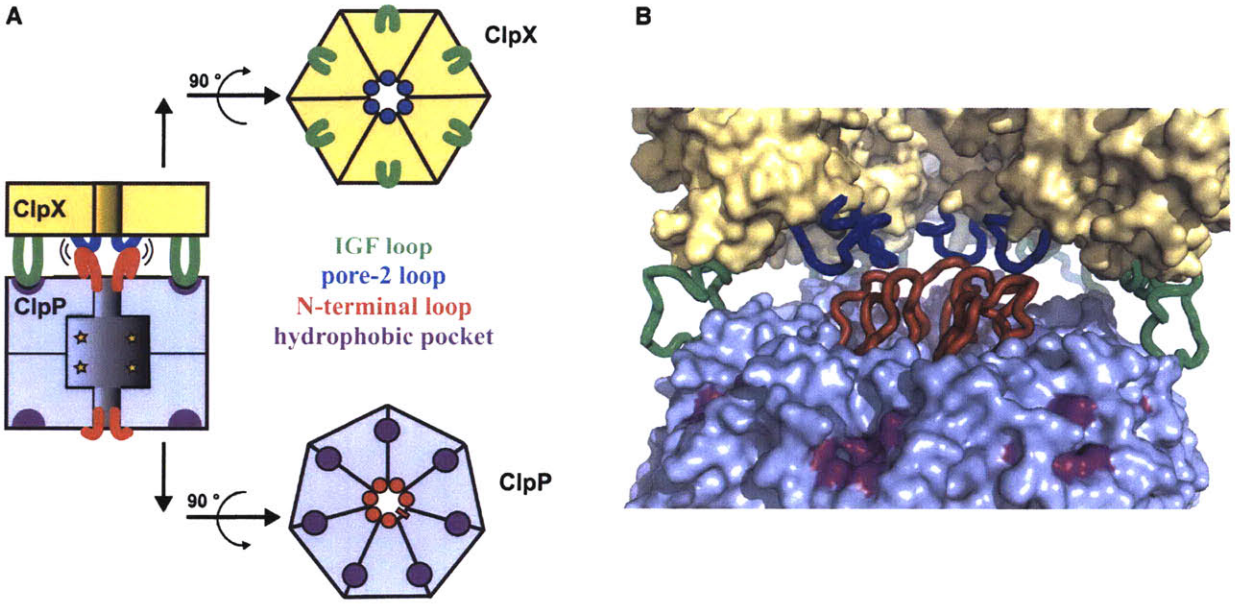

Figure 5. ClpX and ClpP interaction determinants (Martin et al. 2007). (A) Schematic depicting peripheral interactions between the hydrophobic pocket of ClpP (purple) and IGF loops of ClpX (green). Axial interactions involve N-

terminal loops of ClpP (red) and pore-2 loops of ClpX (blue). (B) Model of the ClpXP complex showing both types of interactions.

Coaxial stacking of the ClpX and ClpP rings forms a continuous translocation channel (Figure 5), and functionally important contacts between ClpX and ClpP are made at the sites where the pores of the two rings contact each other. Several results suggest that the pore-2 loops of ClpX and the N-terminal loops of ClpP are responsible for at least some of these "axial" contacts: (*i*) deletion of the ClpX pore-2 loops reduces ClpP affinity ~15-fold but does not prevent functional complex formation (Martin et al. 2007); (*ii*) deletion of N-terminal residues of ClpP weakens or eliminates ClpX binding (Kang et al. 2004; Gribun et al. 2005; Bewley et al. 2009); and (*iii*) cysteines introduced into the pore-2 loop of ClpX and the N-terminal loop of ClpP can form a disulfide bond in a nucleotide-dependent fashion (Martin et al. 2007).

Several results show that the nucleotide state of ClpX influences interactions with ClpP and *vice versa*. First, ATP or ATPγS (a slowly hydrolyzed analog) is required for ClpX binding to ClpP; no binding is observed without nucleotide or with ADP alone (Kim et al. 2000; Joshi et al. 2004). Second, ClpP binding to ClpX reduces the basal rate of ATP hydrolysis in a manner that depends on the presence of the pore-2 loops of the ATPase (Kim et al. 2000; Martin et al. 2007). Third, substrate-dependent changes in the rate of ATP hydrolysis by ClpX alter its apparent affinity for ClpP (Joshi et al. 2004). Because of the staggered arrangement of subunits in ClpX hexamers, only a subset of the pore-2 loops are close to the bottom of the translocation channel and thus potentially in positions to contact ClpP (Glynn

et al. 2009). Moreover, the positions of these loops are expected to change through the ATPase cycle, which could allow dynamic communication between the ATPase and peptidase.

SUBSTRATE SELECTION BY ClpXP

Proteome-scale experiments in *E. coli* using a proteolytically inactive ClpP variant to trap ClpXP substrates resulted in the identification of five classes of peptide sequences that can target proteins for ClpXP degradation (Flynn et al. 2003). In general, these sequences were short (3-10 residues), contained only a few residues that appeared critical for recognition, and were located near the N- or C-terminus of the parent protein. Together with previous studies of ClpX targeting (Levchenko et al. 1997; Gottesman et al. 1998; Gonciarz-Swiatek et al. 1999), this work suggested that ClpX recognizes short, exposed peptide tags at the extreme ends of a substrate.

The best-studied ClpXP recognition motif is the ssrA tag. *In vivo*, this 11-residue sequence (AANDENYALAA in *E. coli*) is appended to the C-terminus of nascent polypeptides when protein synthesis on the ribosome stalls (Keiler et al. 1996). Tagging occurs co-translationally in a process mediated by alanine-charged tmRNA, which functions first as a tRNA to add the alanine to the nascent chain and then as an mRNA to direct synthesis of the ANDENYALAA portion of the tag. These events allow normal termination of translation, ribosome recycling, and release of the nascent polypeptide as an ssrA-tagged molecule.

Degradation of ssrA-tagged proteins in the cytoplasm of *E. coli* is carried out largely by ClpXP (Farrell et al. 2005; Lies et al. 2008), preventing deleterious effects due to the accumulation of incomplete and potential harmful polypeptides. From a biochemical perspective, the ssrA tag has been very useful because appending it to the C-terminus of nearly any protein by cloning makes the tagged protein a substrate for ClpXP degradation (Gottesman et al. 1998; Kim et al. 2000; Kenniston et al. 2003). Mutagenesis experiments have shown that the C-terminal LAA residues of the ssrA tag are most important for recognition by ClpX and degradation by ClpXP (Flynn et al. 2001). Indeed, a number of natural ClpXP substrates have similar genetically encoded recognition tags. For example, *E. coli* RecN (a protein induced as part of the SOS repsonse to DNA damage) is synthesized with a C-terminal LAA sequence, which targets it for constitutive degradation by ClpXP (Neher et al. 2006). Thus, RecN can only be maintained at modest steady-state intracellular levels if it is transcribed and translated very efficiently.

Sometimes, more than one peptide tag is required for efficient ClpXP degradation. For example, the N-terminal residues of λO — a tetrameric protein needed for replication initiation by phage λ — is targeted for ClpXP degradation by its N-terminal residues (Gonciarz-Swiatek et al. 1999). Appending these residues from λO to a monomeric substrate results in relatively inefficient degradation because K_M is high, whereas appending them to a dimeric substrate reduces K_M and increases the second-order rate constants for degradation (Farrell et al. 2007). In this instance, multivalent contacts between the two λO tags in the dimer and sites in the ClpX hexamer probably result in stronger multimer binding. Similarly, ClpX binds tetrameric forms of MuA transposase more tightly than the

monomer (Abdelhakim et al. 2008). MuA tetramerizes during the process of DNA transposition, allowing specific targeting of the multimeric transpososome for disassembly by ClpX. Indeed, if the hyper-stable complex of MuA with recombined DNA cannot be dissociated, then subsequent replication of phage Mu is blocked. In both the λO and MuA examples, multivalent recognition provides a way to target ClpX to substrate multimers with higher efficiency than to individual subunits. In principle, degradation tags for ClpXP could also be masked in an oligomer and only become accessible upon dissociation, allowing individual subunits but not complexes to be targeted for degradation (Baker et al. 2006).

Multivalent substrate delivery is generally more efficient because binding of the first tag occurs in a bimolecular reaction, whereas the second tag binds in a unimolecular reaction with a lower entropic cost. Adaptors use this tactic to enhance degradation of heterologous proteins by binding to both the substrate and to the protease. For example, SspB is a dimeric adaptor protein, initially discovered as a factor that enhanced ClpXP degradation of ssrA-tagged substrates (Levchenko et al. 2000). One part of each SspB subunit binds to the AANDENY portion of the ssrA tag (Levchenko et al. 2000; Flynn et al. 2001; Levchenko et al. 2003; Song et al. 2003). At the same time, a flexible C-terminal tail in each SspB subunit binds to ClpX (Figure 6). Formation of this ternary complex increases the local concentration of the substrate relative to the protease, decreasing K_M and increasing the ClpXP degradation rate at low concentrations of ssrA-tagged substrates.

Adaptors can also reorganize the landscape of substrates to be degraded. For example, some ClpXP substrates in the cell are degraded at enhanced rates in the presence of SspB, whereas

others are degraded more slowly (Flynn et al. 2004). One model to explain this finding is that the tails of SspB compete with certain substrates for binding to ClpX. Biochemical experiments using purified proteins *in vitro* show that ClpAP degrades ssrA-tagged substrates at rates similar to or faster than ClpXP (Gottesman et al. 1998; Weber-Ban et al. 1999; Kim et al. 2000). In an *E. coli* cell, however, ClpAP plays only a minor role in degrading ssrA-tagged substrates (Farrell et al. 2005; Lies et al. 2008). Some of this difference appears to be mediated by SspB, as binding of this adaptor to the ssrA tag of model substrates enhances degradation by ClpXP but slows degradation by ClpAP (Flynn et al. 2001).

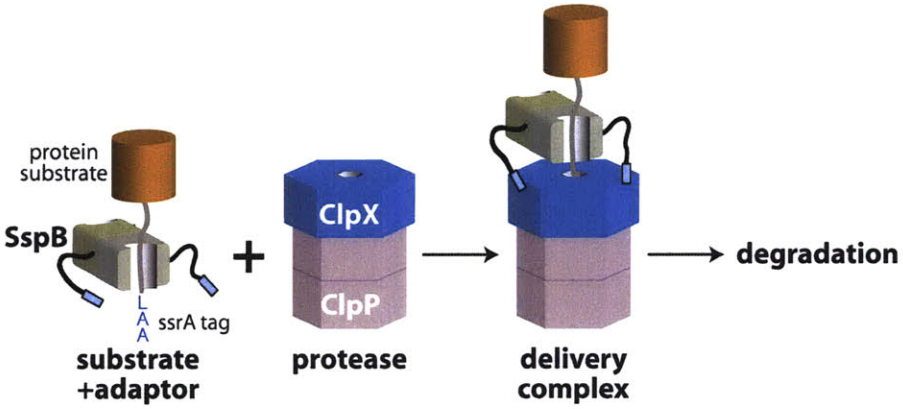

Figure 6. The SspB•substrate complex binds ClpXP to form a ternary complex that leads to efficient protein degradation (McGinness et al. 2006).

ClpP MATURATION AND REGULATION OF INTRINSIC ACTIVITY

How is the proteolytic activity of ClpP repressed in the free enzyme and activated by ClpX binding? Like many serine proteases (Stroud et al. 1977), *E. coli* ClpP is initially synthesized as a proenzyme with a 14-residue N-terminal extension (Maurizi et al. 1990). Assembly of the complete ClpP tetradecamer appears to be required before autoproteolytic

removal of the propeptide can occur. It is not clear how the propeptide limits ClpP activity or what specific signals trigger its cleavage, but this requirement for maturation probably prevents ClpP from degrading substrates prior to formation of its compartmental barrel-shaped structure which can limit substrate access to the active sites.

A somewhat different strategy appears to be used for controlling the activity of human mitochondrial ClpP. In this case, the free enzyme forms a single heptameric ring with extremely low peptidase activity and no protease activity, and the presence of ClpX is required for assembly of the active double-ring structure (Kang et al. 2005). These observations suggest that the Ser-His-Asp catalytic triads in an isolated heptameric ring of human ClpP are present in a non-functional conformation and that allosteric changes in structure, stabilized by ClpX binding, are required for activation. It is possible that single rings of *E. coli* ClpP are also inactive, and thus that assembly of the 14-mer is a prerequisite for activity.

Studies with other compartmental peptidases and AAA+ proteases have documented two distinct mechanisms by which binding of the AAA+ ATPase can control the activity of the peptidase. One of these mechanisms, involving active site rearrangement, is used by the AAA+ HslUV protease. HslU is a hexameric AAA+ ATPase, which shares significant sequence and structural homology with ClpX; HslV is its partner compartmental peptidase (Kessel et al. 1996). Although HslV forms an enclosed proteolytic chamber like ClpP, these enzymes differ in almost all other ways. For example, HslV does not have a Ser-His-Asp catalytic triad and instead uses its N-terminal threonine as the attacking nucleophile in

peptide-bond cleavage. Moreover, the three-dimensional structures of HslV and ClpP subunits are completely different, and the fully assembled HslV enzyme is a 12-mer consisting of two hexameric rings. Importantly, the isolated $HslV_{12}$ enzyme has no detectable peptidase activity, and its active-site threonines do not react with peptide vinyl-sulfone inhibitors (Yoo et al. 1996; Bogyo et al. 1997). In HslUV complexes, by contrast, robust peptidase activity in observed and the active-site threonines react readily with vinyl-sulfone inhibitors. These results suggested that the active sites in free $HslV_{12}$ are either completely inaccessible or are not in catalytically functional conformations. The latter model is strongly supported by comparisons of the crystal structures of $HslV_{12}$, free or in complex with two $HslU_6$ molecules, which show that the active sites only adopt a functional conformation in the HslUV complex (Sousa et al. 2002). Furthermore, in complexes of $HslV_{12}$ containing just a single hexamer of HslU, only the active-site threonines in the HslV ring in direct contact with HslU were reactive with vinyl-sulfone inhibitors, demonstrating that each HslV ring is activated independently rather than cooperatively by HslU binding (Kwon et al. 2003).

A second mechanism that has been documented for control of compartmental peptidase activity is gate opening. The best example involves the 20S proteasome core particle, which is composed of four stacked heptameric rings with two types of subunits in an $\alpha_7\beta_7\beta_7\alpha_7$ arrangement (Murata et al. 2009). The 20S proteasome can degrade depeptide substrates efficiently but has almost no activity against larger peptides (Groll et al. 2003). In the free 20S enzyme, flexible N-terminal sequences in each flanking α_7 ring protrude into the axial entry channels, effectively blocking entry of small peptides 3-4 amino acids long and

deletion of these N-terminal α sequences allows faster degradation (Groll et al. 2000). Crystal and cryo-EM structures of the core particle in complex with 11S non-AAA+ activators reveal rearrangements that result in pore or gate opening (Rabl et al. 2008). Together, these results support a model in which the N-termini of the α subunits physically gate access of polypeptides to the lumen of the degradation chamber.

RESEARCH APPROACH

Since the publication of the first ClpP crystal structure (Wang et al., 1997), workers in the field have generally assumed that a gating mechanism is used to control substrate access to the degradation chamber in the free enzyme. Somewhat surprisingly, however, many aspects of this model had not been investigated, and an allosteric model had not been ruled out. Indeed, recent studies appeared to provide some evidence for allosteric activation, as deleting ClpP residues that form part of the axial channel resulted in defects in hydrolysis of the acyl-enzyme intermediate (Jennings et al. 2008). It is also possible that the ClpP propeptide inhibits activity in an allosteric fashion, and an allosteric model provides one explanation for the observation that DFP-modified ClpP binds ClpX with higher affinity than unmodified ClpP (Joshi et al. 2004). As a result, I became interested in understanding how ClpP activity is regulated in the absence and presence of ClpX.

I approached this question using genetic and biochemical experiments. In Chapter 2, I present experiments involving peptide degradation and active-site modification that support an important role for the N-terminal residues of ClpP in gating substrate entry. These studies also showed that sequence determinants in α-helix A, which links the pore residues

of ClpP to the peripheral binding clefts, play critical roles in binding ClpX. Interestingly, ClpP mutations that severely impaired ClpX binding had relatively small effects on ClpA interactions, suggesting that these AAA+ ATPases must interact with ClpP in somewhat different ways. Finally, I found that sequences that form the axial channel of ClpP play roles in determining the specificity of polypeptide entry into the peptidase chamber during ClpXP degradation.

In Chapter 3, I describe a genetic selection for dominant-negative ClpP mutations that impair wild-type ClpXP function. Using this selection, I identified point mutations in multiple regions of the three-dimensional structure that prevent or diminish function. The majority of mutations mapped to the N-terminal channel residues and α-helix A, the peripheral IGF-binding clefts, the ring-ring interface of ClpP, and active sites. I characterized several of these mutants, and discuss potential mechanisms to explain the phenotypes observed. In the first appendix to Chapter 3, I describe a selection for ClpX mutations that restore the ability to interact functionally with a ClpP mutant that fails to bind wild-type ClpX and preliminary results of this selection. The second appendix describes characterization of two mutations in the peripheral IGF-binding clefts.

REFERENCES

Abdelhakim, A. H., Oakes, E. C., Sauer, R. T. and Baker, T. A. (2008). "Unique contacts direct high-priority recognition of the tetrameric Mu transposase-DNA complex by the AAA+ unfoldase ClpX." Mol Cell **30**(1): 39-50.

Ades, S. E. (2004). "Control of the alternative sigma factor sigmaE in Escherichia coli." Curr Opin Microbiol **7**(2): 157-62.

Bajorek, M. and Glickman, M. H. (2004). "Keepers at the final gates: regulatory complexes and gating of the proteasome channel." Cell Mol Life Sci **61**(13): 1579-88.

Baker, T. A. and Sauer, R. T. (2006). "ATP-dependent proteases of bacteria: recognition logic and operating principles." Trends Biochem Sci **31**(12): 647-53.

Bewley, M. C., Graziano, V., Griffin, K. and Flanagan, J. M. (2006). "The asymmetry in the mature amino-terminus of ClpP facilitates a local symmetry match in ClpAP and ClpXP complexes." J Struct Biol **153**(2): 113-28.

Bewley, M. C., Graziano, V., Griffin, K. and Flanagan, J. M. (2009). "Turned on for degradation: ATPase-independent degradation by ClpP." J Struct Biol **165**(2): 118-25.

Biondi, E. G., Reisinger, S. J., Skerker, J. M., Arif, M., Perchuk, B. S., Ryan, K. R. and Laub, M. T. (2006). "Regulation of the bacterial cell cycle by an integrated genetic circuit." Nature **444**(7121): 899-904.

Bogyo, M., McMaster, J. S., Gaczynska, M., Tortorella, D., Goldberg, A. L. and Ploegh, H. (1997). "Covalent modification of the active site threonine of proteasomal beta subunits and the Escherichia coli homolog HslV by a new class of inhibitors." Proc Natl Acad Sci U S A **94**(13): 6629-34.

Bolon, D. N., Grant, R. A., Baker, T. A. and Sauer, R. T. (2004). "Nucleotide-dependent substrate handoff from the SspB adaptor to the AAA+ ClpXP protease." Mol Cell **16**(3): 343-50.

Brown, M. S., Ye, J., Rawson, R. B. and Goldstein, J. L. (2000). "Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans." Cell **100**(4): 391-8.

Brown, P. J., Hardy, G. G., Trimble, M. J. and Brun, Y. V. (2009). "Complex regulatory pathways coordinate cell-cycle progression and development in Caulobacter crescentus." Adv Microb Physiol **54**: 1-101.

Burton, R. E., Siddiqui, S. M., Kim, Y. I., Baker, T. A. and Sauer, R. T. (2001). "Effects of protein stability and structure on substrate processing by the ClpXP unfolding and degradation machine." Embo J **20**(12): 3092-100.

Choi, K. H. and Licht, S. (2005). "Control of peptide product sizes by the energy-dependent protease ClpAP." Biochemistry **44**(42): 13921-31.

Doyle, S. M. and Wickner, S. (2009). "Hsp104 and ClpB: protein disaggregating machines." Trends Biochem Sci **34**(1): 40-8.

Farrell, C. M., Baker, T. A. and Sauer, R. T. (2007). "Altered specificity of a AAA+ protease." Mol Cell **25**(1): 161-6.

Farrell, C. M., Grossman, A. D. and Sauer, R. T. (2005). "Cytoplasmic degradation of ssrA-tagged proteins." Mol Microbiol **57**(6): 1750-61.

Flynn, J. M., Levchenko, I., Sauer, R. T. and Baker, T. A. (2004). "Modulating substrate choice: the SspB adaptor delivers a regulator of the extracytoplasmic-stress response to the AAA+ protease ClpXP for degradation." Genes Dev **18**(18): 2292-301.

Flynn, J. M., Levchenko, I., Seidel, M., Wickner, S. H., Sauer, R. T. and Baker, T. A. (2001). "Overlapping recognition determinants within the ssrA degradation tag allow modulation of proteolysis." Proc Natl Acad Sci U S A **98**(19): 10584-9.

Flynn, J. M., Neher, S. B., Kim, Y. I., Sauer, R. T. and Baker, T. A. (2003). "Proteomic discovery of cellular substrates of the ClpXP protease reveals five classes of ClpX-recognition signals." Mol Cell **11**(3): 671-83.

Glynn, S. E., Martin, A., Nager, A. R., Baker, T. A. and Sauer, R. T. (2009). "Structures of asymmetric ClpX hexamers reveal nucleotide-dependent motions in a AAA+ protein-unfolding machine." Cell **139**(4): 744-56.

Gonciarz-Swiatek, M., Wawrzynow, A., Um, S. J., Learn, B. A., McMacken, R., Kelley, W. L., Georgopoulos, C., Sliekers, O. and Zylicz, M. (1999). "Recognition, targeting, and hydrolysis of the lambda O replication protein by the ClpP/ClpX protease." J Biol Chem **274**(20): 13999-4005.

Gottesman, S. (1996). "Proteases and their targets in Escherichia coli." Annu Rev Genet **30**: 465-506.

Gottesman, S., Roche, E., Zhou, Y. and Sauer, R. T. (1998). "The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system." Genes Dev **12**(9): 1338-47.

Gribun, A., Kimber, M. S., Ching, R., Sprangers, R., Fiebig, K. M. and Houry, W. A. (2005). "The ClpP double ring tetradecameric protease exhibits plastic ring-ring interactions, and the N termini of its subunits form flexible loops that are essential for ClpXP and ClpAP complex formation." J Biol Chem **280**(16): 16185-96.

Grimaud, R., Kessel, M., Beuron, F., Steven, A. C. and Maurizi, M. R. (1998). "Enzymatic and structural similarities between the Escherichia coli ATP-dependent proteases, ClpXP and ClpAP." J Biol Chem **273**(20): 12476-81.

