Role of communication between subunits and enzymes in ClpXP-mediated substrate unfolding and degradation

by

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ABSTRACT

Chaperones and proteases play important roles in quality control by helping proteins fold, by dismantling hyper-stable complexes, and by degrading unwanted proteins. The highly conserved AAA+ Clp/Hsp100 proteins are ATPases which function as disassembly chaperones as well as essential components of energy-dependent proteases. For example, the ClpX ATPase disassembles macromolecular complexes and combines with the ClpP peptidase to form ClpXP, a molecular machine with structural and functional similarity to the eukaryotic 26S proteasome. ClpXP consists of hexameric ClpX rings stacked coaxially against the double-ring ClpP14 peptidase. ClpXP's peptidase active sites reside in a sequestered chamber accessible through a narrow channel which excludes native, folded substrates. ClpX binds specific substrates, unfolds them in a reaction requiring ATP hydrolysis, and then translocates them into ClpP for degradation. When ClpP is absent, ClpX unfolds and releases specific substrates, an activity that can disassemble otherwise stable complexes.

Although ClpX-mediated substrate unfolding and ClpXP-mediated degradation have been studied extensively, the role of communication between subunits within a ClpX hexamer or between enzymes in ClpXP has not been addressed. I initially screened numerous ClpX point mutants for their ability to support host-cell lysis by bacteriophage Mu, and then purified several mutants for functional characterization in vitro. Three of these mutants contained substitutions at intersubunit interfaces; these variants bound substrate well, but displayed unusual changes in ATP hydrolysis in response to substrate binding and had low protein unfolding activity. These results suggest that communication across these subunit-subunit interfaces coordinates and regulates nucleotide hydrolysis in response to substrate binding and is critical for efficient substrate unfolding. I also discovered that ClpX and ClpP communicate with each other during substrate processing and degradation. ClpX binds ClpP more strongly during substrate unfolding than during translocation, by a mechanism that is linked to the ATP hydrolysis rate. Interaction between ClpX and DFP-modified ClpP, mimicking a peptide-cleavage intermediate, is strengthened significantly suggesting that ClpP also communicates its functional state to ClpX. Overall, this work suggests that communication within the ClpX hexamer and between enzymes in ClpXP modulates and coordinates substrate processing.

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CHAPTER ONE

Clp/Hsp100 proteins: Disassembly chaperones and protease complexes vital to cellular quality control
Proteins carry out critical macromolecular functions in every living cell. In order to work properly, however, proteins must be correctly folded. Within the crowded cellular environment, there is ample opportunity for proteins to become misfolded or aggregated. It is not surprising, therefore, that cells have evolved molecular systems to help proteins fold properly and to help take apart aggregates. Hsp70 and Hsp60 proteins help proteins fold and much is known about the activities of these protein families. Dismantling of protein aggregates is carried out by members of the more recently discovered Clp/Hsp100 family.

**Hsp70 and Hsp60 chaperone systems**

Classical chaperone systems, like DnaK/DnaJ (Hsp70/Hsp40) and GroEL/GroES (Hsp60/Hsp10), function to assist protein folding. Prokaryotic DnaK/DnaJ and GroEL/GroES are heat shock proteins which are transcriptionally upregulated (Cowing *et al.*, 1985; Yamamori and Yura, 1980) in response to diverse kinds of cellular stress in addition to high temperature (VanBogelen *et al.*, 1987). *Escherichia coli* DnaK and GroEL, which are the main chaperone components of each system, bind hydrophobic sequences (Houry *et al.*, 1999; Rudiger *et al.*, 1997) presumably exposed because a given protein is unfolded or improperly folded. DnaK tends to bind extended stretches of amino acids, while GroEL binds more compact shapes (Flynn *et al.*, 1993; Hayer-Hartl *et al.*, 1994; Landry *et al.*, 1992). The DnaK system is believed important for nascent polypeptide folding and for protein translocation across membranes (Frydman *et al.*, 1994; Gaitanaris *et al.*, 1994; Langer *et al.*, 1992a; Petit *et al.*, 1994; Wild *et al.*, 1992; Wild *et al.*, 1996), whereas the GroEL system is thought by some to be more important for the refolding of misfolded proteins (Gordon *et al.*, 1994; Viitanen *et al.*, 1992). Substrates bound by the DnaK system are maintained in a soluble folding-competent state by repeated cycles of
binding to the exposed hydrophobic patches and release (Buchberger et al., 1996; Szabo et al., 1994). In contrast, GroEL is believed to help bound proteins fold by providing a protected environment within an encapsulated chamber where aggregation cannot occur (Fenton and Horwich, 1997; Ranson et al., 1995; Walter et al., 1996; Zahn et al., 1996).