Groll, M., Bajorek, M., Kohler, A., Moroder, L., Rubin, D. M., Huber, R., Glickman, M. H. and Finley, D. (2000). "A gated channel into the proteasome core particle." Nat Struct Biol **7**(11): 1062-7.

Groll, M. and Huber, R. (2003). "Substrate access and processing by the 20S proteasome core particle." Int J Biochem Cell Biol **35**(5): 606-16.

Guisbert, E., Yura, T., Rhodius, V. A. and Gross, C. A. (2008). "Convergence of molecular, modeling, and systems approaches for an understanding of the Escherichia coli heat shock response." Microbiol Mol Biol Rev **72**(3): 545-54.

Hanson, P. I. and Whiteheart, S. W. (2005). "AAA+ proteins: have engine, will work." Nat Rev Mol Cell Biol **6**(7): 519-29.

Herman, C., Thevenet, D., D'Ari, R. and Bouloc, P. (1995). "Degradation of sigma 32, the heat shock regulator in Escherichia coli, is governed by HflB." Proc Natl Acad Sci U S A **92**(8): 3516-20.

Hersch, G. L., Burton, R. E., Bolon, D. N., Baker, T. A. and Sauer, R. T. (2005). "Asymmetric interactions of ATP with the AAA+ ClpX6 unfoldase: allosteric control of a protein machine." Cell **121**(7): 1017-27.

Hinnerwisch, J., Fenton, W. A., Furtak, K. J., Farr, G. W. and Horwich, A. L. (2005). "Loops in the central channel of ClpA chaperone mediate protein binding, unfolding, and translocation." Cell **121**(7): 1029-41.

Holzer, H. and Heinrich, P. C. (1980). "Control of proteolysis." Annu Rev Biochem **49**: 63-91.

Jennings, L. D., Bohon, J., Chance, M. R. and Licht, S. (2008). "The ClpP N-terminus coordinates substrate access with protease active site reactivity." Biochemistry **47**(42): 11031-40.

Joshi, S. A., Hersch, G. L., Baker, T. A. and Sauer, R. T. (2004). "Communication between ClpX and ClpP during substrate processing and degradation." Nat Struct Mol Biol **11**(5): 404-11.

Kang, S. G., Dimitrova, M. N., Ortega, J., Ginsburg, A. and Maurizi, M. R. (2005). "Human mitochondrial ClpP is a stable heptamer that assembles into a tetradecamer in the presence of ClpX." J Biol Chem **280**(42): 35424-32.

Kang, S. G., Maurizi, M. R., Thompson, M., Mueser, T. and Ahvazi, B. (2004). "Crystallography and mutagenesis point to an essential role for the N-terminus of human mitochondrial ClpP." J Struct Biol **148**(3): 338-52.

Keiler, K. C., Waller, P. R. and Sauer, R. T. (1996). "Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA." Science **271**(5251): 990-3.

Kenniston, J. A., Baker, T. A., Fernandez, J. M. and Sauer, R. T. (2003). "Linkage between ATP consumption and mechanical unfolding during the protein processing reactions of an AAA+ degradation machine." Cell **114**(4): 511-20.

Kenniston, J. A., Baker, T. A. and Sauer, R. T. (2005). "Partitioning between unfolding and release of native domains during ClpXP degradation determines substrate selectivity and partial processing." Proc Natl Acad Sci U S A **102**(5): 1390-5.

Kenniston, J. A., Burton, R. E., Siddiqui, S. M., Baker, T. A. and Sauer, R. T. (2004). "Effects of local protein stability and the geometric position of the substrate degradation tag on the efficiency of ClpXP denaturation and degradation." J Struct Biol **146**(1-2): 130-40.

Kessel, M., Wu, W., Gottesman, S., Kocsis, E., Steven, A. C. and Maurizi, M. R. (1996). "Six-fold rotational symmetry of ClpQ, the E. coli homolog of the 20S proteasome, and its ATP-dependent activator, ClpY." FEBS Lett **398**(2-3): 274-8.

Kim, Y. I., Burton, R. E., Burton, B. M., Sauer, R. T. and Baker, T. A. (2000). "Dynamics of substrate denaturation and translocation by the ClpXP degradation machine." Mol Cell **5**(4): 639-48.

Kim, Y. I., Levchenko, I., Fraczkowska, K., Woodruff, R. V., Sauer, R. T. and Baker, T. A. (2001). "Molecular determinants of complex formation between Clp/Hsp100 ATPases and the ClpP peptidase." Nat Struct Biol **8**(3): 230-3.

Kwon, A. R., Kessler, B. M., Overkleeft, H. S. and McKay, D. B. (2003). "Structure and reactivity of an asymmetric complex between HslV and I-domain deleted HslU, a prokaryotic homolog of the eukaryotic proteasome." J Mol Biol **330**(2): 185-95.

Levchenko, I., Grant, R. A., Wah, D. A., Sauer, R. T. and Baker, T. A. (2003). "Structure of a delivery protein for an AAA+ protease in complex with a peptide degradation tag." Mol Cell **12**(2): 365-72.

Levchenko, I., Luo, L. and Baker, T. A. (1995). "Disassembly of the Mu transposase tetramer by the ClpX chaperone." Genes Dev **9**(19): 2399-408.

Levchenko, I., Seidel, M., Sauer, R. T. and Baker, T. A. (2000). "A specificity-enhancing factor for the ClpXP degradation machine." Science **289**(5488): 2354-6.

Levchenko, I., Yamauchi, M. and Baker, T. A. (1997). "ClpX and MuB interact with overlapping regions of Mu transposase: implications for control of the transposition pathway." Genes Dev **11**(12): 1561-72.

Lies, M. and Maurizi, M. R. (2008). "Turnover of endogenous SsrA-tagged proteins mediated by ATP-dependent proteases in Escherichia coli." J Biol Chem **283**(34): 22918-29.

Little, J. W. (1991). "Mechanism of specific LexA cleavage: autodigestion and the role of RecA coprotease." Biochimie **73**(4): 411-21.

Martin, A., Baker, T. A. and Sauer, R. T. (2005). "Rebuilt AAA + motors reveal operating principles for ATP-fuelled machines." Nature **437**(7062): 1115-20.

Martin, A., Baker, T. A. and Sauer, R. T. (2007). "Distinct static and dynamic interactions control ATPase-peptidase communication in a AAA+ protease." Mol Cell **27**(1): 41-52.

Martin, A., Baker, T. A. and Sauer, R. T. (2008a). "Diverse pore loops of the AAA+ ClpX machine mediate unassisted and adaptor-dependent recognition of ssrA-tagged substrates." Mol Cell **29**(4): 441-50.

Martin, A., Baker, T. A. and Sauer, R. T. (2008b). "Protein unfolding by a AAA+ protease is dependent on ATP-hydrolysis rates and substrate energy landscapes." Nat Struct Mol Biol **15**(2): 139-45.

Maurizi, M. R., Clark, W. P., Katayama, Y., Rudikoff, S., Pumphrey, J., Bowers, B. and Gottesman, S. (1990). "Sequence and structure of Clp P, the proteolytic component of the ATP-dependent Clp protease of Escherichia coli." J Biol Chem **265**(21): 12536-45.

McGinness, K. E., Baker, T. A. and Sauer, R. T. (2006). "Engineering controllable protein degradation." Mol Cell **22**(5): 701-7.

Murata, S., Yashiroda, H. and Tanaka, K. (2009). "Molecular mechanisms of proteasome assembly." Nat Rev Mol Cell Biol **10**(2): 104-15.

Nakayama, K. I. and Nakayama, K. (2006). "Ubiquitin ligases: cell-cycle control and cancer." Nat Rev Cancer **6**(5): 369-81.

Nasmyth, K. (2005). "How do so few control so many?" Cell **120**(6): 739-46.

Neher, S. B., Villen, J., Oakes, E. C., Bakalarski, C. E., Sauer, R. T., Gygi, S. P. and Baker, T. A. (2006). "Proteomic profiling of ClpXP substrates after DNA damage reveals extensive instability within SOS regulon." Mol Cell **22**(2): 193-204.

Neuwald, A. F., Aravind, L., Spouge, J. L. and Koonin, E. V. (1999). "AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes." Genome Res **9**(1): 27-43.

Ortega, J., Singh, S. K., Ishikawa, T., Maurizi, M. R. and Steven, A. C. (2000). "Visualization of substrate binding and translocation by the ATP-dependent protease, ClpXP." Mol Cell **6**(6): 1515-21.

Pop, C. and Salvesen, G. S. (2009). "Human caspases: activation, specificity, and regulation." J Biol Chem **284**(33): 21777-81.

Rabl, J., Smith, D. M., Yu, Y., Chang, S. C., Goldberg, A. L. and Cheng, Y. (2008). "Mechanism of gate opening in the 20S proteasome by the proteasomal ATPases." Mol Cell **30**(3): 360-8.

Sauer, R. T., Bolon, D. N., Burton, B. M., Burton, R. E., Flynn, J. M., Grant, R. A., Hersch, G. L., Joshi, S. A., Kenniston, J. A., Levchenko, I., Neher, S. B., Oakes, E. S., Siddiqui, S. M., Wah, D. A. and Baker, T. A. (2004). "Sculpting the proteome with AAA(+) proteases and disassembly machines." Cell **119**(1): 9-18.

Schirmer, E. C., Glover, J. R., Singer, M. A. and Lindquist, S. (1996). "HSP100/Clp proteins: a common mechanism explains diverse functions." Trends Biochem Sci **21**(8): 289-96.

Schrader, E. K., Harstad, K. G. and Matouschek, A. (2009). "Targeting proteins for degradation." Nat Chem Biol **5**(11): 815-22.

Song, H. K. and Eck, M. J. (2003). "Structural basis of degradation signal recognition by SspB, a specificity-enhancing factor for the ClpXP proteolytic machine." Mol Cell **12**(1): 75-86.

Sousa, M. C., Kessler, B. M., Overkleeft, H. S. and McKay, D. B. (2002). "Crystal structure of HslUV complexed with a vinyl sulfone inhibitor: corroboration of a proposed mechanism of allosteric activation of HslV by HslU." J Mol Biol **318**(3): 779-85.

Sousa, M. C. and McKay, D. B. (2001). "Structure of Haemophilus influenzae HslV protein at 1.9 A resolution, revealing a cation-binding site near the catalytic site." Acta Crystallogr D Biol Crystallogr **57**(Pt 12): 1950-4.

Striebel, F., Kress, W. and Weber-Ban, E. (2009). "Controlled destruction: AAA+ ATPases in protein degradation from bacteria to eukaryotes." Curr Opin Struct Biol **19**(2): 209-17.

Stroud, R. M., Kossiakoff, A. A. and Chambers, J. L. (1977). "Mechanisms of zymogen activation." Annu Rev Biophys Bioeng **6**: 177-93.

Thompson, M. W. and Maurizi, M. R. (1994a). "Activity and specificity of Escherichia coli ClpAP protease in cleaving model peptide substrates." J Biol Chem **269**(27): 18201-8.

Thompson, M. W., Singh, S. K. and Maurizi, M. R. (1994b). "Processive degradation of proteins by the ATP-dependent Clp protease from Escherichia coli. Requirement for the multiple array of active sites in ClpP but not ATP hydrolysis." J Biol Chem **269**(27): 18209-15.

Turner, G. C. and Varshavsky, A. (2000). "Detecting and measuring cotranslational protein degradation in vivo." Science **289**(5487): 2117-20.

Vembar, S. S. and Brodsky, J. L. (2008). "One step at a time: endoplasmic reticulum-associated degradation." Nat Rev Mol Cell Biol **9**(12): 944-57.

Walsh, N. P., Alba, B. M., Bose, B., Gross, C. A. and Sauer, R. T. (2003). "OMP peptide signals initiate the envelope-stress response by activating DegS protease via relief of inhibition mediated by its PDZ domain." Cell **113**(1): 61-71.

Wang, J., Hartling, J. A. and Flanagan, J. M. (1997). "The structure of ClpP at 2.3 A resolution suggests a model for ATP-dependent proteolysis." Cell **91**(4): 447-56.

Weber-Ban, E. U., Reid, B. G., Miranker, A. D. and Horwich, A. L. (1999). "Global unfolding of a substrate protein by the Hsp100 chaperone ClpA." Nature **401**(6748): 90-3.

Wickner, S., Maurizi, M. R. and Gottesman, S. (1999). "Posttranslational quality control: folding, refolding, and degrading proteins." Science **286**(5446): 1888-93.

Wolf, D. H. (2004). "From lysosome to proteasome: the power of yeast in the dissection of proteinase function in cellular regulation and waste disposal." Cell Mol Life Sci **61**(13): 1601-14.

Yoo, S. J., Seol, J. H., Shin, D. H., Rohrwild, M., Kang, M. S., Tanaka, K., Goldberg, A. L. and Chung, C. H. (1996). "Purification and characterization of the heat shock proteins HslV and HslU that form a new ATP-dependent protease in Escherichia coli." J Biol Chem **271**(24): 14035-40.

CHAPTER TWO:

Control of substrate gating and translocation into ClpP by channel residues and ClpX binding

ABSTRACT

ClpP is a self-compartmentalized protease, which has very limited degradation activity unless it associates with ClpX or ClpA to form the AAA+ ClpXP or ClpAP proteases. Here, we show that ClpX binding stimulates ClpP cleavage of peptides larger than a few amino acids and enhances ClpP active-site modification. Stimulation requires ATP binding but not hydrolysis by ClpX and increases with molecular weight in a manner consistent with diffusion of peptides or inhibitors into the ClpP proteolytic chamber. Amino-acid substitutions in the axial channel or helix A of ClpP interfere with proper substrate gating in the free enzyme and weaken ClpX binding. For example, the I19A mutation in helix A results in a ClpP enzyme that degrades unfolded proteins and eliminates ClpX binding. Channel mutations in ClpP also prevent ClpXP translocation of certain amino-acid sequences, suggesting that the channel plays an active role in translocation specificity. These results support a model in which the channel residues of free ClpP exclude efficient entry of all but the smallest peptides into the degradation chamber. ClpX binding relieves these inhibitory interactions, presumably by opening the channel pore, and sets up additional interactions required for functional communication with ClpP and robust translocation.

INTRODUCTION

Intracellular protein degradation is an important facet of proteome maintenance (Lopez-Otin et al. 2002; Weichart et al. 2003; Haynes et al. 2007; Hengge et al. 2009; Powers et al. 2009), and misregulation of proteolysis can lead to severe cellular defects (Turk 2006). One strategy that helps to ensure that only the proper intracellular proteins are degraded is to sequester proteolytic enzymes within organelles, such as lysosomes or vacuoles (Ivanova et al. 2008; Hengge et al. 2009). In such cases, only specific proteins that are transported into the organelle and subsequently recognized by the protease will be degraded. A different but related strategy is to assemble cytoplasmic proteases into self-compartmentalized structures in which the proteolytic active sites are sequestered within the lumen of a barrel-shaped chamber, which is only accessible through narrow axial channels or pores (Sauer et al. 2004; Striebel et al. 2009). In this case, proteins that are substrates of the protease are typically recognized by an associated AAA+ ATPase, unfolded if necessary, and then translocated into the proteolytic chamber (Sauer et al. 2004).

ClpP is a self-compartmentalized protease, formed by the stacking of two heptameric rings (Maurizi et al. 1990; Wang et al. 1997). It associates with AAA+ hexamers of either ClpX or ClpA to form the ATP-dependent proteases, ClpXP or ClpAP (Yu et al. 2007; Striebel et al. 2009). Similarly, the HslUV protease consists of a compartmentalized protease (HslV) and a AAA+ ATPase (HslU), whereas the 26S proteasome is composed of the compartmentalized 20S proteasome core and the 19S regulatory complex (Pickart et al. 2004). By themselves, the free forms of ClpP, HslV, and the 20S proteasome core do not degrade native or denatured proteins (Thompson et al. 1994a; Thompson et al. 1994b; Yu et al. 2007). Thus,

mechanisms must exist to limit the destructive potential of these isolated enzymes. The importance of proper regulation of proteolytic activity is highlighted by the action of acyldepsipeptide antibiotics, which kill bacteria by binding ClpP, preventing association with ClpX or ClpA, and endowing ClpP with the ability to degrade unfolded polypeptides, including nascent chains (Brotz-Oesterhelt et al. 2005; Kirstein et al. 2009).

Two distinct mechanisms can limit the activity of self-compartmentalized proteases in the absence of their AAA+ partners. One mechanism involves active-site rearrangment. For example, when HslU is absent, the active-site residues of HslV assume an inactive conformation and fail to cleave even small peptide substrates or to react with peptide vinyl-sulfone inhibitors (Yoo et al. 1996; Bogyo et al. 1997; Sousa et al. 2000; Sousa et al. 2002). In this instance, binding of an HslU ring to an HslV ring is required to remodel the peptidase active sites (Sousa et al. 2002; Kwon et al. 2003). The second mechanism involves gating or controlling substrate access to the proteolytic chamber of the compartmental peptidase. For example, crystal structures show that the active sites in the chamber of the isolated 20S proteasome are arranged in a functional conformation but substrate access to these sites is severely limited by residues which sterically block the entrance pore (Groll et al. 2000; Groll et al. 2003; Rabl et al. 2008). This blockade is relieved by structural rearrangements that accompany binding of the 20S core to the 19S regulatory complex or to non-ATPase regulators such as PA26, allowing substrate access and degradation (Smith et al. 2005; Smith et al. 2007).

We are interested in the mechanism(s) that repress the proteolytic activity of the isolated ClpP enzyme and that allow activation by ClpX. *E. coli* ClpP is initially expressed as a proenzyme, which is autoproteolytically processed to remove an N-terminal propeptide (Maurizi et al. 1990). Crystallographic studies of the mature ClpP tetradecamer reveal canonical Ser-His-Asp catalytic triads and a properly formed oxyanion hole (Wang et al. 1997; Kang et al. 2004; Gribun et al. 2005; Bewley et al. 2006; Szyk et al. 2006; Ingvarsson et al. 2007; Kim et al. 2008), which appear to be functional as free ClpP can degrade small peptides and reacts with diisopropylfluorophosphate, an active-site inhibitor (Thompson et al. 1994a; Thompson et al. 1994b). The axial channel of free ClpP is formed by N-terminal stem-loop structures, with the stems forming the rim of the pore and the loops forming the channel (Wang et al. 1997; Kang et al. 2004; Gribun et al. 2005; Bewley et al. 2006; Szyk et al. 2006; Ingvarsson et al. 2007; Kim et al. 2008). This channel is too narrow to admit native proteins and even large peptides are degraded very slowly (Grimaud et al. 1998; Maurizi et al. 1998). However, deletion of segments of the pore and channel allow degradation of unfolded proteins, which are not degraded by wild-type ClpP alone (Bewley et al. 2009). Moreover, ClpP degradation of large peptides can be stimulated substantially by ClpA binding (Thompson et al. 1994b). All of these results are consistent with regulation of ClpP proteolytic activity by a simple gating mechanism. However, recent studies suggest that allosteric regulation of the active-site conformation of ClpP by ClpX or ClpA binding may also be required under some circumstances to allow hydrolysis of the acyl intermediate in peptide-bond cleavage (Jennings et al. 2008).

Electron microscopy (EM) shows that hexameric rings of ClpX or ClpA stack coaxially with the heptameric rings of ClpP, aligning the central translocation channel of the AAA+ ATPase with the ClpP pore (Grimaud et al. 1998; Ortega et al. 2000). There are no crystal structures of ClpXP or ClpAP, however, and the resolution of the EM structures are insufficient to observe atomic details. It is known that formation of these active proteolytic complexes requires ATP or ATPγS, affects the rate of ATP hydrolysis, and requires conserved IGF/IGL motifs located in loops on the AAA+ ring that appear to dock into hydrophobic clefts on the periphery of the ClpP ring (Kessel et al. 1995; Grimaud et al. 1998; Kim et al. 2001; Singh et al. 2001; Joshi et al. 2004; Martin et al. 2007; Bewley et al. 2009). Amino acids that form the axial pore and channel of ClpP also appear to play roles in recognition of the AAA+ rings of ClpX and ClpA (Kang et al. 2004; Gribun et al. 2005; Bewley et al. 2006; Martin et al. 2008a; Bewley et al. 2009).

In this paper, we test predictions of the pore-gating model for ClpP and investigate the role of ClpX in controlling gating. We find that ClpX binding stimulates ClpP cleavage of peptide substrates larger than a few amino acids in a reaction that does not require ATP hydrolysis. Moreover, this stimulatory effect increases as a function of peptide molecular weight, as expected if ClpX binding increases the rate at which peptides diffuse into ClpP. ClpX binding also stimulates active-site modification of ClpP by fluorophosphates, but only to a level expected from faster diffusion of the inhibitor into the ClpP chamber. The ability of wild-type ClpP to exclude large peptides depends on interactions mediated by the channel region of the pore and by conserved residues in α-helix A. Indeed, a single mutation in helix A results in a ClpP enzyme that degrades unfolded proteins in the absence of a AAA+

partner. Mutations in the ClpP channel and helix A also weaken ClpX binding and affect communication during the degradation of protein substrates. Importantly, we find that ClpP channel mutations can prevent ClpXP translocation of certain amino-acid sequences, suggesting that the wild-type ClpP channel plays an active role in the specificity of the translocation reaction. Overall, our results support a model in which the channel residues in free ClpP prevent efficient entry of all but the smallest peptides into the degradation chamber. ClpX binding relieves these inhibitory interactions, presumably by opening the channel, and sets up additional interactions with ClpP that are required for functional communication and robust translocation.

RESULTS

Active-site reactivity of ClpP

In an initial set of experiments, we used the rhodamine-labeled fluorophosphate inhibitor developed by Cravatt and colleagues (Rh-FP; M_R 845 Da) (Liu et al. 1999) to probe the reactivity of the active-site serines of *E. coli* ClpP, both alone and in complex with *E. coli* ClpX. For these experiments, the fluorescent inhibitor was incubated with ClpP, with ClpP plus ClpX•ATPγS, or with ClpP plus ClpX•ADP for different times before quenching the reaction. Samples were then analyzed by SDS PAGE and fluorimetry. In the ClpX•ATPγS experiment, the rate of active-site modification by Rh-FP was ~3-fold faster than for ClpP alone (Figure 1A). The rate of modification with ClpX•ADP, which does not bind ClpP, was the same as that measured for ClpP alone. Thus, ClpX binding modestly enhances the rate of active-site modification of ClpP. Importantly, this result is very different from that observed for HslV, where no active-site modification was observed in the absence of HslU•ATP

(Bogyo et al. 1997), and is consistent with crystallographic results that reveal a well-formed catalytic triad and oxyanion hole in the structure of ClpP alone (Wang et al. 1997). The modest ClpX stimulation of ClpP active-site reactivity probably occurs because ClpX binding enhances the rate at which the inhibitor diffuses into the ClpP chamber.

ClpX stimulates ClpP peptide cleavage in a size-dependent fashion

Early biochemical studies demonstrated that ClpA•ATPγS did not stimulate ClpP cleavage of very small peptides but did stimulate cleavage of longer peptides (Thompson et al. 1994b). To test this possibility for ClpX, we assayed the dependence of ClpP cleavage of a dipeptide substrate (succinyl-LY-AMC; M_R 552 Da), a decapeptide substrate (M_R 1219 Da), and a 20-residue substrate (M_R 2404 Da). The 10- and 20-residue substrates were flanked by a fluorophore (2-aminobenzoic acid) and quencher (3-nitrotyrosine) to allow cleavage to be detected by increased fluorescence. The rates of dipeptide cleavage by ClpP alone or in the presence of ClpX•ATPγS were within error (Figure 1B). By contrast, ClpXP•ATPγS cleaved the decapeptide ~9-fold faster and the 20-residue substrate ~40-fold faster than ClpP by itself (Figure 1B).

ClpX can use ATPγS hydrolysis, which occurs 10- to 20-fold more slowly than ATP hydrolysis, to power protein unfolding, translocation, and degradation (Burton et al. 2003; Martin et al. 2008b). To test if nucleotide hydrolysis by ClpX was required for enhanced ClpP cleavage of the decapeptide substrate, we also determined rates in the presence of ClpX•ATP and $ClpX^{E285Q}$•ATPγS. The $ClpX^{E285Q}$ mutant harbors a substitution for a highly conserved glutamate in the Walker B motif and is defective in ATP hydrolysis (Hersch et al.

2005). In both cases, decapeptide cleavage occurred at similar rates to those observed with ClpX•ATPγS (Figure 1C). No stimulation of cleavage was observed with ClpX•ADP (Figure 1C). We conclude that nucleotide hydrolysis is not essential for ClpX stimulation of peptide degradation by ClpP.

Figure 1D shows that activity stimulation by ClpX increases as a function of the molecular weight of the compound that needs to enter ClpP. These findings, in conjunction with previous results, support a model in which ClpX binding to ClpP facilitates faster diffusion of Rh-FP, the decapeptide substrate, and the 20-residue substrate into the proteolytic chamber of ClpP. Because the dipeptide substrate seems to enter the chambers of ClpP and ClpXP at comparable rates, it appears that the pore in isolated ClpP only restricts entry of compounds with a higher molecular weight or larger radius of gyration. It might be argued that changes in the diffusion rate of the dipeptide are masked by ClpX stimulation of ClpP active-site reactivity. If this were true, however, then the dipeptide would have to diffuse more slowly into ClpXP than into ClpP, making it difficult to explain the results with the 10- and 20-residue peptides.

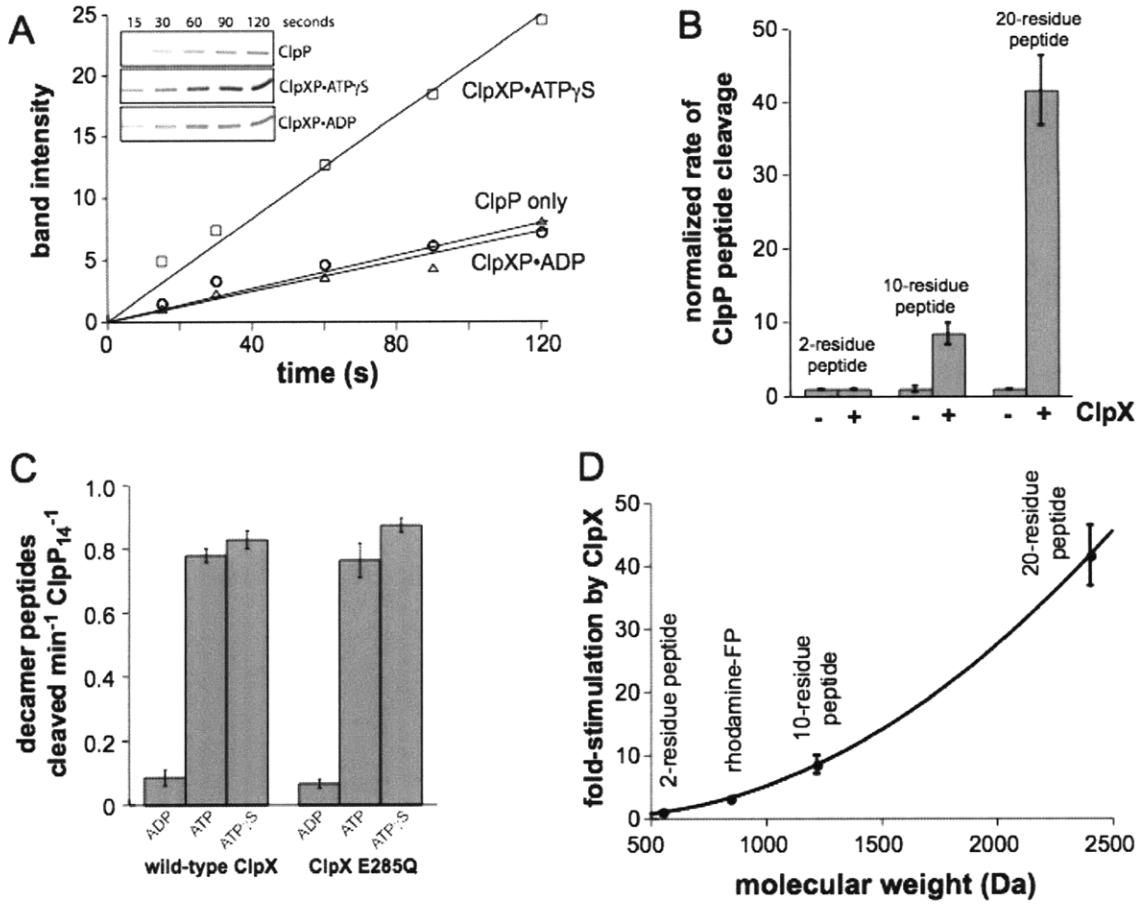

Figure 1. Changes in ClpP activity in response to ClpX binding. (A) Rate of ClpP reactivity with the active-site inhibitor rhodamine-FP. The inset gel shows the fluorescence of rhodamine-FP modified ClpP as a function of time after addition of the inhibitor with or without ClpX and ATPγS/ADP. The graph represents quantification of band intensities from the gel. (B) Rates of ClpP cleavage for a dipeptide (succinyl-LY-AMC), a decapeptide (Abz-KASPVSLGY^{NO2}D), and a 20-residue peptide (Abz-ASSHATRQLSGLKIHSNLY^{NO2}H) were determined in the presence and absence of ClpX•ATPγS and were normalized to the rate observed for ClpP alone. (C) Rates of ClpP cleavage of the decapeptide were determined in the presence of wild-type ClpX or the ATPase defective ClpX^{E185Q} mutant and different nucleotides. (D) ClpX•ATPγS stimulation of the rate of ClpP peptide cleavage or active-site modification is plotted as a function of the molecular weight of the peptide substrate or the active-site inhibitor. The solid line is a fit to a polynomial function. In panels B-D, the data are averages of triplicate experiments and the error bars represent one standard deviation from the mean.

Substitution mutations in the ClpP channel activate peptide cleavage

Truncated variants of ClpP, lacking 7-14 N-terminal residues of the mature enzyme, show faster degradation of large peptides (Jennings et al. 2008; Bewley et al. 2009). We sought to determine whether substitutions for residues 8-15, which form the channel loop of the pore (Figure 2A), could also alter ClpP's ability to discriminate against longer peptides. Indeed, when we replaced residues 8-15 with eight glycines, this mutant (GGGGGGGG^{15}) cleaved the decapeptide at a rate ~8-fold faster than that observed with wild-type ClpP (Figure 2B) but cleaved the dipeptide at a comparable rate (Figure 2C). Mutations in the N-terminal loop can alter ClpP processing (Bewley et al. 2006). However, the GGGGGGGG^{15} mutant was properly processed by the criteria that it had the same mobility as wild-type ClpP during SDS PAGE (data not shown) and the expected molecular weight in MALDI-TOF mass spectrometry experiments (calculated 22141 Da; observed 22166Da).

Residues 8-15 of wild-type *E. coli* ClpP include four highly conserved charged residues (EQTSRGER^{15}). To probe the importance of these charged channel residues, we constructed and purified mutants in which these charges were reversed (RQTSEGRE^{15}), were replaced by glycines (GQTSGGGG^{15}), or were replaced by alanines (AQTSAGAA^{15}). Each of these mutants was properly processed, behaved like wild-type ClpP during purification, and exhibited similar rates of degradation of the succinyl-LY-AMC dipeptide (Figure 2C) but cleaved the decapeptide ~10-fold faster than wild type (Figure 2B). These results suggest that proper regulation of access to the proteolytic chamber of ClpP requires interactions mediated by specific charged residues in the wild-type channel.

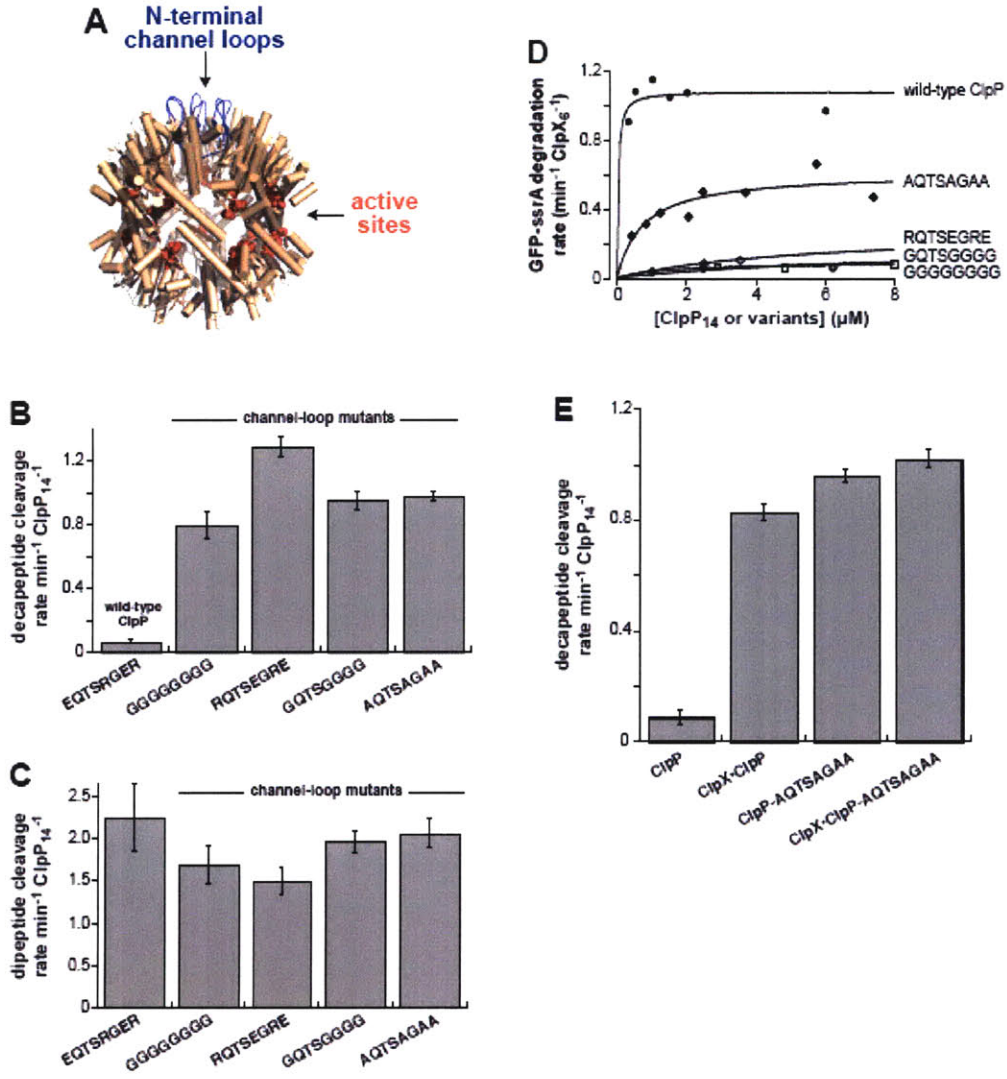

Figure 2. Activity of ClpP variants with mutations in the N-terminal channel loops. (A) Side view of the crystal structure of ClpP (pdb code 1YG6) showing the location of the N-terminal channel loops (residues 8-15) in blue and the Ser-His-Asp catalytic triad in red. (B) Rates of decapeptide (15 μM) cleavage by wild-type ClpP and the channel variants (0.3 μM). The channel-loop sequence for each mutant is shown. Values are averages (n=3) ± 1 SD. (C) Rates of succinyl-LY-AMC (50 μM) cleavage by ClpP and the channel variants (0.1 μM). Values are averages (n=3) ± 1 SD. (D) Rates of ClpXP degradation of GFP-ssrA (5 μM). Reactions contained ClpX (0.1 μM hexamer) and different quantities of wild-type ClpP or the channel variants. Solid lines are fits to a hyperbolic function. (E) Non-additive effects of ClpX (6.8 μM hexamer plus 1 mM ATPγS) and the AQTSAGAA[15] channel mutations on ClpP (0.3 μM tetradecamer) cleavage of the decapeptide (15 μM). Values are averages (n=3) ± 1 SD.

Channel residues influence ClpX binding and ClpXP degradation

To assess the effects of our channel mutations on ClpX interactions, we titrated increasing quantities of the mutant variants or wild-type ClpP against a fixed concentration of ClpX and assayed degradation of GFP-ssrA. The ssrA tag targets substrates to the axial pore of ClpX, and unfolding and subsequent degradation of GFP-ssrA require ATP hydrolysis (Kim et al. 2000; Siddiqui et al. 2004; Martin et al. 2008b). As assayed by loss of native fluorescence, GFP-ssrA degradation was observed in the experiments using wild-type ClpP or AQTSAGAA^{15} ClpP but was not detected using the RQTSEGRE^{15}, GQTSGGGG^{15}, and GGGGGGGG^{15} ClpP variants at the highest concentrations tested (Figure 2D). Moreover, substantially higher concentrations of the AQTSAGAA^{15} mutant than of wild-type ClpP were required for half-maximal stimulation of proteolysis, and the GFP-ssrA degradation rate at saturation was approximately 2-fold slower for the AQTSAGAA^{15} mutant than for wild-type ClpP (Figure 2D). We conclude that residues in the ClpP channel play important roles both in binding ClpX and in determining the maximal rate of degradation of native protein substrates.

ClpP-channel residues facilitate translocation of specific substrate sequences

Peptides with a C-terminal ssrA tag, a preceding guest region of variable sequence, and an N-terminal module with a cleavage site flanked by a fluorophore and quencher provide a convenient assay for ATP-dependent degradation that requires ClpXP engagement and translocation but not protein unfolding (Barkow et al. 2009). It seemed possible that degradation of an ssrA-tagged peptide might be more permissive than degradation of GFP-ssrA. Hence, we titrated increasing ClpX against a fixed concentration of the ClpP channel

variants or the wild-type enzyme and assayed degradation of a 33-residue peptide containing a YGYGYGYGYG guest sequence ($[YG]_5$-ssrA). To restrict degradation of this substrate via passive diffusion into the ClpP proteolytic chamber, we added the SspB protein, which binds part of the ssrA tag and restricts the bound peptide from entering ClpP unless ClpX is present and can interact functionally with ClpP (Flynn et al. 2001; Levchenko et al. 2003; Song et al. 2003; Barkow et al. 2009). At the highest ClpX concentrations tested (8 μM), RQTSEGRE[15] and GQTSGGGG[15] ClpP showed no increase in degradation of the $[YG]_5$-ssrA peptide (Figure 3A), mirroring the results obtained using GFP-ssrA as the substrate. As expected, degradation of this peptide by ClpP or the AQTSAGAA[15] mutant increased as a function of ClpX concentration and then saturated (Figure 3A). Compared to wild-type ClpP, half-maximal stimulation of degradation of the $[YG]_5$-ssrA substrate by AQTSAGAA[15] ClpP required an approximate ~10-fold higher concentration of ClpX and the degradation rate was ~6-fold slower when ClpX was saturating (Figure 3A).

Intriguingly, AQTSAGAA[15] ClpXP degraded native GFP-ssrA at ~50% of the wild-type rate but only degraded the unfolded $[YG]_5$-ssrA peptide at ~20% of the corresponding wild-type velocity (Figures 2D & 3A). We also tested degradation of additional ssrA-tagged peptides with different guest sequences for degradation by AQTSAGAA[15] ClpXP as well as wild-type ClpXP (Figure 3B). Surprisingly, AQTSAGAA[15] ClpXP showed almost no degradation of peptides with 10 glutamic acids, 10 lysines, or four glycines in the guest region. Somewhat higher rates were observed for peptides with guest regions containing 10 glutamines, 10 arginines, or six alanines (Figure 3B). Peptides with guest regions containing proline or repeats of tyrosine-glycine or phenyalanine-glycine were degraded at the highest

rates by AQTSAGAA^{15} ClpXP. In all cases, wild-type ClpXP degraded the same peptides substantially faster (Figure 3B). Thus, mutating the charged residues in the wild-type ClpP channel to alanines seems to restrict translocation of charged, polar, and small amino acids more than it affects translocation of more hydrophobic side chains. Therefore, the chemical identity of the amino acids that form the wild-type channel appear to play roles in determining translocation specificity.

Non-additivity of ClpP channel mutations and ClpX binding

ClpX binding stimulated degradation of the decapeptide substrate by wild-type ClpP roughly 10-fold (Figure 1B) and the AQTSAGAA^{15} channel mutations resulted in a similar increase in decapeptide cleavage by the free mutant enzyme compared to wild-type ClpP (Figure 2B). However, when we titrated increasing ClpX against a constant quantity of AQTSAGAA^{15} ClpP and assayed decapeptide degradation in the presence of ATPγS, no increase in cleavage was observed (Figure 2E), although based on experiments in the previous section, ClpX binds to AQTSAGAA^{15} ClpP (Figure 2D). This observed lack of additivity in decapeptide cleavage could be explained if the channel mutations and ClpX binding affect ClpP gating by similar mechanisms.

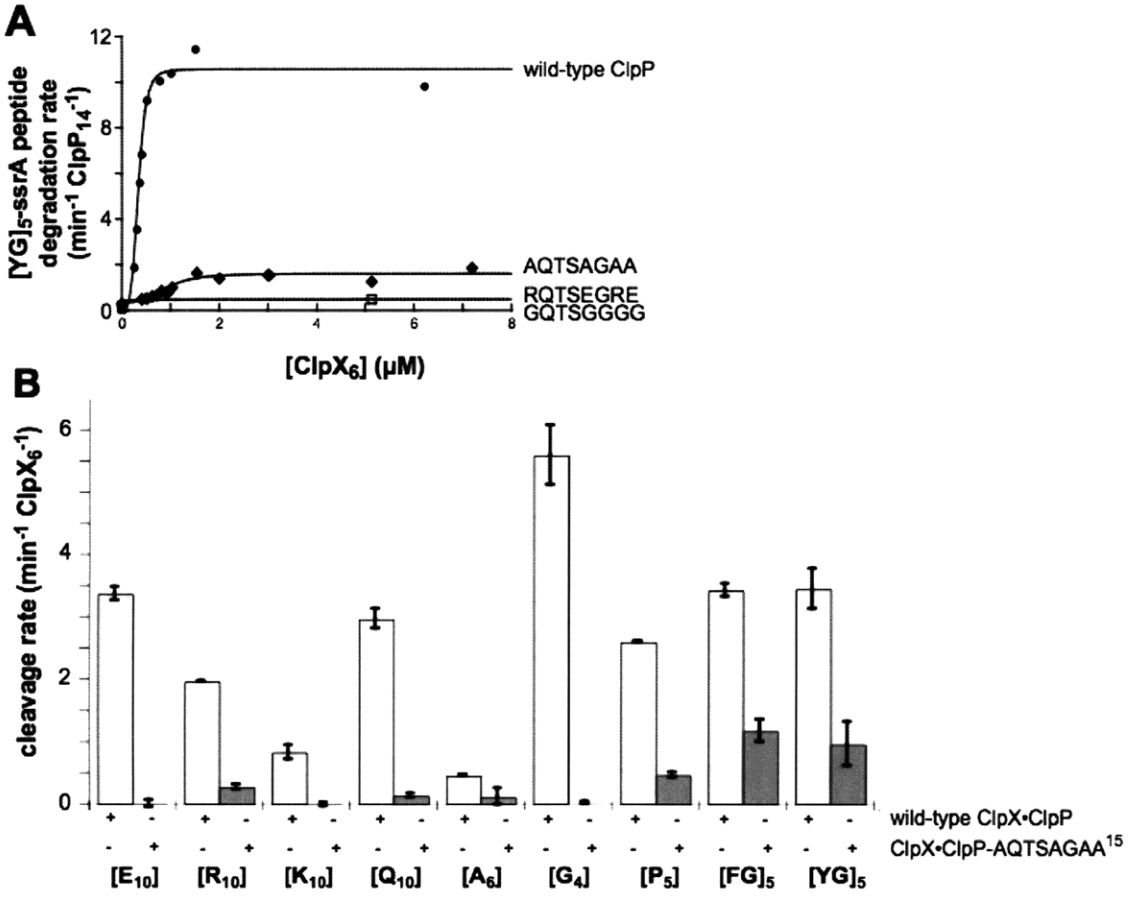

Figure 3. Degradation of peptides containing an N-terminal Abz-FAPHMALVPY^{NO2}, a guest region, and a C-terminal KKAANDENYALAA (ssrA tag underlined). (A) Rates of ClpXP degradation of a 33 residue ssrA-tagged peptide (8 μM) with a $[YG]_5$ guest region. Each reaction contained $ClpP_{14}$ or mutant variants (0.15 μM), SspB (8 μM), and different quantities of wild-type ClpX. Solid lines are fits to the Hill equation. (B) Rates of ClpX•ClpP or ClpX•ClpP-AQTSAGAA^{15} of ssrA-tagged peptide substrates with different guest regions. The rates shown were corrected by subtracting background cleavage by ClpP or ClpP-AQTSAGAA^{15} only. Values are an average of two determinations. Guest regions: $[E_{10}]$, 10 glutamic acids; $[R_{10}]$, 10 arginines; $[K_{10}]$, 10 lysines; $[Q_{10}]$, 10 glutamines, $[A_6]$, six alanines; $[G_4]$, four glycines; $[P_5]$, five prolines; $[FG]_5$, five phenylalanine-glycine repeats; and $[YG_5]$, five tyrosine-glycine repeats. All reactions contained 8 μM peptide, 8 μM SspB, 0.15 μM $ClpX_6$, and 4 μM $ClpP_{14}$.

Helix-A residues play roles in substrate gating

In a screen for dominant-negative ClpP mutations that prevent or reduce wild-type ClpXP activity, we isolated I19T and S21Y mutations in helix A (see Chapter 3). This helix, which consists of residues 19-25, is immediately proximal to the N-terminal channel loops and pore in the ClpP structure (Figure 4A), and Ile^{19} and Leu^{24} in helix A form a hydrophobic cluster with Phe^{49} and pore-stem residues Pro^{4} and Val^{6} (Figure 4B). To investigate the role of helix A in ClpP activity, we constructed alanine-substitution mutations at each helix-A residue, purified the mutant enzymes, and assayed peptidase activity. All of these mutants showed wild-type levels of succinyl-LY-AMC cleavage (data not shown), but decapeptide rates differed substantially (Figure 4C). Relative to wild-type ClpP, for example, the R22A and K25A mutations caused small increases (~1.5 fold) in decapeptide cleavage, the Y20A, S21A, and L23A mutations caused modest increases (~5 fold), and the I19A and L24A mutations caused large increases (~20 fold). We also constructed and purified mutants with more conservative leucine and valine substitutions for Ile^{19}. Decapeptide cleavage was increased ~6 fold by the I19L mutation and ~16 fold by the I19V mutation (Figure 4D). Thus, even subtle changes in the stereochemistry of the side chain of residue 19 alter ClpP gating.

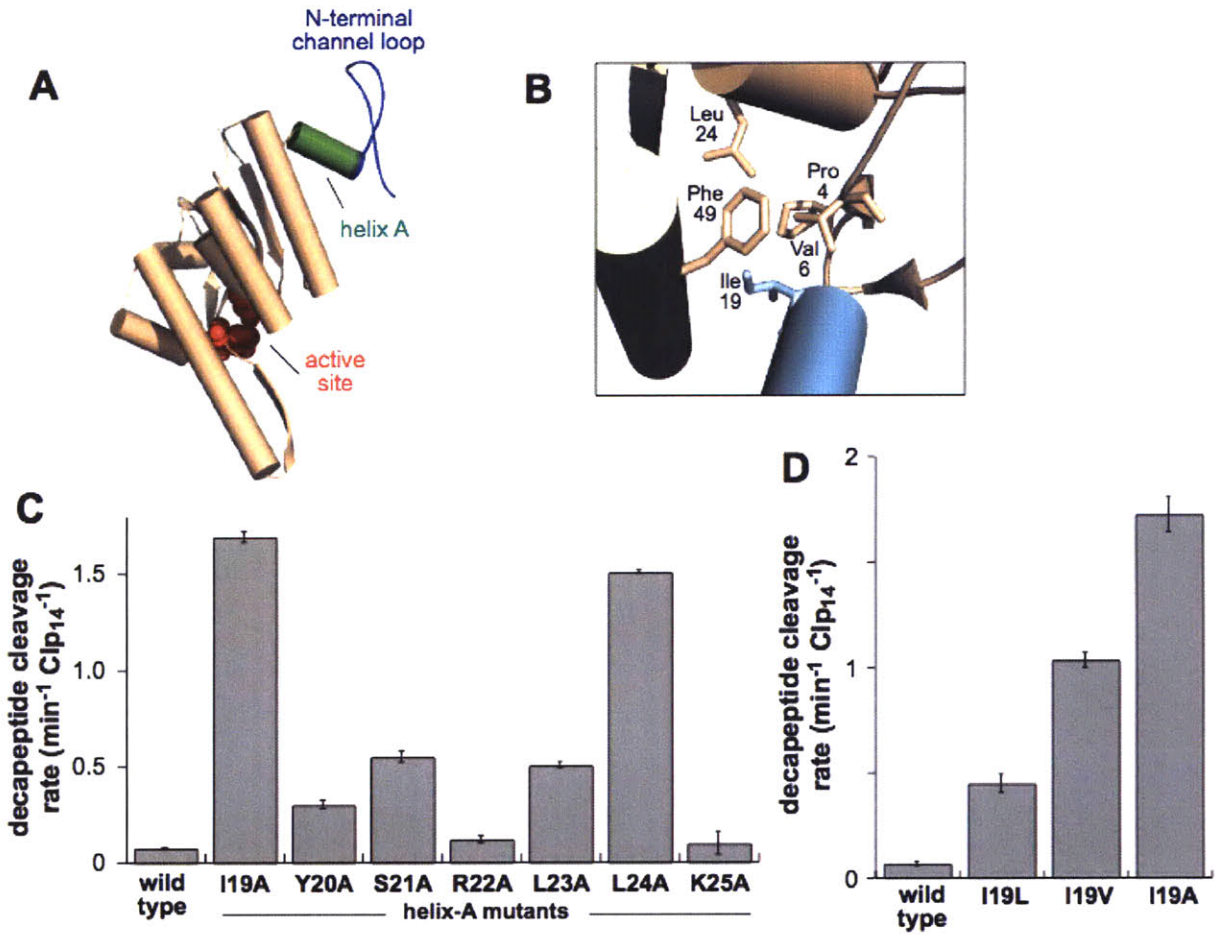

Figure 4. Helix A plays a role in regulating peptide degradation. (A) Structure of a ClpP subunit (pdb code 1YG6) showing residues 8-15 of the N-terminal channel loop, (blue), helix A (green), and the catalytic triad (red). (B) Pro4, Val6, and Ile19 from one subunit pack in a hydrophobic cluster with residues Leu24 and Phe49 from a neighboring ClpP subunit. (C) Rates of decapeptide (15 μM) degradation for wild-type $ClpP_{14}$ and helix-A mutants (0.3 μM). (D) Decapeptide cleavage by Ile19 mutants (conditions as in panel C). Values shown in panels C and D are averages (n=3); error bars represent one standard deviation.

We used trypsin to probe effects of the helix-A alanine-substitution mutations on the conformation of the ClpP channel, which contains potential sites of tryptic cleavage after Arg^{12} and Arg^{15}. Incubation of trypsin with a wild-type ClpP variant bearing a C-terminal His_6-tag resulted in formation of a stably truncated fragment within 30 minutes, as assayed

by SDS PAGE (Figure 5A). Control experiments with ClpP lacking a His_6-tag revealed that tryptic cleavage removed this C-terminal affinity tag (data not shown). The Y20A, S21A, R22A, L23A, and K25A mutants behaved like the wild-type control after incubation with trypsin (Figure 5A). By contrast, the I19A and L24A mutations resulted in enhanced trypsin susceptibility, as shown by lower molecular weight products following SDS PAGE (Figure 5A). Edman sequencing of these smaller fragments revealed that the I19A mutant was cleaved after Arg^{12}, whereas the L24A mutant was cleaved after Arg^{15} (data not shown). Thus, the I19A and L24A mutations, which resulted in the largest increases in decapeptide cleavage, also increase the susceptibility of the N-terminal ClpP channel to tryptic cleavage. These results support a model in which packing interactions mediated by the wild-type side chains of Ile^{19} and Leu^{24} stabilize a stem-loop conformation of the N-terminal channel that restricts passage of all but the smallest peptides into free ClpP.

If the helix-A mutations increase decapeptide-cleavage activity solely by influencing the conformation of the ClpP channel and pore, then the effects of a double mutation involving helix A and the channel should not be additive. Indeed, when we constructed and purified an $L24A/GGGGGGGG^{15}$ variant of ClpP, this double mutant was no more active in cleaving the decapeptide than the parental L24A or $GGGGGGGG^{15}$ mutants (Figure 5B). These results strongly suggest that mutations in both helix A and in the ClpP channel disrupt local conformations that are required to restrict diffusive passage of the decapeptide substrate into the ClpP proteolytic chamber.

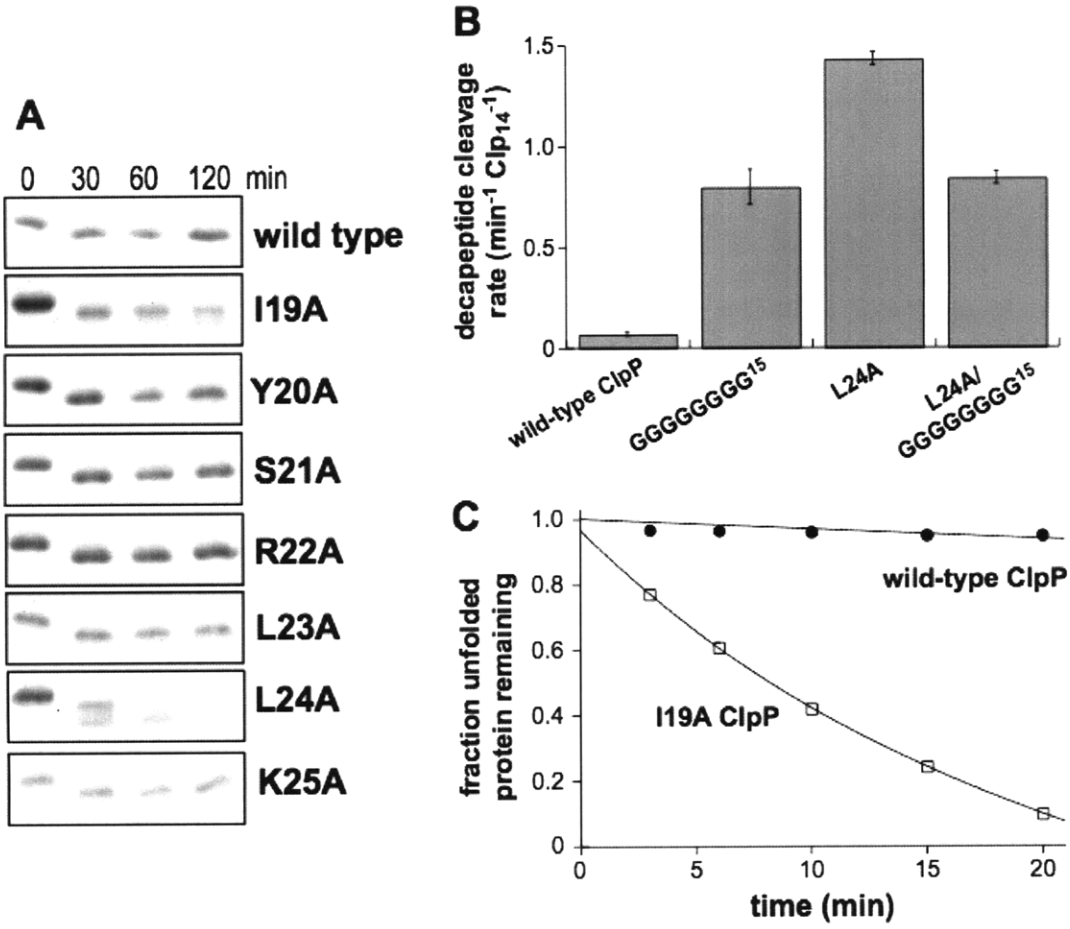

Figure 5. Properties of channel-loop and helix-A mutants. (A) Analysis by SDS-PAGE of time courses of digestion (30 °C) of wild-type $ClpP_{14}$ or variants (0.7 μM) with trypsin (0.5 μM). The initial shift in mobility observed for all proteins is caused by cleavage of the C-terminal His_6 tag. (B) Rates of decapeptide (15 μM) cleavage by single and double ClpP mutants (4 μM tetradecamer) containing the $GGGGGGGG^{15}$ channel-loop substitution and/or the L24A helix-A mutation. Values are averages (n=3); error bars represent one standard deviation. (C) Degradation of the carboxymethylated ^{35}S-titin-I27-ssrA protein (5 μM) by wild-type $ClpP_{14}$ or the I19A mutant (1 μM). Values are averages (n=2). The solid lines are fits to an exponential function.

Deletion of the N-terminal 10, 14, or 17 residues of mature ClpP allows these mutants to degrade α-casein, a natively unfolded protein (Bewley et al. 2009). To test if a helix-A mutation also allows ClpP to degrade unfolded proteins, we assayed degradation of a carboxymethylated and ^{35}S-labeled variant of the I27 domain of human titin (Kenniston et al. 2003). Indeed, this 113-residue unfolded substrate was degraded far more rapidly by I19A ClpP than by wild-type ClpP (Figure 5C). The I19A ClpP mutant did not degrade the native titin I27 domain (data not shown). These results show that a single amino-acid substitution in ClpP is sufficient to deregulate gating, allowing the mutant to degrade unfolded proteins, and highlight the important role played by I19 in controlling access to the ClpP chamber.

In studies of a ClpP mutant lacking seven N-terminal amino acids, Jennings et al. (2008) observed non-linear cleavage kinetics of the succinyl-LY-AMC dipeptide, caused by accumulation of an acylated enzyme intermediate. Using this substrate and their assay conditions, our I19A and L24A ClpP mutants failed to show comparable behavior, suggesting that accumulation of acylated ClpP is probably a consequence of the deletion mutation used and not a general consequence of unregulated gating. Maurizi and colleagues also showed that wild-type ClpP cleaves specific peptides with turnover numbers of ~10,000 min^{-1}, a result inconsistent with slow hydrolysis of the acyl intermediate in peptide-bond hydrolysis for wild-type ClpP (Thompson et al. 1994a).

Helix-A mutations disrupt ClpX binding

Deletion and substitution mutations affecting residues that form the ClpP pore and channel have been shown to weaken or prevent ClpX binding (Kang et al. 2004; Gribun et al. 2005;

Jennings et al. 2008; Bewley et al. 2009). To test if the alanine-substitution mutations in helix A have similar effects, we combined increasing quantities of these mutants with a fixed concentration of ClpX and assayed for ATP-dependent degradation of GFP-ssrA. The I19A, Y20A, S21A, R22A, and L24A mutants showed very low substrate cleavage at the highest ClpP concentrations tested, suggesting substantial defects in ClpX binding, whereas the L23A and K25A mutants showed only modest decreases in apparent ClpX affinity (Figure 6A & 6B).

Importantly, the effects of helix-A mutations on ClpX interactions were relatively poorly correlated with decapeptide cleavage or trypsin susceptibility. For the I19A and L24A mutations, for example, all three assays showed large changes compared to the wild-type controls. For the S21A and R22A mutations, by contrast, large effects on apparent ClpX binding were coupled with relatively small effects on decapeptide cleavage and no changes in trypsin susceptibility. Similarly, the L23A mutant bound ClpX ($K_{app} \approx 0.2\ \mu M$) far better than the R22A mutant ($K_{app} > 10\ \mu M$), but both mutations resulted in similar rates of decapeptide cleavage (Figure 6A). Thus, these results suggest that helix-A mutations, such as S21A and R22A, do not affect ClpX binding solely through indirect effects on ClpP-channel residues but also influence ClpX interactions directly.

We also combined the R22A and S21A mutants with ClpA and assayed GFP-ssrA degradation. Interestingly, half-maximal degradation of GFP-ssrA by ClpAP required only ~6-fold higher concentrations of the R22A mutant than of wild-type ClpP and both the wild-type and mutant complexes degraded this native substrate at similar rates under conditions

of ClpP saturation (Figure 6C). By contrast, combining the S21A mutant with ClpA resulted in GFP-ssrA degradation at a maximal rate half that of the wild-type enzyme complex (Figure 6D). These results suggest that the Arg^{22} and Ser^{21} side chains of ClpP play far more important roles in binding ClpX than in binding ClpA.

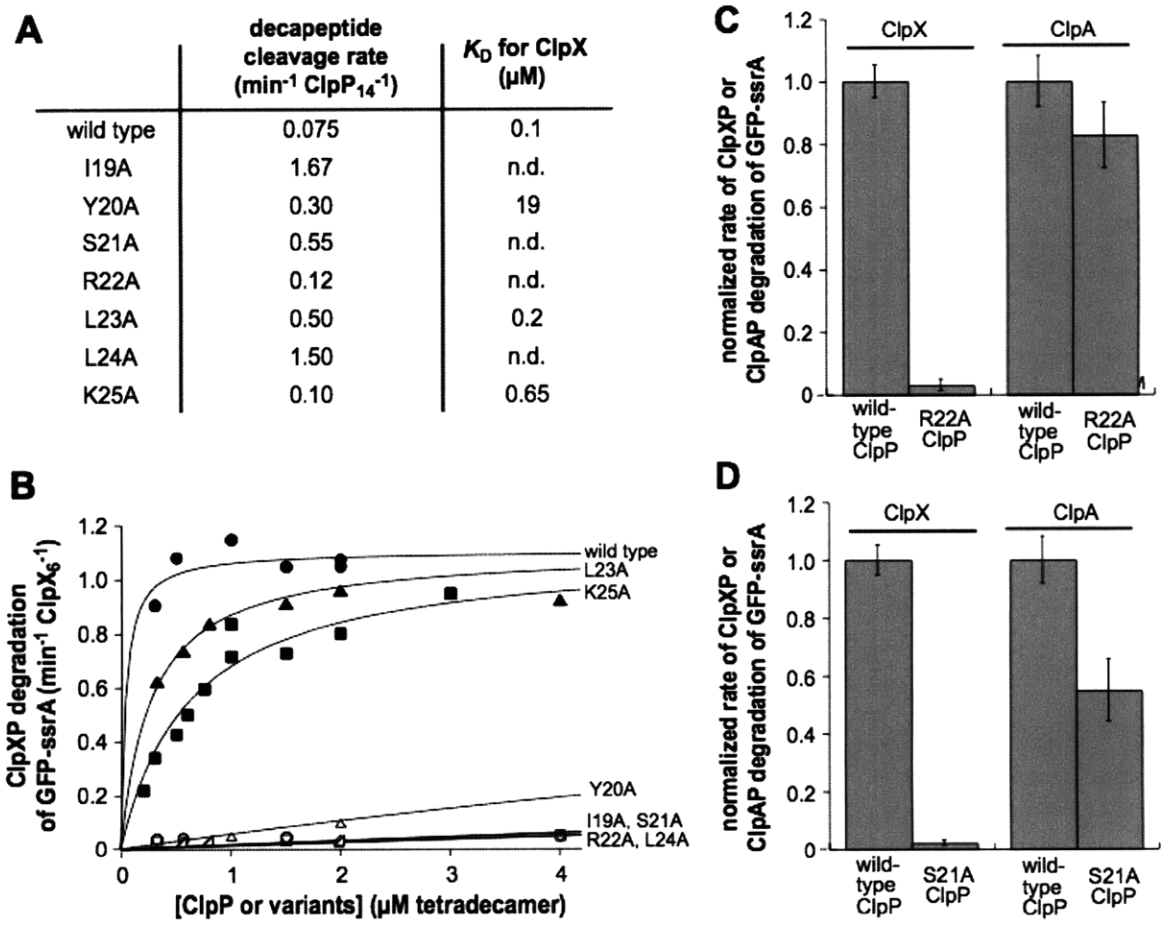

Figure 6. Interaction of ClpP variants with ClpX or ClpA. (A) Decapeptide cleavage activity of isolated ClpP or its helix-A mutants and the affinity of these enzymes for ClpX, as measured by the GFP-ssrA degradation assays shown in panel B. n.d., affinity was too weak to determine. (B) Rates of ClpXP degradation of GFP-ssrA (5 μM) were measured as a function of the concentration of ClpP or helix-A mutants using a covalently linked ClpX-ΔN trimer (0.1 μM pseudo-hexamer equivalents). The solid lines are fits to a hyperbolic function. (C, D) Normalized rates of ClpXP or ClpAP degradation of GFP-ssrA were determined using wild-type ClpP, R22A ClpP, or S21A ClpP (4 μM tetradecamer) in combination with the ClpX-ΔN trimer (0.1 μM pseudo-hexamer equivalents) or ClpA (50 nM hexamer). Values were divided by the rate observed using wild-type ClpP.

DISCUSSION

Previous studies demonstrated that ClpP alone has a very limited ability to degrade peptide substrates larger than a few amino acids, but this activity can be stimulated substantially by ClpA (Thompson et al. 1994b). The work reported here shows that ClpX binding to ClpP also enhances its rate of polypeptide degradation. For both ClpX and ClpA, the magnitude of the rate enhancement increases with the molecular weight of the peptide substrate and requires ATP binding by the AAA+ enzyme but not hydrolysis. These results in combination with crystal structures of ClpP and studies of ClpP mutants suggest that simple ATP-dependent binding of either ClpX or ClpA to ClpP induces conformational rearrangements that allow unfolded peptide and polypeptide substrates to diffuse through the axial channel and into the proteolytic chamber of ClpP. Although ClpX and ClpA are both AAA+ ATPases, they differ markedly in size, sequence, substrate specificity, and some aspects of their interactions with ClpP (Gottesman et al. 1990; Gottesman et al. 1993; Singh et al. 2000; Flynn et al. 2001; Kim et al. 2001; Sharma et al. 2005). Thus, their common ability to activate ClpP peptide cleavage was not a foregone conclusion.

The majority of ClpP molecules in *E. coli* appear to be bound to ClpX or ClpA, but measurements of intracellular abundance and affinity calculations suggest that some ClpP is present as the free enzyme (Farrell et al. 2005). The inability of this uncomplexed ClpP to degrade unstructured polypeptides is likely to be important in avoiding rogue degradation of such sequences in the cell. For example, unstructured regions can be essential for protein function, native and denatured proteins are often in dynamic equilibrium, and nascent polypeptides might be degraded immediately after synthesis. Indeed, acyldepsipeptide

antibiotics kill *E. coli* and other strains of bacteria by activating ClpP polypeptide degradation and preventing its binding to ClpX/ClpA (Kirstein et al. 2009). Our results indicate that restriction of the polypeptide cleavage activity of free ClpP depends both on the identity of residues in the wild-type channel and in the neighboring helix A.

ClpX- or ClpA-dependent remodeling of the ClpP channel is probably required to allow efficient ATP-fueled degradation by these AAA+ proteases. Degradation of unfolded polypeptides by ClpXP or ClpAP, which is independent of ATP hydrolysis, is a useful biochemical assay but is likely to be biologically irrelevant. This conclusion follows from the facts that only ATP-bound forms of ClpX or ClpA bind ClpP and that ATP hydrolysis by these complexes is both constitutive and stimulated by substrates (Joshi et al. 2004; Sauer et al. 2004; Hersch et al. 2005). In fact, wild-type ClpXP hydrolyzes roughly 100 ATPs during degradation of a single molecule of the unfolded titin I27 domain (Kenniston et al. 2003).

Deletion of N-terminal sequences that form the ClpP channel has been shown to activate polypeptide cleavage, and crystal structures of mutants lacking 14 or 17 N-terminal residues reveal altered positions for some of the remaining pore/channel residues (Jennings et al. 2008; Bewley et al. 2009). Our results show that amino-acid substitutions in helix A (e.g., I19A) and in the channel (e.g. AQTSAGAA[15]) have similar effects to those caused by large N-terminal deletions in terms of allowing ClpP degradation of polypeptide substrates. These results suggest that relatively small perturbations in the structure of ClpP are sufficient to permit more efficient polypeptide degradation. Systems of this type, in which many different

mutations lead to similar gain-of-function phenotypes, generally occur via an increase in the population of an active conformation because a competing inactive conformation is destabilized. For ClpP, it seems likely that "restrictive" and "permissive" conformations of the channel are in dynamic equilibrium in the wild-type enzyme, with the permissive conformation being present in just a small fraction of enzymes. By this model, any mutation that destabilized the restrictive conformation would increase the population of enzymes with permissive channels.

In the simplest allosteric model, activating ClpP mutations and the binding of ClpX or ClpA might stabilize the same permissive ClpP conformation. In this case, however, activating mutations should enhance binding of the AAA+ ATPases to ClpP, because less binding energy would be required to drive the conformational change. This result is not observed. Indeed, our work and previous studies (Bewley et al. 2009) show that activating channel deletion and substitution mutations reduce ClpP affinity for ClpX and ClpA. Although it is formally possible that all of the mutations that activate polypeptide cleavage by ClpP also involve side chains that directly contact the AAA+ ATPases in the ClpXP or ClpAP complexes, this explanation seems unlikely. The observed results could also be explained if there were multiple "permissive" conformations of the ClpP channel and ClpX or ClpA binding stabilized different conformations than the activating mutations. High resolution structures of ClpP in complex with ClpX or ClpA will be needed to resolve this issue.

There is abundant evidence that ClpX and ClpA interact with ClpP channel residues. For example, both double-mutant cycle analysis and crosslinking support the existence of

contacts between the axial pore-2 loops of ClpX and channel residues of ClpP (Martin et al. 2007). We find that certain helix-A mutations (S21A; R22A) also reduce ClpX affinity. Although some of these helix-A effects could be indirectly caused by remodeling of channel residues, our results are most consistent with direct effects on ClpX binding. For example, the R22A mutation causes a larger defect in ClpX binding than ClpA binding, activates ClpP polypeptide cleavage only modestly, and does not alter the sensitivity of ClpP channel residues to tryptic cleavage. In the ClpP structure, helix A connects the pore and channel residues to residues that form the hydrophobic clefts, which serve as docking sites for the IGF/IGL motifs of ClpX and ClpA. It remains to be determined if these docking interactions transmit a signal via helix A that remodels the channel residues of ClpP or if other binding interactions are responsible for this activity. In this regard, interactions between the pore-2 loops of ClpX and ClpP are not required for activation of polypeptide cleavage (Martin et al. 2007).

Our results show that changes in the sequence of the ClpP channel prevent translocation of certain highly charged sequences during ATP-dependent degradation by ClpXP. Specifically, replacing Glu^{8}, Arg^{12}, Glu^{14}, and Arg^{15} in the wild-type ClpP channel with alanines resulted in a mutant, which in combination with ClpX, could degrade ssrA-tagged peptides with stretches of non-polar residues (FGFGFGFGFG) but could not degrade otherwise identical peptides containing runs of glutamic acids, lysines, or glcines. These findings suggest that the wild-type ClpP channel facilitates translocation of diverse sequences, including highly charged amino acids, during normal ClpXP degradation. It is unlikely that such an activity would be required if the channel dimensions were wide enough

to allow efficient solvation of the translocating polypeptide. Because the residues corresponding to Glu^{8}, Arg^{12}, Glu^{14}, and Arg^{15} in *E. coli* ClpP are highly conserved in orthologs, one function of these side chains may be to neutralize charges on translocating polypeptides. Thus, the channel of ClpP appears to play an active rather than passive role in energy dependent degradation.

MATERIALS AND METHODS

Proteins and peptides

The *E. coli* ClpP variants used in these studies had C-terminal His_6 tags (Kim et al. 2000), were generated by inverse PCR mutagenesis, and were expressed from multi-copy, IPTG-inducible pQE70 vectors, and purified as described by Kim *et al.* (2000). Mutant names refer to the altered amino-acid position(s) in the mature form of *E. coli* ClpP. Wild-type *E. coli* ClpX, $ClpX^{E185Q}$, and covalently linked wild-type ClpX trimers lacking the N-domain were expressed and purified as described (Kenniston et al. 2003; Hersch et al. 2005; Martin et al. 2005).

Succinyl-LY-AMC was purchased from Sigma. The Abz-KASPVSLGY^{NO2}D decapeptide (where Abz is the fluorophore 2-aminobenzoic acid and Y^{NO2} is the quencher 3-nitrotyrosine) was a gift from B. Cezairliyan (MIT). The 20-mer peptide, Abz-ASSHATRQLSGLKIHSNLY^{NO2}H, was a gift from Eyal Gur (MIT). SsrA-tagged peptides were gifts from Igor Levchenko and Sarah Barkow (MIT). Each peptide consisted of an N-terminal cleavage module (Abz-FAPHMALVPY^{NO2}), a guest region ($[YG]_5$, $[FG]_5$, $[E_{10}]$, $[R_{10}]$, $[K_{10}]$, $[Q_{10}]$, $[A_6]$, $[P_5]$ or $[G_4]$) and a C-terminal sequence (KKANDENYALAA)

containing the ssrA tag (Barkow et al. 2009). GFP-ssrA was purifed as described (Burton et al. 2001). *E. coli* SspB was a gift from S. Barkow (MIT).

Assays

Degradation assays were performed at 30 °C in PD buffer, which consists of 25 mM Hepes (pH 7.6), 100 mM KCl, 20 mM $MgCl_2$, 1 mM EDTA, and 10% glycerol, and were generally monitored by changes in fluorescence using a QM-2000-4SE spectrofluorimeter (Photon Technology International). When ClpX was present in assays, it was was preincubated with 1 mM nucleotide (ATP, ATPγS, or ADP) for at least 1 minute prior to addition of ClpP and substrate. Succinyl-LY-AMC cleavage assays (excitation 345 nm; emission 440 nm) contained 50 μM substrate and 0.1 μM $ClpP_{14}$ with or without 1 uM $ClpX_6$. Decapeptide cleavage assays (excitation 320 nm; emission 420 nM) contained 15 μM substrate and 0.3 μM $ClpP_{14}$ with or without 0.5 μM $ClpX_6$. Degradation of the 20-mer peptide (excitation 320 nm; emission 420 nM) was assayed using 15 μM substrate and 0.1 μM $ClpP_{14}$ with 1 mM ATPγS, plus or minus 0.5 μM $ClpX_6$. The assays described above used peptide substrate concentrations substantially below K_M for degradation by $ClpP_{14}$, as shown by linear changes in rate versus substrate concentration plots. Degradation of ssrA-tagged peptides (excitation 320 nm; emission 420 nm) was assayed using 8 μM substrate, 8 μM *E. coli* SspB, 4 μM $ClpP_{14}$, 0.15 μM $ClpX_6$, and an ATP regeneration mix (Kenniston et al. 2003). Control experiments lacking ClpX were performed to test background peptide degradation by free ClpP. Under these conditions, the substrate concentration is well above K_M. For GFP-ssrA degradation assays (excitation 467 nm; emission 511 nm), each reaction contained 5 μM substrate (K_M 1-2 μM), a covalently linked ClpX-ΔN trimer (0.1 μM

pseudo-hexamer equivalents) or $ClpA_6$ (50 nM), an ATP regeneration mix, and increasing concentrations of ClpP or ClpP mutants. For ClpX-independent degradation of the unfolded protein, carboxymethylated [^{35}S]-titin-I27-ssrA, 5 μM substrate was incubated with 1 μM $ClpP_{14}$ or I19A-$ClpP_{14}$ and degradation was monitored by the release of acid-soluble peptides (Kenniston et al. 2003).

Active-site modification of $ClpP_{14}$ (0.5 μM) with rhodamine-FP (200 μM; a gift of B. Cravatt, Scripps) was performed in PD buffer at 0° C with or without $ClpX_6$ (1 μM), ADP (5 μM), or ATPγS (5 μM). Reactions were quenched in 2% SDS, separated by SDS-PAGE on 12% gels, and fluorescently modified ClpP was quantified using a Typhoon fluorimager and ImageQuant software. Tryptic digests were performed in PD buffer at 30 °C using wild-type or mutant $ClpP_{14}$ (0.7 μM) and porcine pancreas trypsin (0.5 μM; Sigma). At different times, reactions were quenched by boiling in 2% SDS and 1 mM phenylmethanesulphonyl fluoride, and aliquots were subjected to 12% SDS-PAGE and then stained with Coomassie Blue.

REFERENCES

Barkow, S. R., Levchenko, I., Baker, T. A. and Sauer, R. T. (2009). "Polypeptide translocation by the AAA+ ClpXP protease machine." Chem Biol **16**(6): 605-12.

Bewley, M. C., Graziano, V., Griffin, K. and Flanagan, J. M. (2006). "The asymmetry in the mature amino-terminus of ClpP facilitates a local symmetry match in ClpAP and ClpXP complexes." J Struct Biol **153**(2): 113-28.

Bewley, M. C., Graziano, V., Griffin, K. and Flanagan, J. M. (2009). "Turned on for degradation: ATPase-independent degradation by ClpP." J Struct Biol **165**(2): 118-25.

Bogyo, M., McMaster, J. S., Gaczynska, M., Tortorella, D., Goldberg, A. L. and Ploegh, H. (1997). "Covalent modification of the active site threonine of proteasomal beta subunits and the Escherichia coli homolog HslV by a new class of inhibitors." Proc Natl Acad Sci U S A **94**(13): 6629-34.

Brotz-Oesterhelt, H., Beyer, D., Kroll, H. P., Endermann, R., Ladel, C., Schroeder, W., Hinzen, B., Raddatz, S., Paulsen, H., Henninger, K., Bandow, J. E., Sahl, H. G. and Labischinski, H. (2005). "Dysregulation of bacterial proteolytic machinery by a new class of antibiotics." Nat Med **11**(10): 1082-7.

Burton, R. E., Baker, T. A. and Sauer, R. T. (2003). "Energy-dependent degradation: Linkage between ClpX-catalyzed nucleotide hydrolysis and protein-substrate processing." Protein Sci **12**(5): 893-902.

Burton, R. E., Siddiqui, S. M., Kim, Y. I., Baker, T. A. and Sauer, R. T. (2001). "Effects of protein stability and structure on substrate processing by the ClpXP unfolding and degradation machine." Embo J **20**(12): 3092-100.

Farrell, C. M., Grossman, A. D. and Sauer, R. T. (2005). "Cytoplasmic degradation of ssrA-tagged proteins." Mol Microbiol **57**(6): 1750-61.

Flynn, J. M., Levchenko, I., Seidel, M., Wickner, S. H., Sauer, R. T. and Baker, T. A. (2001). "Overlapping recognition determinants within the ssrA degradation tag allow modulation of proteolysis." Proc Natl Acad Sci U S A **98**(19): 10584-9.

Gottesman, S., Clark, W. P., de Crecy-Lagard, V. and Maurizi, M. R. (1993). "ClpX, an alternative subunit for the ATP-dependent Clp protease of Escherichia coli. Sequence and in vivo activities." J Biol Chem **268**(30): 22618-26.

Gottesman, S., Clark, W. P. and Maurizi, M. R. (1990). "The ATP-dependent Clp protease of Escherichia coli. Sequence of clpA and identification of a Clp-specific substrate." J Biol Chem **265**(14): 7886-93.

Gribun, A., Kimber, M. S., Ching, R., Sprangers, R., Fiebig, K. M. and Houry, W. A. (2005). "The ClpP double ring tetradecameric protease exhibits plastic ring-ring interactions, and the N termini of its subunits form flexible loops that are essential for ClpXP and ClpAP complex formation." J Biol Chem **280**(16): 16185-96.

Grimaud, R., Kessel, M., Beuron, F., Steven, A. C. and Maurizi, M. R. (1998). "Enzymatic and structural similarities between the Escherichia coli ATP-dependent proteases, ClpXP and ClpAP." J Biol Chem **273**(20): 12476-81.

Groll, M., Bajorek, M., Kohler, A., Moroder, L., Rubin, D. M., Huber, R., Glickman, M. H. and Finley, D. (2000). "A gated channel into the proteasome core particle." Nat Struct Biol **7**(11): 1062-7.

Groll, M. and Huber, R. (2003). "Substrate access and processing by the 20S proteasome core particle." Int J Biochem Cell Biol **35**(5): 606-16.

Haynes, C. M., Petrova, K., Benedetti, C., Yang, Y. and Ron, D. (2007). "ClpP mediates activation of a mitochondrial unfolded protein response in C. elegans." Dev Cell **13**(4): 467-80.

Hengge, R. and Turgay, K. (2009). "Editorial overview: Proteolysis in prokaryotes - from molecular machines to a systems perspective." Res Microbiol.

Hersch, G. L., Burton, R. E., Bolon, D. N., Baker, T. A. and Sauer, R. T. (2005). "Asymmetric interactions of ATP with the AAA+ ClpX6 unfoldase: allosteric control of a protein machine." Cell **121**(7): 1017-27.

Ingvarsson, H., Mate, M. J., Hogbom, M., Portnoi, D., Benaroudj, N., Alzari, P. M., Ortiz-Lombardia, M. and Unge, T. (2007). "Insights into the inter-ring plasticity of caseinolytic proteases from the X-ray structure of Mycobacterium tuberculosis ClpP1." Acta Crystallogr D Biol Crystallogr **63**(Pt 2): 249-59.

Ivanova, S., Repnik, U., Bojic, L., Petelin, A., Turk, V. and Turk, B. (2008). "Lysosomes in apoptosis." Methods Enzymol **442**: 183-99.

Jennings, L. D., Bohon, J., Chance, M. R. and Licht, S. (2008). "The ClpP N-terminus coordinates substrate access with protease active site reactivity." Biochemistry **47**(42): 11031-40.

Joshi, S. A., Hersch, G. L., Baker, T. A. and Sauer, R. T. (2004). "Communication between ClpX and ClpP during substrate processing and degradation." Nat Struct Mol Biol **11**(5): 404-11.

Kang, S. G., Maurizi, M. R., Thompson, M., Mueser, T. and Ahvazi, B. (2004). "Crystallography and mutagenesis point to an essential role for the N-terminus of human mitochondrial ClpP." J Struct Biol **148**(3): 338-52.

Kenniston, J. A., Baker, T. A., Fernandez, J. M. and Sauer, R. T. (2003). "Linkage between ATP consumption and mechanical unfolding during the protein processing reactions of an AAA+ degradation machine." Cell **114**(4): 511-20.

Kessel, M., Maurizi, M. R., Kim, B., Kocsis, E., Trus, B. L., Singh, S. K. and Steven, A. C. (1995). "Homology in structural organization between E. coli ClpAP protease and the eukaryotic 26 S proteasome." J Mol Biol **250**(5): 587-94.

Kim, D. Y. and Kim, K. K. (2008). "The structural basis for the activation and peptide recognition of bacterial ClpP." J Mol Biol **379**(4): 760-71.

Kim, Y. I., Burton, R. E., Burton, B. M., Sauer, R. T. and Baker, T. A. (2000). "Dynamics of substrate denaturation and translocation by the ClpXP degradation machine." Mol Cell **5**(4): 639-48.

Kim, Y. I., Levchenko, I., Fraczkowska, K., Woodruff, R. V., Sauer, R. T. and Baker, T. A. (2001). "Molecular determinants of complex formation between Clp/Hsp100 ATPases and the ClpP peptidase." Nat Struct Biol **8**(3): 230-3.

Kirstein, J., Hoffmann, A., Lilie, H., Schmidt, R., Rubsamen-Waigmann, H., Brotz-Oesterhelt, H., Mogk, A. and Turgay, K. (2009). "The antibiotic ADEP reprogrammes ClpP, switching it from a regulated to an uncontrolled protease." EMBO Molecular Medicine **1**(1): 37-49.

Kwon, A. R., Kessler, B. M., Overkleeft, H. S. and McKay, D. B. (2003). "Structure and reactivity of an asymmetric complex between HslV and I-domain deleted HslU, a prokaryotic homolog of the eukaryotic proteasome." J Mol Biol **330**(2): 185-95.

Levchenko, I., Grant, R. A., Wah, D. A., Sauer, R. T. and Baker, T. A. (2003). "Structure of a delivery protein for an AAA+ protease in complex with a peptide degradation tag." Mol Cell **12**(2): 365-72.

Liu, Y., Patricelli, M. P. and Cravatt, B. F. (1999). "Activity-based protein profiling: the serine hydrolases." Proc Natl Acad Sci U S A **96**(26): 14694-9.

Lopez-Otin, C. and Overall, C. M. (2002). "Protease degradomics: a new challenge for proteomics." Nat Rev Mol Cell Biol **3**(7): 509-19.

Martin, A., Baker, T. A. and Sauer, R. T. (2005). "Rebuilt AAA + motors reveal operating principles for ATP-fuelled machines." Nature **437**(7062): 1115-20.

Martin, A., Baker, T. A. and Sauer, R. T. (2007). "Distinct static and dynamic interactions control ATPase-peptidase communication in a AAA+ protease." Mol Cell **27**(1): 41-52.

Martin, A., Baker, T. A. and Sauer, R. T. (2008a). "Diverse pore loops of the AAA+ ClpX machine mediate unassisted and adaptor-dependent recognition of ssrA-tagged substrates." Mol Cell **29**(4): 441-50.

Martin, A., Baker, T. A. and Sauer, R. T. (2008b). "Protein unfolding by a AAA+ protease is dependent on ATP-hydrolysis rates and substrate energy landscapes." Nat Struct Mol Biol **15**(2): 139-45.

Maurizi, M. R., Clark, W. P., Katayama, Y., Rudikoff, S., Pumphrey, J., Bowers, B. and Gottesman, S. (1990). "Sequence and structure of Clp P, the proteolytic component of the ATP-dependent Clp protease of Escherichia coli." J Biol Chem **265**(21): 12536-45.

Maurizi, M. R., Singh, S. K., Thompson, M. W., Kessel, M. and Ginsburg, A. (1998). "Molecular properties of ClpAP protease of Escherichia coli: ATP-dependent association of ClpA and clpP." Biochemistry **37**(21): 7778-86.

Ortega, J., Singh, S. K., Ishikawa, T., Maurizi, M. R. and Steven, A. C. (2000). "Visualization of substrate binding and translocation by the ATP-dependent protease, ClpXP." Mol Cell **6**(6): 1515-21.

Pickart, C. M. and Cohen, R. E. (2004). "Proteasomes and their kin: proteases in the machine age." Nat Rev Mol Cell Biol **5**(3): 177-87.

Powers, E. T., Morimoto, R. I., Dillin, A., Kelly, J. W. and Balch, W. E. (2009). "Biological and chemical approaches to diseases of proteostasis deficiency." Annu Rev Biochem **78**: 959-91.

Rabl, J., Smith, D. M., Yu, Y., Chang, S. C., Goldberg, A. L. and Cheng, Y. (2008). "Mechanism of gate opening in the 20S proteasome by the proteasomal ATPases." Mol Cell **30**(3): 360-8.

Sauer, R. T., Bolon, D. N., Burton, B. M., Burton, R. E., Flynn, J. M., Grant, R. A., Hersch, G. L., Joshi, S. A., Kenniston, J. A., Levchenko, I., Neher, S. B., Oakes, E. S., Siddiqui, S. M., Wah, D. A. and Baker, T. A. (2004). "Sculpting the proteome with AAA(+) proteases and disassembly machines." Cell **119**(1): 9-18.

Sharma, S., Hoskins, J. R. and Wickner, S. (2005). "Binding and degradation of heterodimeric substrates by ClpAP and ClpXP." J Biol Chem **280**(7): 5449-55.

Siddiqui, S. M., Sauer, R. T. and Baker, T. A. (2004). "Role of the processing pore of the ClpX AAA+ ATPase in the recognition and engagement of specific protein substrates." Genes Dev **18**(4): 369-74.

Singh, S. K., Grimaud, R., Hoskins, J. R., Wickner, S. and Maurizi, M. R. (2000). "Unfolding and internalization of proteins by the ATP-dependent proteases ClpXP and ClpAP." Proc Natl Acad Sci U S A **97**(16): 8898-903.

Singh, S. K., Rozycki, J., Ortega, J., Ishikawa, T., Lo, J., Steven, A. C. and Maurizi, M. R. (2001). "Functional domains of the ClpA and ClpX molecular chaperones identified by limited proteolysis and deletion analysis." J Biol Chem **276**(31): 29420-9.

Smith, D. M., Chang, S. C., Park, S., Finley, D., Cheng, Y. and Goldberg, A. L. (2007). "Docking of the proteasomal ATPases' carboxyl termini in the 20S proteasome's alpha ring opens the gate for substrate entry." Mol Cell **27**(5): 731-44.

Smith, D. M., Kafri, G., Cheng, Y., Ng, D., Walz, T. and Goldberg, A. L. (2005). "ATP binding to PAN or the 26S ATPases causes association with the 20S proteasome, gate opening, and translocation of unfolded proteins." Mol Cell **20**(5): 687-98.

Song, H. K. and Eck, M. J. (2003). "Structural basis of degradation signal recognition by SspB, a specificity-enhancing factor for the ClpXP proteolytic machine." Mol Cell **12**(1): 75-86.

Sousa, M. C., Kessler, B. M., Overkleeft, H. S. and McKay, D. B. (2002). "Crystal structure of HslUV complexed with a vinyl sulfone inhibitor: corroboration of a proposed mechanism of allosteric activation of HslV by HslU." J Mol Biol **318**(3): 779-85.

Sousa, M. C., Trame, C. B., Tsuruta, H., Wilbanks, S. M., Reddy, V. S. and McKay, D. B. (2000). "Crystal and solution structures of an HslUV protease-chaperone complex." Cell **103**(4): 633-43.

Striebel, F., Kress, W. and Weber-Ban, E. (2009). "Controlled destruction: AAA+ ATPases in protein degradation from bacteria to eukaryotes." Curr Opin Struct Biol **19**(2): 209-17.

Szyk, A. and Maurizi, M. R. (2006). "Crystal structure at 1.9A of E. coli ClpP with a peptide covalently bound at the active site." J Struct Biol **156**(1): 165-74.

Thompson, M. W. and Maurizi, M. R. (1994a). "Activity and specificity of Escherichia coli ClpAP protease in cleaving model peptide substrates." J Biol Chem **269**(27): 18201-8.

Thompson, M. W., Singh, S. K. and Maurizi, M. R. (1994b). "Processive degradation of proteins by the ATP-dependent Clp protease from Escherichia coli. Requirement for the multiple array of active sites in ClpP but not ATP hydrolysis." J Biol Chem **269**(27): 18209-15.

Turk, B. (2006). "Targeting proteases: successes, failures and future prospects." Nat Rev Drug Discov **5**(9): 785-99.

Wang, J., Hartling, J. A. and Flanagan, J. M. (1997). "The structure of ClpP at 2.3 A resolution suggests a model for ATP-dependent proteolysis." Cell **91**(4): 447-56.

Weichart, D., Querfurth, N., Dreger, M. and Hengge-Aronis, R. (2003). "Global role for ClpP-containing proteases in stationary-phase adaptation of Escherichia coli." J Bacteriol **185**(1): 115-25.

Yoo, S. J., Seol, J. H., Shin, D. H., Rohrwild, M., Kang, M. S., Tanaka, K., Goldberg, A. L. and Chung, C. H. (1996). "Purification and characterization of the heat shock proteins HslV

and HslU that form a new ATP-dependent protease in Escherichia coli." J Biol Chem **271**(24): 14035-40.

Yu, A. Y. and Houry, W. A. (2007). "ClpP: a distinctive family of cylindrical energy-dependent serine proteases." FEBS Lett **581**(19): 3749-57.

CHAPTER THREE:

Dominant Negative Selection for ClpP Mutants that Prevent Degradation of an ssrA-tagged Substrate by ClpXP

INTRODUCTION

The proteolytic complex formed by ClpX and ClpP represents an important example in which two large protein machines must work together to ensure the selective degradation of target proteins. To probe determinants in ClpP that are important for its collaborative function with ClpX, I carried out a genetic selection for dominant negative ClpP mutations that prevented ClpXP degradation of an ssrA-tagged protein substrate.

ClpP serves as a good model system in which to try to understand the structural and functional features that are required for the activity of a compartmental peptidase. The crystal structure of this homo-tetradecameric serine protease has been solved by itself (Wang et al. 1997; Bewley et al. 2006), in an inhibitor-bound state (Szyk et al. 2006), and in a peptide-bound state (Kim et al. 2008). Numerous studies of peptide and protein degradation by ClpP and ClpXP have also been reported (Thompson et al. 1994; Gottesman et al. 1998; Sauer et al. 2004). Although there are no high-resolution structures of the ClpXP complex, recent structures of the AAA+ ClpX hexamer in nucleotide-free and nucleotide-bound conformations provide some insight into the molecular motions that may be involved in substrate unfolding and translocation (Glynn et al. 2009).

Biochemical studies suggest that communication between ClpX and ClpP occurs bidirectionally. For human ClpXP, assembly of the complete ClpP tetradecamer from two heptameric rings requires the presence of ClpX (Kang et al. 2005). Thus, binding of a ClpX hexamer to one face of a heptameric ClpP ring appears to alter ClpP•ClpP interactions on the opposing face of the ring. For *E. coli* ClpXP, formation of the complex results in a

slowing of the rate of ATP hydrolysis by ClpX (Joshi et al. 2004). In addition, ClpXP complex formation increases the rate at which model peptides are cleaved by ClpP. DFP-modified ClpP, which is thought to mimic the acyl intermediate in peptide-bond hydrolysis, binds ClpX more tightly than unmodified ClpP (Joshi et al. 2004). Furthermore, ClpXP affinity changes in a substrate-dependent fashion that appears to be linked to the rate at which ClpX hydrolyzes ATP during protein unfolding and translocation. Thus, communication between ClpX and ClpP begins upon complex formation, occurs in a dynamic and substrate-specific fashion, and is mediated by a symmetry mismatched interface.

Crystal structures of ClpP in which the active sites are modified by a chloromethylketone (CMK) or with peptides bound in the active sites show subtle structural variations when compared to the free enzyme (Szyk et al. 2006; Kim et al. 2008). The most striking difference between these structures and free ClpP is a narrowing of the diameter of the degradation chamber by ~1.5 Å. These results suggest that substrate binding stabilizes the active sites of ClpP in a conformation competent to perform peptide-hydrolysis chemistry (Szyk et al. 2006; Kim et al. 2008). Experiments showing positive cooperativity in the degradation of small peptides by ClpP support this model (Thompson et al. 1994).

The CMK-modified and peptide-bound structures of ClpP provide additional insights concerning the selectivity and processivity of polypeptide degradation. The P1 binding pocket for the S1 side chain of the substrate is larger in ClpP than in many other serine proteases (e.g., chymotrypsin and subtilisin), explaining why ClpP can accommodate

residues ranging from glycine to tyrosine at this position. Short bound peptides form the third strand of an anti-parallel β sheet with strands β2′ and β6 of ClpP (Kim et al. 2008). Extending these peptides by molecular modeling suggests that longer polypeptides might wrap around the interior of the degradation lumen with 6-8 extended amino acids spanning the ~25 Å spacing between active sites. This "molecular ruler" binding mode in combination with broad cleavage specificity may explain why ClpP cleavage of polypeptides with completely different sequences generates nearly identical distributions of products in terms of peptide length (Choi et al. 2005).

Almost all mutagenesis of ClpP reported to date has been based on the crystal structure (Kang et al. 2004; Gribun et al. 2005; Bewley et al. 2006; Bewley et al. 2009). I wished to probe the sequence determinants of ClpP and ClpXP activity in a less-biased fashion by selecting for ClpP variants whose overexpression, in the presence of wild-type ClpX and ClpP, could prevent efficient degradation of an ssrA-tagged substrate *in vivo*. Dominant-negative mutants of this type should be capable of folding and forming tetradecamers and thus any defects should affect other aspects of ClpP function required for ClpXP proteolysis.

RESULTS AND DISCUSSION

Selection Strategy

To identify residues of ClpP required for function with ClpX, I designed a genetic system in which a protein with a selectable function would only be present at high steady-state levels *in vivo* if it was not degraded by ClpXP. For the selectable protein, I chose chloramphenicol acetyl transferase (CAT), which mediates resistance to the antibiotic chloramphenicol and

assembles into a trimeric enzyme with unstructured C-terminal tails (Leslie 1990). To make this enzyme a substrate for ClpXP degradation, I fused its coding sequence to a sequence encoding a C-terminal ssrA to generate a CATssrA gene.

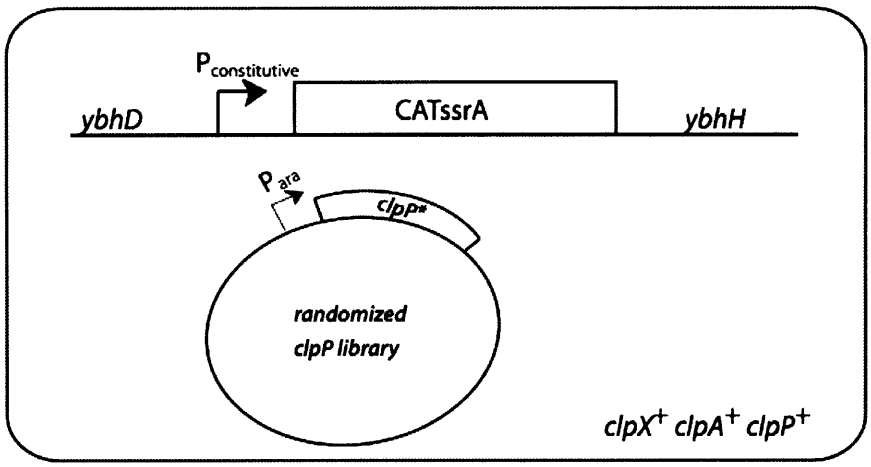

Figure 1. Strategy used to select for dominant-negative ClpP mutants. CATssrA is constitutively expressed but degraded by ClpXP in an otherwise wild-type *E. coli* strain. Following introduction of a randomized *clpP** library under arabinose-promoter control, dominant-negative ClpP* variants should inactivate ClpXP and prevent CATssrA degradation, resulting in chloramphenicol resistance.

The gene encoding CATssrA was placed under transcriptional control of a synthetic constitutive promoter (Jensen et al. 1998) and was integrated between two non-essential genes, *ybhD* and *ybhH*, in the *E. coli* chromosome. When this CATssrA strain also harbored wild-type copies of the *clpX, clpA,* and *clpP* genes, cells were killed by addition of chloramphenicol (Figure 2A). When the genes for either *clpX* or *clpP* were deleted, however, the CATssrA strain became resistant to chloramphenicol (Figure 2A). These results suggest that CATssrA is synthesized efficiently but is degraded in a *clpX*- and *clpP*-dependent fashion (Figure 2A). Importantly, deletion of *clpA* did not confer

chloramphenicol resistance to the CATssrA strain. Although ClpAP degrades ssrA-tagged proteins *in vitro*, the presence of the SspB and ClpS adaptor proteins appear to repress ClpAP degradation of such substrates *in vivo* (Gottesman et al. 1998; Farrell et al. 2005; Lies et al. 2008). Taken together, these results are consisent with a model in which ClpXP is responsible for the *in vivo* degradation of CATssrA.

To confirm that CATssrA was degraded in a ClpP-dependent reaction, I monitored CATssrA protein levels in a Δ*clpP* strain in which a His_6-tagged ClpP variant could be expressed in an arabinose-dependent fashion (Figure 2B). In a mock induction without arabinose (Figure 2B, left panel), no accumulation of ClpP-His_6 was detected as determined by a Western blot probed with an α-His_6 antibody, but CATssrA (which has an internal His_6 tag) accumulated. Following addition of arabinose (Figure 2B, right panel), CATssrA levels were undetectable 20 minutes after induction. Thus, CATssrA appears to be efficiently degraded in a reaction mediated by ClpP.

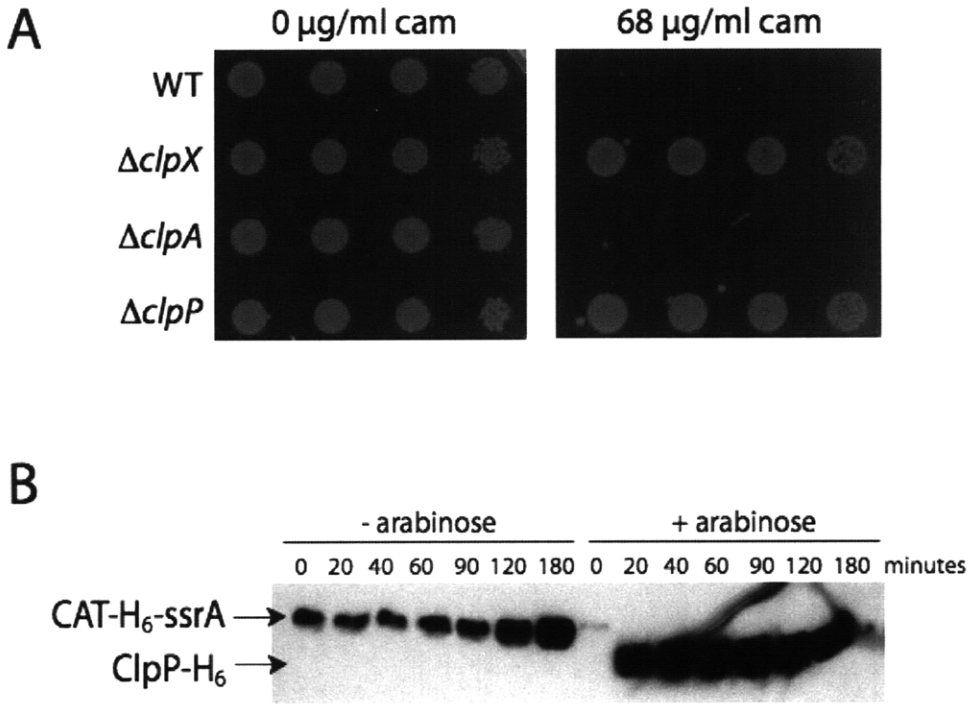

Figure 2. (A) CATssrA strains in which *clpX* or *clpP* were deleted are resistant to chloramphenicol. Deletion of *clpA* did not result in chloramphenicol resistance. Spot tests are 10-fold serial dilutions, with the most concentrated samples on the left. (B) In a *clpP*-deletion strain, Western blots using anti-His_6 antibodies show that CATssrA (internal His_6 tag) accumulates when ClpP-His_6 is absent but does not accumulate when ClpP-His_6 is expressed from an arabinose-inducible promoter. The numbers above each lane are times, in minutes, following a mock induction or the addition of 0.2% arabinose.

Selection Results

A randomized library of *clpP* mutants on a low-copy plasmid vector was generated by error-prone PCR. The total libray size was 1.7×10^6, and sequencing of selected variants revealed an average of ~1 mutation per *clpP* gene. Following addition of 0.2% arabinose, Western blots showed that plasmid-expressed ClpP levels were 6-10 times higher than levels from the chromosomal gene (data not shown). I selected for dominant-negative ClpP* mutants by growth in the presence of chloramphenicol. To confirm that the drug-resistant phenotypes

arose from mutant ClpPs, plasmids were purified after the initial selection, retransformed into fresh selection strains, and reselected on LB-chloramphenicol plates. Each clone was also tested to ensure that the chloramphenicol-resistance phenotype was arabinose-dependent.

My selection identified 52 unique *clpP** variants. Of these variants, 37 contained single mutations and 15 contained multiple mutations. Table 1 lists the single mutations and indicates their anticipated contribution to ClpP function. The majority of single mutations mapped to one of five ClpP areas: the N-terminal channel and pore, α-helix A, the hydrophobic clefts, the ring-ring interface, and the catalytic triad and substrate-binding region. Table 2 lists the amino-acid substitutions in *clpP** variants with multiple mutations.

Residue	Functional Consequence	Conservation	Consensus (%)
P4R	N-terminal loop	10	Pro (94)
V6G	N-terminal loop	7	Val (86)
V6A	N-terminal loop	7	Val (86)
I19T	Helix A	6	Ile (87)
S21Y	Helix A	10	Ser (86)
S21P	Helix A	10	Ser (86)
V28P	Hydrophobic Cleft	9	Ile (77)
M39K	unknown	5	Met (39)
Q46R	unknown	10	Gln (95)
L48P	Hydrophobic Cleft	10	Leu (93)
E51G	unknown	4	Glu (71)
S75F	unknown	8	Ala (73)
S75P	unknown	8	Ala (73)
T79I	unknown	10	Thr (95)
S97P	Active Site	10	Ser (99)
L114P	Hydrophobic Cleft	6	Leu (95)
L114R	Hydrophobic Cleft	6	Leu (95)
M120T	unknown	9	Met (84)
Q123R*	Active Site	11	Gln (100)
P124S*	Active Site	10	Pro (97)
G126S	Ring-Ring Interface	10	Gly (88)
Q129R	Ring-Ring Interface	0	Gln (56)
G130D	Ring-Ring Interface	3	Gly (92)
D134G	Ring-Ring Interface	9	Asp (88)
D134N	Ring-Ring Interface	9	Asp (88)
I135T	Ring-Ring Interface	9	Ile (83)
I137T	Ring-Ring Interface	5	Ile (90)
E141K	Active Site	4	Glu (70)
E141G	Active Site	4	Glu (70)
L143P	Active Site	5	Leu (78)
K144E	Ring-Ring Interface	4	Lys (40)
L152P	unknown	8	Ile (53)
D167G	unknown	5	Asp (84)
E169K	Ring-Ring Interface	9	Asp (52)
D171G	Active Site	10	Asp (98)
F173L	unknown	7	Phe (70)
F173V	unknown	7	Phe (70)

Table 1. Single dominant negative mutations in ClpP. Conservation scores, consensus identity, and % were derived from a multiple sequence alignment of bacterial ClpP proteins (Waterhouse et al. 2009). Colors indicated likely functional significance. Mutants denoted by an asterisk are involved in both ring-ring interactions and the active sites.

	Mutations
1	P4Q, Q129R
2	E8K, I63V, T79A, E181G
3	E14G, I19T, Q123K
4	K25M, I142V
5	M39L, E51G
6	L42R, S160L
7	I63V, D167N
8	F82L, T133S
9	Q94R, N150D
10	K108E, I137S, K144R
11	Y128C, L152P
12	I135T, V185A, T190A
13	E136G, L203P
14	I137N, T157A
15	I188G, K144E

Table 2. Non-single site mutants of ClpP identified in the dominant negative selection.

N-terminal Mutations

As discussed in chapter 2, crystallographic and mutagenesis studies have suggested important roles for N-terminal ClpP residues in forming the channel and pore into the degradation chamber and in binding ClpX and/or ClpA (Kang et al. 2004; Bewley et al. 2006; Bewley et al. 2009). I recovered the P4R, V6A, and V6G substitutions in my selection for dominant-negative mutations (Table 1). In the ClpP structure, Pro4 and Val6 form part of the stem region of the channel pore and pack together in a conserved hydrophobic cluster with Ile19, Leu24, and Phe49. Bewley *et al.* (2006) previously showed that the P4A and V6A mutations caused aberrant processing of the ClpP propeptide, eliminated detectable cleavage of casein by ClpP alone, and showed no detectable interactions with ClpX. Nevertheless, the purified P4A and V6A mutants showed normal

cleavage of a model dipeptide, and the V6A crystal structure revealed a 14-mer with well-formed active sites but with rearrangements in the only portion of the channel visible in electron-density maps (residues 15-17; Bewley et al. 2006).

In principle, dominant negative ClpP mutants could act in two ways: (*i*) 14-mers formed just by mutant subunits could bind ClpX in an inactive complex and thus prevent formation of wild-type ClpXP complexes; or (*ii*) wild-type and mutant subunits could assemble into mixed 14-mers that were unable to bind ClpX or formed inactive complexes with ClpX. Because a 14-mer formed just with P4A or V6A subunits failed to bind ClpX (Bewley et al. 2006), it seems likely that the dominant-negative phenotype of the P4R, V6A, and V6G mutants results from formation of mixed multimers with wild-type ClpP subunits.

Helix-A Mutations

In Chapter 2, I showed that mutations in helix A affected gating through the axial pore and ClpX binding. I isolated three dominant-negative mutations (I19T, S21Y, and S21P) in helix A (Table 1). The purified I19T mutant had biochemical properties similar to the I19A and I19V mutants characterized in Chapter 2. Specifically, I19T ClpP had no defects processing its propeptide fragment (as confirmed by mass spectrometry; not shown) and had trypsin-accessible N-termini (Figure 2A). In addition, I19T ClpP showed ~15-fold faster cleavage of a decapeptide (Figure 2B) than wild-type ClpP and also independently degraded carboxymethylated titin, an unfolded protein substrate (Figure 2C). I19T ClpP also showed defects in ClpX interactions, as this mutant failed to alter the ATPase rate of ClpX (Figure 2D). I did not purify the S21Y or S21P variants but suspect that these mutants (like S21A)

would also show defects in gating and in ClpX binding. Similar to mutations in the N-terminal stem loop, it seems likely that the dominant-negative phenotypes of the helix-A mutations are caused by mixed multimer formation with wild-type ClpP.

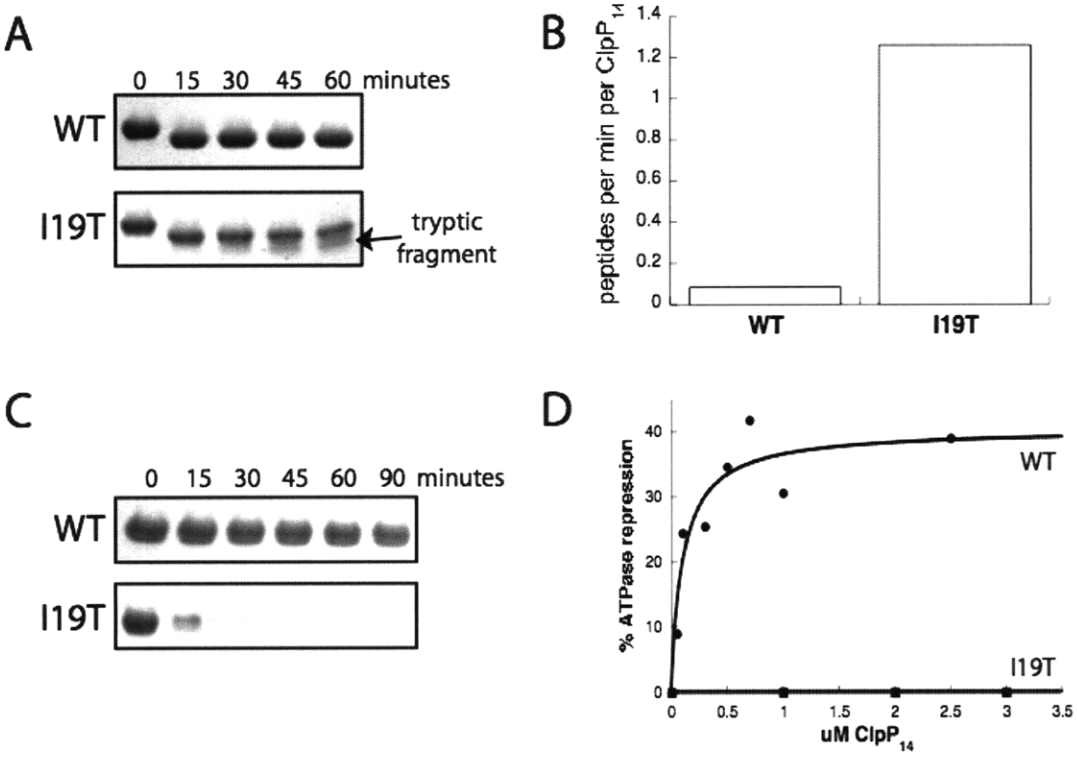

Figure 3. Comparative activities of wild type and I19T ClpP. (A) Limited tryptic digests, (B) decapeptide cleavage activity, (C) degradation carboxymethylated λO-I27, and (D) ClpX ATPase response to ClpP.

Hydrophobic-cleft Mutations

The IGF loops of ClpX are thought to dock into hydrophobic clefts on the periphery of the ClpP ring (Kim et al. 2001). Figure 4A shows a surface representation of a ClpP ring with cleft residues colored in purple and blue. Four single dominant-negative mutations affected residues in or near the cleft (V28P, L48P, L114P, L114R; Table 1). Another cleft mutation

(F82L; Table 2) was recovered with one additional mutation (T133S), which is likely to be silent based on phylogenetic comparisons.

I purified the V28P mutant from a *clpP*-deletion strain and found that it was properly processed as determined by mass spectrometry (data not shown). Thus, this mutant must be active in autoprocessing of the 14-residue propeptide. In addition, the purified V28P enzyme degraded a decapeptide at approximately 60% of the wild-type ClpP rate (Figure 4B), showing that it is catalytically active. These assays indicate the V28P mutant is properly folded. I detected no interaction between V28P ClpP and ClpX in pull-down assays (not shown) and observed a very weak interaction in ClpX- and nucleotide-dependent stimulation of decapeptide cleavage (Figure 4C). Moreover, I did not detect any degradation of GFP-ssrA when ClpX was combined with the V28P mutant (data not shown). To test for potential degradation *in vivo*, I monitored CATssrA protein levels in a *clpX*$^+$ Δ*clpP* strain transformed with a plasmid encoding V28P ClpP. As shown in Figure 4D, roughly comparable amounts of CATssrA accumulated in the absence of V28P ClpP and when expression of this mutant was induced by addition of arabinose. These combined results indicate that the major effect of the V28P mutation is a substantial decrease in its affinity for ClpX.

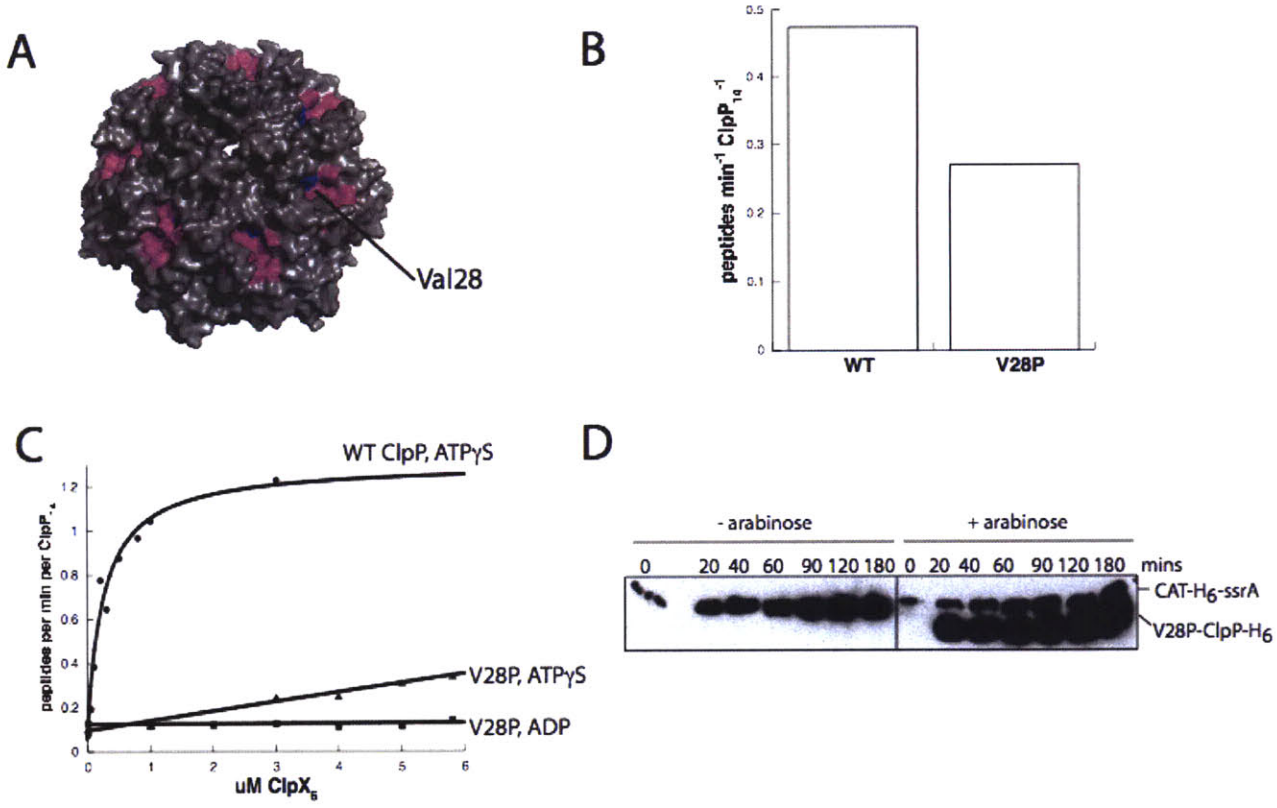

Figure 4. (A) Location of Val28 (blue) on a surface depiction of a ClpP ring (PDB 1YG6). Residues L48, Y60, Y62, F82, I90, M92, F112, L114, and L189 of the hydrophobic cleft are colored purple. (B) Activity of wild-type ClpP and the V28P mutant in cleavage of the Abz-KASPVSLGY^{NO2}D decapeptide. (C) Activation of V28P decapeptide cleavage by ClpX occurs with ATPγS (triangles) but not ADP (squares). ClpX stimulation of wild-type ClpP (circles) was performed with ATPγS. (C) Western blots show that CATssrA accumulates to roughly similar levels *in vivo* in the absence of V28P ClpP and following arabinose-mediated overexpression of this mutant (for comparison, see Figure 2B).

Only one cleft mutation (F112A) has previously been characterized biochemically. Bewley et al. (2006) reported that this mutation prevented interactions with ClpX, but I found that the F112A mutant behaved similarly to wild type ClpP in ClpX-interaction assays (Appendix B). Nevertheless, the biochemical properties of the V28P mutant and the recovery of several different dominant-negative cleft mutations suggest that these amino-

acid substitutions inactivate wild-type ClpP by formation of mixed oligomers, which bind ClpX very poorly.

Ring-ring Interface Mutations

Nine of the single dominant-negative mutants (G126S, Q129R, G130D, D134G, D134N, I135T, I137T, K144E, and E169K; Table 1) altered residues in or near the interface between the two heptameric rings of ClpP. I attempted to purify the D134G mutant but it had very low solubility and I did not obtain pure protein. I suspect that the mutant subunits in this class interact with wild-type subunits to form single heptameric rings, which cannot then assemble into stable tetradecamers. Single rings of human ClpP are poorly active in peptide cleavage and require the presence of ClpX to form stable tetradecamers (Kang et al. 2005). Thus, some ClpX binding energy must be used to drive conformational changes that allow stable ring-ring interactions. If mixed heptamers containing wild-type and mutant subunits of *E. coli* ClpP cannot form tetradecamers, then ClpX may be unable to bind or $ClpX_6ClpP_7$ complexes may be proteolytically inactive. If $ClpX_6ClpP_7$ complexes assembled and had unregulated proteolytic activity, I would have expected mutants in this class to display expression-linked growth defects but none were observed.

Active-site Mutations

Seven of the dominant negative mutations (S97P, Q123R, P124S, E141K, E141G, L143P, and D171G; Table 1) altered residues in the catalytic triad or in the substrate-binding pocket. Previous studies have shown that the S97A ClpP mutant, which removes the active-site serine, binds ClpX and can trap intact substrates in its chamber (Flynn et al. 2003; Joshi et

al. 2004). I argued above that the dominant-negative phenotypes of other classes of mutants must arise because of formation of mixed multimers with wild-type subunits. Thus, it seems unlikely that the active-site mutants are dominant because they form homo-tetradecamers that compete with wild-type tetradecamers for ClpX binding. However, formation of 14-mers containing a mixture of wild-type subunits and subunits with active-site mutations would still have some wild-type active sites. Why then would these enzymes be inactive? One possibility is that these mutations cause subtle changes in ClpP folding that are propagated to and inactivate the wild-type active sites. Another possibility is that mixed tetradecamers in combination with ClpX do cleave substrates but at a rate that is too slow to remove CATssrA efficiently from cells. In support of this model, Thompson et al. (1994) showed that ClpP enzymes in which most active sites were modified with DFP cleaved substrates more slowly and released higher molecular weight products than the wild-type enzyme. Nevertheless, further studies will be needed to clarify the mechanism by which active-site mutations result in a dominant-negative phenotype.

Unclassified Mutations

Eleven mutations (M39K, Q46R, E51G, S75F, S75P, T79I, M120T, L152P, D167G, F173L, and F173V; Table 1) could not be easily classified and affected side chains that were generally buried within individual ClpP subunits. In mixed multimers with wild-type subunits, these mutant subunits may cause changes in folding that inactivate the good subunits or act in a fashion like that suggested above for the active-site mutants. Attempts to purify S75P ClpP yielded low concentrations of soluble protein, supporting the hypothesis

that, at least this mutant, simultaneously was defective in ClpP folding and inactivated wild type ClpP.

Future Studies

The selection described in this chapter identified numerous single substitution mutations in ClpP that inhibit the ability of wild-type ClpP to collaborate with ClpX in degradation of an ssrA-tagged substrate. Based on biochemical characterization of a few mutants and mapping of the positions of other mutations onto the wild-type structure, plausible mechanisms of inactivation exist for mutations that affect the N-terminal channel, helix A, and the hydrophobic cleft. Namely, mixed tetradecamers of these mutants and wild-type ClpP would be expected to bind poorly to ClpX. For mutations that affect residues in or near the ring-ring interface of ClpP, it also seems reasonable that the mutant subunits would sequester wild-type subunits into inactive heptameric rings. Nevertheless, it will be important to test this model directly by characterizing the oligomeric state of interface mutants. It is also possible, for example, that mutants in this class might form tetradecamers but be impaired in other functions such as peptide egress, as some studies suggest that cleavage products exit the ClpP degradation chamber through transient openings in the interface region (Gribun et al. 2005).

It is less clear how mutations that affect active-site or peptide-binding residues in ClpP give rise to the observed dominant negative phenotypes. One possibility is that mixed multimers of these mutant subunits with wild-type subunits simply have a level of activity that is too low to effectively remove CATssrA from cells. If this model is correct, then cells

expressing these mutants may have higher levels of CATssA, which could be detected by Western blots or by improved growth in the presence of low concentrations of chloramphenicol. Alternatively, allowing wild-type and an excess of mutant ClpP subunits to exchange *in vitro* could reveal if mixed multimers have any detectable activity.

MATERIALS AND METHODS

Strain construction. Strains were derivatives of *E. coli* X90 [F'*lacI*^{q}*lac' pro'/ara Δ(lac-pro) nalA argE(am) rif*^{R}*thi-1*]. Chromosomal insertions of CATssrA were constructed by genetic recombineering (Sawitzke et al. 2007). A synthetic constitutive promoter, *cp25* (Jensen et al. 1998), was fused by PCR to a gene encoding chloramphenicol acetyl transferase gene followed by a two residue Leu-Arg, a His_6 sequence, and the ssrA tag to generate the CATssrA gene. CATssrA was initially cloned upstream of a kanamycin-resistance cassette in a modified pBluescript vector described in Farrell *et al.* (2005). Integration events were selected by kanamycin resistance, confirmed by colony PCR, and the *kan* marker was then removed using FLP recombinase (Sawitzke et al. 2007). All deletion strains were constructed by phage P1 transduction from donor strains X90 *clpA::kan*, X90 *clpP::kan*, or X90 *clpX::kan*. Knockouts were confirmed by colony PCR and western blotting using antibodies against ClpX, ClpP, or ClpA. Deletion strains were screened for chloramphenicol resistance by spotting 10-fold dilutions of saturated cultures onto LB plates with or without 68 μg mL^{-1} chloramphenicol.

Determination of protein levels by western blot. Anti-His_6 western blots were performed to monitor the cellular amounts of CATssrA and ClpP-His_6 in whole cell lysates using the protocol described by Farrell *et al.* (2005).

Selection. A randomized *clpP* library was generated by error-prone PCR using Taq polymerase in 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton-X100 with 4 mM $MgCl_2$, and 0.25 mM $MnCl_2$. (Cadwell et al. 1994). The amplified library was cloned into a modified pBAD vector (Tet^R; pBR322 *ori*) with XhoI/NcoI cleavage sites. The plasmid library was purified by the maxiprep method after initial amplification by transformation into X90 cells and selection in liquid LB broth containing tetracycline (12 μg mL^{-1}). The frequency of mutagenesis was determined by sequencing the *clpP* genes of randomly selected 15 colonies. For the selection, ten 30-μL aliquots of electrocompetent ML183 (X90 CATssrA) were transformed with the mutagenic library, allowed to recover and express tetracycline-resistance for 1 hour at 37 °C, and plated onto LB agar plates containing 0.2% arabinose, tetracycline, and chloramphenicol. All colonies were picked and screened on LB agar with tetracycline and chloramphenicol in the presence or absence of 0.2% arabinose to ensure all phenotypes were dependent on expression of the mutagenic *clpP**. Colonies were also purified and retransformed into fresh ML183 cells and plated on LB agar containing arabinose, tetracycline, and chloramphenicol. All clones with arabinose-dependent and plasmid-dependent phenotypes were sequenced.

Biochemistry. I19T and V28P ClpP were expressed in JK10 (BL21 λDE3 *ΔclpP*) cells and purified as described in chapter 2. Limited tryptic digests of ClpP, degradation of the Abz-

KASPVSLGY^{NO_2}D decapeptide by ClpP alone or with ClpX•ATPγS or ClpX•ADP, and ClpX ATPase assays were performed as described in Chapter 2. Carboxymethylated λO-I27 was purified and unfolded as described (Kenniston et al. 2003). Degradation reactions were performed as described in chapter 2 using 40 μM λO-I27 and 10 μM ClpP.

REFERENCES

Bewley, M. C., Graziano, V., Griffin, K. and Flanagan, J. M. (2006). "The asymmetry in the mature amino-terminus of ClpP facilitates a local symmetry match in ClpAP and ClpXP complexes." J Struct Biol **153**(2): 113-28.

Bewley, M. C., Graziano, V., Griffin, K. and Flanagan, J. M. (2009). "Turned on for degradation: ATPase-independent degradation by ClpP." J Struct Biol **165**(2): 118-25.

Cadwell, R. C. and Joyce, G. F. (1994). "Mutagenic PCR." PCR Methods Appl **3**(6): S136-40.

Choi, K. H. and Licht, S. (2005). "Control of peptide product sizes by the energy-dependent protease ClpAP." Biochemistry **44**(42): 13921-31.

Farrell, C. M., Grossman, A. D. and Sauer, R. T. (2005). "Cytoplasmic degradation of ssrA-tagged proteins." Mol Microbiol **57**(6): 1750-61.

Flynn, J. M., Neher, S. B., Kim, Y. I., Sauer, R. T. and Baker, T. A. (2003). "Proteomic discovery of cellular substrates of the ClpXP protease reveals five classes of ClpX-recognition signals." Mol Cell **11**(3): 671-83.

Glynn, S. E., Martin, A., Nager, A. R., Baker, T. A. and Sauer, R. T. (2009). "Structures of asymmetric ClpX hexamers reveal nucleotide-dependent motions in a AAA+ protein-unfolding machine." Cell **139**(4): 744-56.

Gottesman, S., Roche, E., Zhou, Y. and Sauer, R. T. (1998). "The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system." Genes Dev **12**(9): 1338-47.

Gribun, A., Kimber, M. S., Ching, R., Sprangers, R., Fiebig, K. M. and Houry, W. A. (2005). "The ClpP double ring tetradecameric protease exhibits plastic ring-ring interactions, and the N termini of its subunits form flexible loops that are essential for ClpXP and ClpAP complex formation." J Biol Chem **280**(16): 16185-96.

Jensen, P. R. and Hammer, K. (1998). "Artificial promoters for metabolic optimization." Biotechnol Bioeng **58**(2-3): 191-5.

Joshi, S. A., Hersch, G. L., Baker, T. A. and Sauer, R. T. (2004). "Communication between ClpX and ClpP during substrate processing and degradation." Nat Struct Mol Biol **11**(5): 404-11.

Kang, S. G., Dimitrova, M. N., Ortega, J., Ginsburg, A. and Maurizi, M. R. (2005). "Human mitochondrial ClpP is a stable heptamer that assembles into a tetradecamer in the presence of ClpX." J Biol Chem **280**(42): 35424-32.

Kang, S. G., Maurizi, M. R., Thompson, M., Mueser, T. and Ahvazi, B. (2004). "Crystallography and mutagenesis point to an essential role for the N-terminus of human mitochondrial ClpP." J Struct Biol **148**(3): 338-52.

Kenniston, J. A., Baker, T. A., Fernandez, J. M. and Sauer, R. T. (2003). "Linkage between ATP consumption and mechanical unfolding during the protein processing reactions of an AAA+ degradation machine." Cell **114**(4): 511-20.

Kim, D. Y. and Kim, K. K. (2008). "The structural basis for the activation and peptide recognition of bacterial ClpP." J Mol Biol **379**(4): 760-71.

Kim, Y. I., Levchenko, I., Fraczkowska, K., Woodruff, R. V., Sauer, R. T. and Baker, T. A. (2001). "Molecular determinants of complex formation between Clp/Hsp100 ATPases and the ClpP peptidase." Nat Struct Biol **8**(3): 230-3.

Leslie, A. G. (1990). "Refined crystal structure of type III chloramphenicol acetyltransferase at 1.75 A resolution." J Mol Biol **213**(1): 167-86.

Lies, M. and Maurizi, M. R. (2008). "Turnover of endogenous SsrA-tagged proteins mediated by ATP-dependent proteases in Escherichia coli." J Biol Chem **283**(34): 22918-29.

Sauer, R. T., Bolon, D. N., Burton, B. M., Burton, R. E., Flynn, J. M., Grant, R. A., Hersch, G. L., Joshi, S. A., Kenniston, J. A., Levchenko, I., Neher, S. B., Oakes, E. S., Siddiqui, S. M., Wah, D. A. and Baker, T. A. (2004). "Sculpting the proteome with AAA(+) proteases and disassembly machines." Cell **119**(1): 9-18.

Sawitzke, J. A., Thomason, L. C., Costantino, N., Bubunenko, M., Datta, S. and Court, D. L. (2007). "Recombineering: in vivo genetic engineering in E. coli, S. enterica, and beyond." Methods Enzymol **421**: 171-99.

Szyk, A. and Maurizi, M. R. (2006). "Crystal structure at 1.9A of E. coli ClpP with a peptide covalently bound at the active site." J Struct Biol **156**(1): 165-74.

Thompson, M. W., Singh, S. K. and Maurizi, M. R. (1994). "Processive degradation of proteins by the ATP-dependent Clp protease from Escherichia coli. Requirement for the multiple array of active sites in ClpP but not ATP hydrolysis." J Biol Chem **269**(27): 18209-15.

Wang, J., Hartling, J. A. and Flanagan, J. M. (1997). "The structure of ClpP at 2.3 A resolution suggests a model for ATP-dependent proteolysis." Cell **91**(4): 447-56.

Waterhouse, A. M., Procter, J. B., Martin, D. M., Clamp, M. and Barton, G. J. (2009). "Jalview Version 2--a multiple sequence alignment editor and analysis workbench." Bioinformatics **25**(9): 1189-91.

APPENDIX A:

Selection for ClpX Variants that Suppress Loss-of-Function S21A ClpP

INTRODUCTION

In Chapter 2, I showed that mutations in α-helix A of ClpP affected substrate gating and interactions with ClpX. The S21A mutation in α-helix A was particularly interesting because this substitution impaired binding to both ClpX and ClpA. The S21Y and S21P mutations were also identified in the dominant negative selection described in Chapter 3, further underscoring the importance of serine 21 for ClpP function in collaboration with ClpX. This residue is highly conserved in multiple sequence alignments of ClpP orthologs and interacts within the N-terminal loop from a neighboring subunit, in one crystal structure in which the N termini of ClpP are ordered (Bewley et al. 2006). I did not detect enhanced susceptibility of the S21A mutant to tryptic cleavage and this variant showed only a modest increase in decapeptide cleavage when compared with wild-type ClpP.

In principle, serine 21 could interact directly with ClpX. Docking of the structures of ClpX and ClpP did not reveal any obvious interactions, but the IGF and pore-2 loops of ClpX are largely disordered in the crystal structure and thus it is difficult to be confident of these model building results. To identify potential contacts between ClpP and ClpX, I designed a selection for suppressor mutations in ClpX that would it allow to function with the S21A ClpP mutant.

SELECTION STRATEGY

My selection for suppressor mutants used an ssrA-tagged mutant variant of the phenylalanyl-tRNA synthetase α subunit (mPheS) as a counter selectable marker (Figure 1). The mPheS gene encodes the A294G mutation, which broadens the substrate specificity and

allows the mutant enzyme to charge cognate tRNAs with *para*-chloro-phenylalanine and *para*-bromo-phenylalanine (Kast 1994). By contrast, wild-type PheS can only charge tRNAs with phenylalanine or *para*-fluoro-phenylalanine. Expression of the mPheS enzyme in the presence of *para*-chloro-phenylalanine (p-Cl-Phe) leads to cell death, probably because incorporation of this amino-acid analog into proteins results in folding defects. Thus, degradation of mPheS-ssrA by a ClpX suppressor mutant and S21A ClpP should allow cell growth on selective media containing p-Cl-Phe. To identify ClpX mutations that functioned only with the mutant ClpP, I knocked out the endogenous *clpP* gene and replaced it with a gene encoding the $clpP^{S21A}$ mutation. This integration maintained the 5′ and 3′ sequences flanking the wild-type gene, and thus $ClpP^{S21A}$ is expressed using the native promoter and ribosome-binding site. To prevent complications caused by mixed oligomerization, I also knocked out the endogenous *clpX* gene in the selection strain.

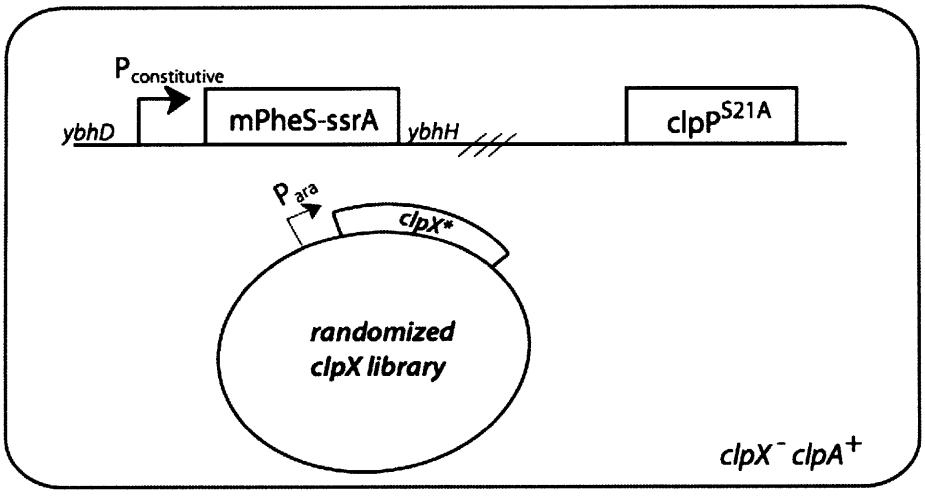

Figure 1. Strategy to identify mutants of ClpX that can operate with S21A ClpP to degrade mPheS-ssrA.

RESULTS AND DISCUSSION

I performed control experiments to ensure that the mutant mPheS-ssrA protein was expressed. As depicted in Figure 2, an *E. coli* strain (X90) carrying the *mPheS-ssrA* and wild-type *clpP* and *clpX* alleles grew, although slowly, in the presence of 20 mM p-Cl-Phe. Cells lacking *mPheS-ssrA* also grow slowly under these conditions (not shown). When the *mPheS-ssrA* strain also harbored a *clpP* deletion, no growth was observed in the presence of p-Cl-Phe (Figure 2). These results suggest that degradation of mPheS-ssrA by ClpXP is required for survival in the presence of p-Cl-Phe. As expected, I also observed no growth of a strain containing $clpP^{S21A}$ rather than wild-type *clpP* when p-Cl-Phe was present (Figure 2). This result suggests that $ClpP^{S21A}$ is nonfunctional in the cell. I attempted to confirm degradation of mPheS-ssrA *in vivo* by western blotting, but the signal was too weak to be confident of the results. Deletion of *clpX* or *clpA* did not alter the growth phenotype of cells with the $clpP^{S21A}$ allele.

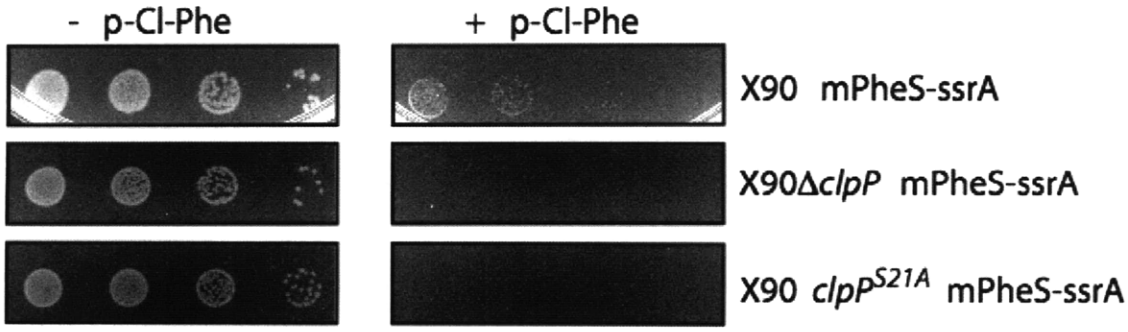

Figure 2. Growth of strains expressing mPheS-ssrA in otherwise wild type, Δ*clpP*, or $clpP^{S21A}$ strains. Spots show 10-fold dilutions of strains in the absence or presence of 20 mM p-Cl-Phe drug, with leftmost spots containing the highest cell densities.

In the selection strain, the chromosomal *clpX* gene was removed and ClpX was expressed under the control of an arabinose promoter from a plasmid that had been mutagenized by error-prone PCR (Figure 1). Using a plasmid library that contained an average of 3.5 mutations per *clpX* gene, I initially recovered ~1000 colonies that grew in the presence of p-Cl-Phe. Of these colonies, 196 were picked at random and screened for an arabinose-dependent phenotype. Sixteen clones displayed the required characteristics and were sequenced. Of these, seven had ClpX protein-coding mutations, listed in Table 1, while the remaining clones had wild-type *clpX* genes. It is possible that these plasmids had mutations in other regions that resulted in overexpression of ClpX. Because growth in the presence of p-Cl-Phe was arabinose dependent, it seems unlikely that the *mPheS-ssrA* allele was in some fashion inactivated. It is formally possible, however, that the $clpP^{S21A}$ allele reverted back to the wild type allele in the nine clones lacking mutations in ClpX.

Mutation(s)	Functional Consequence
S16P	N-domain
I47T, I186T	N-domain & Walker-B
V55A	N-domain
A63T	N-domain
E283A	IGF loop
Q208R	Box VI, downstream of pore-2
L410stop	C-terminal

Table 1. ClpX mutations that appear to suppress S21A ClpP.

Additional studies will be required to determine if the ClpX mutations listed in Table 1 actually allow these enzymes to collaborate functionally with S21A ClpP. Nevertheless,

several of the mutations identified occur in functionally significant regions of ClpX. For example, the I86T mutation alters the Walker-B motif, which contacts bound nucleotide and is necessary for ATP hydrolysis. Moreover, the E283A mutation alters the IGF loop and the Q208R mutation is proximal to the pore-2 loop. Both of these ClpX loops are known to play roles in ClpP binding (Kim et al. 2001; Martin et al. 2007). It is tempting to speculate that these mutations might increase ClpX affinity for S21A ClpP. It is difficult, however, to rationalize why mutations in the N domain of ClpX might affect ClpP interactions, as deletion of this domain does not impair ClpX activity or ClpXP proteolysis of ssrA-tagged substrates (Martin et al. 2005). Work to purify and biochemically characterize these mutants is ongoing.

MATERIALS AND METHODS

Genomic integration of clpP^{S21A}. A two-step recombineering approach (developed by J. H. Davis, personal communication) was used to insert the *clpP*^{S21A} allele into the chromosome. The first step involved a knockout through homologous recombination using a forward primer (5'-CGGTA CAGCA GGTTT TTTCA ATTTT ATCCA GGAGA CGGAA-3') and reverse primer (5'-CGCCC TGGAT AAGTA TAGCG GCACA GTTGC GCCTC TGGCA-3') that were homologous to regions immediately flanking the *clpP* open-reading frame. These primers were used to amplify a kan^{R}-mPheS cassette and the product was transformed into electrocompetent X90 [F'*lacI*^{q}*lac' pro*'/*ara Δ(lac-pro) nalA argE(am) rif*^{R}*thi-1*] cells. Transformants resistant to kanamycin were checked by colony PCR for knock-out of the endogenous *clpP* gene and a knock-in of the Kan^{R}-mPheS marker, which preserves the 5′ and 3′ sequences flanking the original *clpP* gene. The next recombination step utilized

identical primers to amplify *clpP*^{S21A}, and the product was transformed into the electrocompetent Kan^{R}-mPheS strain. In this step, transformants able to grow on *para*-chloro-phenylalanine (Sigma) were selected. Clones obtained after each step of recombineering were tested to confirm the expected genomic structure by colony PCR and the presence or absence of ClpP was assayed by western blotting. The integrated *clpP*^{S21A} allele in the final strain was confirmed by genomic sequencing.

Genomic integration of mPheS-ssrA. The recombineering strategy used to integrate *mPheS-ssrA* was identical to the one described in Chapter 3 (Sawitzke et al. 2007). A PCR product containing mPheS with a C-terminal ssrA tag and kanamycin cassette flanked by FLP recombinase recognition sites was transformed into E. coli strain X90, and integrants were selected by resistance to kanamycin. Colony PCR and genomic sequencing confirmed the final recombination product.

Selection strain construction. Strain ML311 (X90 *clpP*^{S21A} *ΔclpX mPheS-ssrA*) was used for the selection. The *mPheS-ssrA-FLP-kan*^{R} locus was P1 transduced into X90 *clpP*^{S21A} and confirmed by colony PCR. After removing the kanamycin marker via FLP recombinase activity, *clpX* was knocked out by P1 transduction from the donor strain X90 *clpX::kan*^{R}. This strain was confirmed by colony PCR and western blotting against ClpX to check for *clpX* inactivation by insertion of the kanamycin cassette.

Selection. A library of randomized *clpX* genes was generated by error-prone PCR used Taq polymerase for amplification in standard NEB Thermopol reaction buffer (20 mM Tris-HCl,

pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$ 0.1% Triton X-100). The library was cloned into the modified pBAD vector described in Chapter 3 using NdeI and XhoI restriction sites and was amplified by transformation and repurification from X90 cells. Ten 30-μL aliquots of electrocompetent ML311 were transformed with the amplified library, allowed to recover for 1 hour at 37 °C in 1-mL LB/0.2% arabinose, and plated onto YEG media (Wang et al. 2007) containing 20 mM *para*-chloro-phenylalanine, 12 μg mL^{-1} tetracycline, and 0.2% arabinose.

REFERENCES

Bewley, M. C., Graziano, V., Griffin, K. and Flanagan, J. M. (2006). "The asymmetry in the mature amino-terminus of ClpP facilitates a local symmetry match in ClpAP and ClpXP complexes." J Struct Biol **153**(2): 113-28.

Kast, P. (1994). "pKSS--a second-generation general purpose cloning vector for efficient positive selection of recombinant clones." Gene **138**(1-2): 109-14.

Kim, Y. I., Levchenko, I., Fraczkowska, K., Woodruff, R. V., Sauer, R. T. and Baker, T. A. (2001). "Molecular determinants of complex formation between Clp/Hsp100 ATPases and the ClpP peptidase." Nat Struct Biol **8**(3): 230-3.

Martin, A., Baker, T. A. and Sauer, R. T. (2005). "Rebuilt AAA + motors reveal operating principles for ATP-fuelled machines." Nature **437**(7062): 1115-20.

Martin, A., Baker, T. A. and Sauer, R. T. (2007). "Distinct static and dynamic interactions control ATPase-peptidase communication in a AAA+ protease." Mol Cell **27**(1): 41-52.

Sawitzke, J. A., Thomason, L. C., Costantino, N., Bubunenko, M., Datta, S. and Court, D. L. (2007). "Recombineering: in vivo genetic engineering in E. coli, S. enterica, and beyond." Methods Enzymol **421**: 171-99.

Wang, K. H., Sauer, R. T. and Baker, T. A. (2007). "ClpS modulates but is not essential for bacterial N-end rule degradation." Genes Dev **21**(4): 403-8.

APPENDIX B:

Biochemical Characterization of Mutants in the Hydrophobic Pocket of ClpP: F112A ClpP and L114W ClpP

INTRODUCTION

The first evidence that residues within the "IGF" motif of ClpX were important for interactions with ClpP came from the I268E and F270W point mutants, which were unable to bind ClpP but were not impaired in ClpX unfoldase activity (Kim et al. 2001). By docking of a model of the ClpX structure with the crystal structure of ClpP, the likely site of interaction of these IGF residues with ClpP was identified as hydrophobic clefts on the periphery of the ClpP ring. To date, there has been only limited biochemical evidence to support this docking model. As an extension of the experiments with V28P ClpP, described in Chapter 3, I mutagenized two other ClpP residues within the hydrophobic cleft: Phe112 and Leu114. The F112A mutant was initially characterized by others and was reported to be unable to bind ClpX (Bewley et al. 2006). I remade this mutant but found that it behaved similarly to wild-type ClpP in all assays, including ClpXP degradation. I also constructed and characterized the L114W ClpP mutation and found that it does not appear to interact with ClpX.

RESULTS

F112A ClpP

Multiple sequence alignments of ClpP show that a phenylalanine is not strongly conserved at position 112 (Figure 1). For example, the *H. influenzae* ClpP homolog has an alanine at this position, whereas the human enzyme has a histidine. To test whether the *E. coli* protein could accommodate alternative amino acids at position 112, I constructed and purified the F112A mutant.

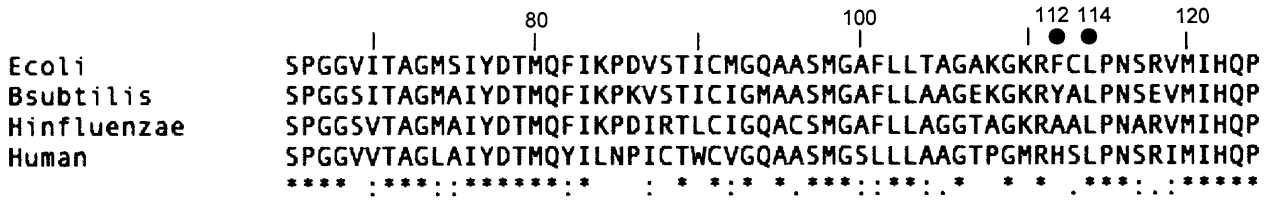

Figure 1. Local multiple sequence alignment of ClpP orthologs, highlighting residues 112 and 114. The numbering is that of the mature *E. coli* protein.

The purified F112A mutant was highly soluble protein and cleaved the Abz-KASPVSLGY^{NO2}D decapeptide at rates comparable to wild type ClpP (data not shown). Moreover, the electrophoretic mobility of F112A ClpP on SDS-PAGE was identical to the wild-type enzyme, suggesting that proenzyme maturation for this mutant is not impaired (data not shown). Both results indicate that the F112A mutation does not impair the peptide-cleavage function of free ClpP, as previously reported (Bewley et al. 2006). By contrast, I did not detect impairment in the interaction of the F112A mutant with ClpX. As shown in Figure 2A, this mutant repressed the rate of ATP hydrolysis by ClpX with an apparent affinity very similar to that of the wild-type ClpP control. Similarly, the F112A mutant and wild-type ClpP behaved almost identically when ClpX was titrated against a constant amount of ClpP or the mutant and changes in the rate of decapeptide cleavage were assayed (Figure 2B). Finally, I found that wild-type ClpP and the F112A mutant were indistinguishable in ClpXP degradation of GFP-ssrA, a native protein substrate (data not shown). Thus, F112A ClpP appears to be functionally equivalent to wild-type ClpP.

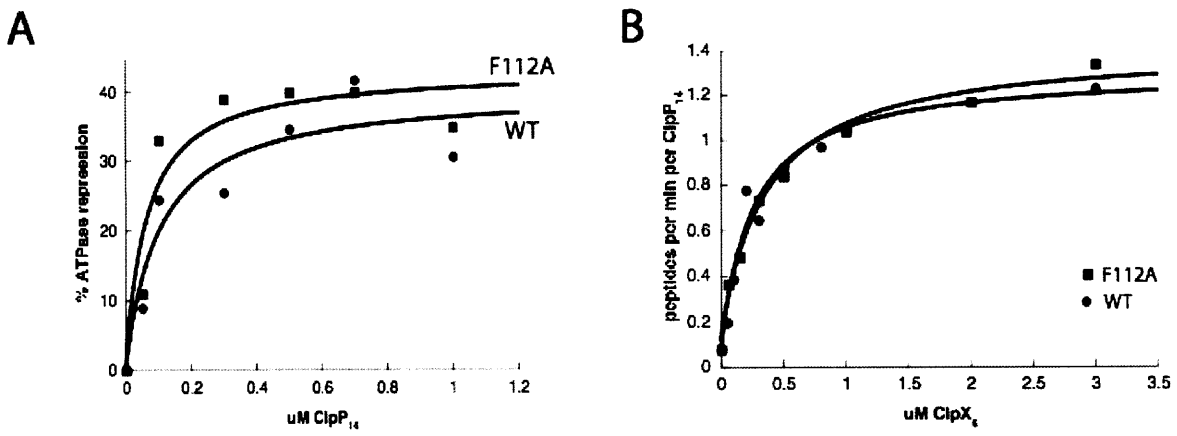

Figure 2. Assays monitoring ClpXP binding. (A) Increasing quantities of wild-type ClpP (circles) or F112A ClpP (squares) were titrated against a fixed quantity of ClpX (0.1 μM hexamer) and changes in the rate of ATP hydrolysis by ClpX were assayed. (B) Increasing quantities of ClpX were titrated against 50 nM wild-type ClpP (circles) or F112A ClpP (squares) and the rate of degradation of the Abz-KASPVSLGY^{NO2}D decapeptide was assayed.

L114W ClpP

Leu114 is also part of the hydrophobic cleft of ClpP but is more highly conserved than Phe112 (Figure 1). I constructed and purified the L114W ClpP mutant, which I hoped might be used to monitor ClpX•ClpP binding by changes in fluorescence. L114W ClpP was less soluble than wild-type ClpP and the purified protein contained a significant population (~50%) of unprocessed proenzyme (Figure 3A). In the absence of ClpX, L114W ClpP cleaved the decapeptide substrate at a rate similar to wild-type ClpP (Figure 3B), suggesting that the observed defect in L114W ClpP maturation does not arise because the mutation prevents peptide cleavage. Addition of ClpX did not cause a significant increase in the rate of decapeptide cleavage by L114W ClpP (Figure 3B). Similarly, no interaction of L114W ClpP with ClpX was detected in assays monitored by changes in ATPase rates (Figure 3C),

or degradation of GFP-ssrA (data not shown). These results suggest that the L114W-ClpP variant is functional as a peptidase but is severely defective in binding ClpX.

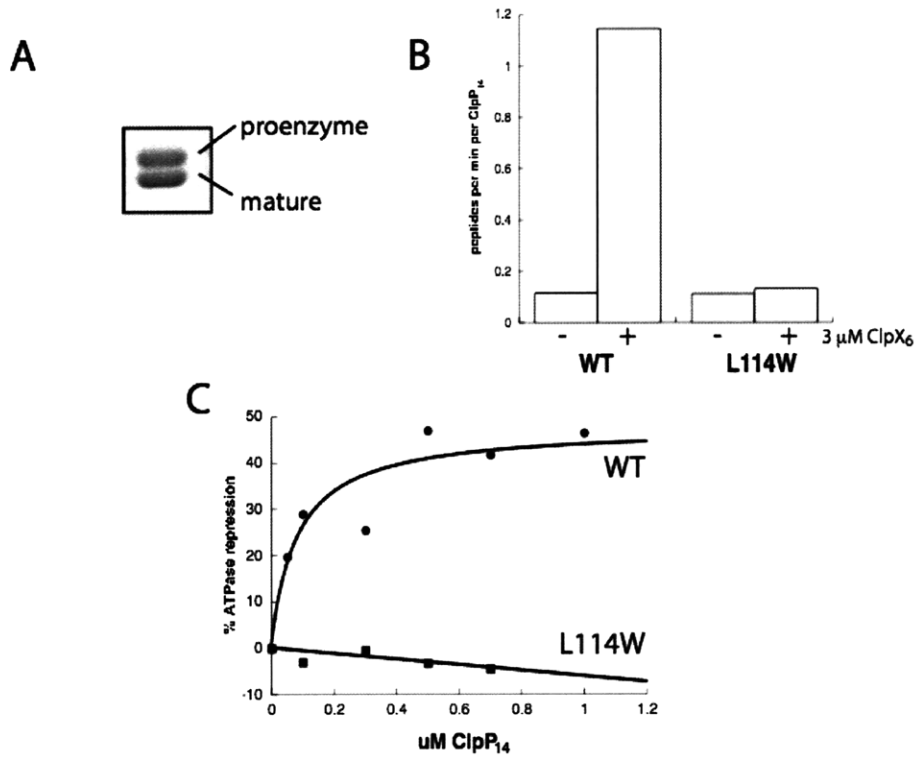

Figure 3. Characterization of L114W ClpP. (A) Mobility on 12% SDS-PAGE shows that ~50% of the purified protein was not processed to the mature enzyme. (B) Wild-type and L114W ClpP (0.2 μM) have the same activity in cleavage of the Abz-KASPVSLGY^{NO2}D decapeptide in the absence of ClpX. Addition of 3 μM ClpX hexamer and ATPγS stimulates peptide cleavage by wild-type ClpP but not by the L114W enzyme. (C) Titration of the L114W mutant (squares) against ClpX (0.3 μM hexamer) did not result in ATPase repression. Circles show the wild-type ClpP control.

DISCUSSION

Mutations at positions 112 and 114 within the hydrophobic cleft of ClpP have different effects on function depending on the assay. The F112A and L114W mutants both had

peptide-hydrolysis activity comparable to wild-type ClpP. For the F112A mutant, there was no defect in proenzyme processing and the protein was highly soluble. Processing of the L114W mutant, by contrast, was incomplete and the protein was poorly soluble. This reduced solubility may reflect the fact that roughly 50% of the subunits still contain the MSYSGERDNFAPHM propeptide, which is reasonably hydrophobic. It is also possible that this mutant has some type of folding defect (L114 is located at a subunit interface in the heptameric ring), but this explanation seems unlikely because L114W had peptide-cleavage activity comparable to wild-type ClpP. Why then is propeptide maturation compromised for this mutant? One possibility is that normal maturation somehow involves stimulation of activity by binding of propeptides, possibly after cleavage, in the hydrophobic clefts.

I found the F112A ClpP was essentially indistinguishable from wild-type ClpP in all of its activities, including functional collaboration with ClpX. This result is supported by multiple sequence alignments, which show that Phe112 is not highly conserved. Bewley *et al.* (2006) reported that F112A ClpP was severely defective in interactions with ClpA, did not support ClpXP degradation of GFP-ssrA, and showed only modest repression of ClpX ATPase activity. I did not assay the interaction of F112A with ClpA and do not know why my results with ClpX differ from those of Bewley *et al.*

The L114W mutation resulted in a ClpP variant defective in binding ClpX. The larger tryptophan side chain at this position may sterically occlude binding of the IGF motif in the hydrophobic cleft. Alternatively, the presence of unprocessed propeptides on many L114W subunits might interfere with ClpX binding in another fashion. Expressing L114W ClpP

without its propeptide sequence and characterizing the fully mutant enzyme would help to decide between these models. At present, I do not know if the heterogeneity of propeptide processing for the L114W mutant is randomly distributed among subunits in each tetradecamer or whether some heptameric rings or 14-mers are fully mature. More stringent purification of this mutant before or after dissociation of 14-mers into individual heptameric rings (Maurizi et al. 1998) might help decide these questions.

MATERIALS AND METHODS

Mutagenesis and purification. The F112A and L114W mutations were constructed by inverse PCR using primers that introduced the necessary coding changes at each position. Both mutant proteins contained C-terminal His_6-tags, were expressed in strain JK10 (BL21 λDE3 Δ*clpP*), and were purified by chromatography on Ni^{++}-NTA and monoQ columns as described previously (Kim et al. 2000).

Assays for ClpP activity. Peptide hydrolysis assays using the fluorogenic decapeptide Abz-KASPVSLGY^{NO2}D were performed as described in Chapter 2. Maturation defects were detected by analyzing purified protein on 12% SDS-PAGE.

Assays for ClpXP activity. Activation of peptide degradation by ClpX was performed as described in Chapter 2, using ATPγS, an ATP analog that supports ClpX binding to ClpP but is slowly hydrolyzed. GFP-ssrA degradation and ATPase assays were also performed as described in Chapter 2.

REFERENCES

Bewley, M. C., Graziano, V., Griffin, K. and Flanagan, J. M. (2006). "The asymmetry in the mature amino-terminus of ClpP facilitates a local symmetry match in ClpAP and ClpXP complexes." J Struct Biol **153**(2): 113-28.

Kim, Y. I., Burton, R. E., Burton, B. M., Sauer, R. T. and Baker, T. A. (2000). "Dynamics of substrate denaturation and translocation by the ClpXP degradation machine." Mol Cell **5**(4): 639-48.

Kim, Y. I., Levchenko, I., Fraczkowska, K., Woodruff, R. V., Sauer, R. T. and Baker, T. A. (2001). "Molecular determinants of complex formation between Clp/Hsp100 ATPases and the ClpP peptidase." Nat Struct Biol **8**(3): 230-3.

Maurizi, M. R., Singh, S. K., Thompson, M. W., Kessel, M. and Ginsburg, A. (1998). "Molecular properties of ClpAP protease of Escherichia coli: ATP-dependent association of ClpA and clpP." Biochemistry **37**(21): 7778-86.