ATP binding and hydrolysis regulates the reaction cycles of both systems. In *E. coli*, the DnaK system involves a nucleotide-exchange factor, GrpE, in addition to the co-chaperone DnaJ, whereas GroEL functions with its co-chaperone, GroES (Friedman et al., 1984; Georgopoulos et al., 1973; Georgopoulos et al., 1972; Sternberg, 1973). DnaK•ATP binds substrate proteins weakly, while DnaK•ADP assumes a different conformation which binds substrates more tightly (Palleros et al., 1993; Palleros et al., 1994; Pierpaoli et al., 1997). DnaJ stimulates DnaK’s ATPase activity (McCarty et al., 1995; Szabo et al., 1994), and GrpE binding causes ADP release from DnaK•ADP•substrate (Szabo et al., 1994). In one model, a DnaK•ATP•substrate complex binds DnaJ and is converted into DnaK•ADP•substrate, whereas in another model this same complex is formed after DnaJ•substrate binds DnaK•ATP (Fink, 1999). GrpE then causes ADP to be released from DnaK•ADP•substrate, and subsequent ATP binding causes both a conformational change and substrate release (Buchberger et al., 1995; Palleros et al., 1993; Theyssen et al., 1996). In addition to being regulated by ATP binding and hydrolysis, GroEL’s reaction cycle is modulated by GroES binding. GroEL is composed of two back-to-back heptameric rings (Braig et al., 1994), which form two cylindrical chambers, each with a single opening at its end. GroES binds to the end of one ring, closing off that GroEL chamber from the cellular environment (Langer et al., 1992b; Xu et al., 1997). Substrate proteins originally bind to hydrophobic surfaces in a GroEL chamber (Braig et al., 1994; Braig et al., 1993; Fenton et al., 1995).
and only have a chance to refold when the inner surfaces of the GroEL cavity become hydrophilic as a result of a dramatic conformational change after GroES has bound (Chen et al., 1994; Roseman et al., 1996; Xu et al., 1997). Differences in affinity between GroES and the different nucleotide-bound GroEL forms (Jackson et al., 1993) and negative allostery in the system (Hayer-Hartl et al., 1995; Todd et al., 1994; Xu et al., 1997) drive the reaction cycle forward.

**Functional overview of the Clp/Hsp100 protein family**

*Cellular roles.* Unlike the Hsp70 and Hsp60 chaperone systems (Goloubinoff et al., 1989; Schroder et al., 1993; Veinger et al., 1998), the Clp/Hsp100 protein family’s main function is to disassemble macromolecular complexes or disaggregate protein aggregates after they have formed. Clp/Hsp100 proteins take apart some multimers by unfolding individual components (Burton et al., 2001a; Kim et al., 2000; Weber-Ban et al., 1999). Some Clp proteins also function as part of ATP-dependent proteases (Figure 1) (Gottesman et al., 1997a; Gottesman et al., 1997b; Wickner et al., 1999). The first energy-dependent protease was discovered in *E. coli*. It was known that when ATP generation was disrupted, protein degradation *in vivo* decreased by 90-95% (Goldberg and St John, 1976). Soon thereafter, an ATP-dependent protease, Lon (protease La), was isolated and identified (Charette et al., 1981; Chung and Goldberg, 1981; Swamy and Goldberg, 1981). However, casein, a model substrate, was still found to be degraded in lysates from cells with a disrupted *lon* gene (Maurizi et al., 1985). Using casein degradation as an assay, the ATP-dependent protease, ClpAP, was discovered (Katayama et al., 1988; Katayama-Fujimura et al., 1987). ClpA was shown to be an ATPase, whereas ClpP had peptidase activity.
Figure 1. Some Clp proteins have dual functions.
Table 1. Clp proteins support various biological processes

<table>
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<th>Stress tolerance</th>
<th>Developmental stage/process</th>
<th>Cell division</th>
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<td>ClpB (heat)</td>
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<td>B. subtilis</td>
<td>ClpP</td>
<td>ClpX; ClpP (sporulation)</td>
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<td>B. thuringiensis</td>
<td>ClpP1</td>
<td>ClpC/ClpP2 (sporulation)</td>
<td>ClpP1</td>
<td>ClpP2</td>
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<td>C. crescentus</td>
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<td>ClpP</td>
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<tr>
<td>E. coli</td>
<td>ClpA/ClpB/ HslU (heat); ClpX/ClpP (many)</td>
<td>ClpX (bacteriophage Mu cell-lysis)</td>
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<td>L. lactis</td>
<td>ClpP (heat, pH)</td>
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<td>L. donovani</td>
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<td>ClpB</td>
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<td>L. monocytogenes</td>
<td>ClpC</td>
<td>ClpE</td>
<td>ClpC/ClpE/ClpP</td>
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<td>P. fluorescens</td>
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<td>S. cerevisiae</td>
<td>Hsp104 (heat)</td>
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<td>ClpX</td>
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<td>S. pneumoniae</td>
<td>ClpC (heat)</td>
<td>ClpC</td>
<td>ClpC/ClpE/ClpC</td>
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<td>S. coelicolor</td>
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<td>ClpP (mycelium differentiation)</td>
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<td>Synechococcus sp. PCC 7942</td>
<td>ClpB/ClpP1 (thermal); ClpP1 (UV-B light)</td>
<td>ClpP</td>
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<td>Y. enterocolitica</td>
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Since the discovery of *E. coli* ClpA, ATPases of the Clp/Hsp100 family have been found to be highly conserved and to exist in all biological kingdoms (Gottesman *et al*., 1990b; Maurizi *et al*., 1990b; Neuwald *et al*., 1999; Schirmer *et al*., 1996; Squires and Squires, 1992). Known Clp ATPases include ClpA, ClpB, ClpC, ClpE, ClpX, and ClpY. Many of these ATPases associate with peptidases to form energy-dependent proteases. Clp/Hsp100 family ATPases and proteases have critical roles in a diverse variety of processes in many different prokaryotic and eukaryotic organisms (Table 1). For example, Clp proteins are key parts of stress tolerance systems. *Lactococcus lactis* ClpP is involved in tolerance to heat and pH changes, and cells lacking ClpP are much less viable (Frees and Ingmer, 1999). *Synechococcus* sp. PCC 7942, a cyanobacterial strain, requires ClpB for survival after heat stress (Eriksson and Clarke, 1996) and ClpP1 for long-term acclimation to different stresses, particularly cold temperatures and exposure to UV-B light (Clarke *et al*., 1998; Porankiewicz *et al*., 1998). Heat stress tolerance in *Arabidopsis thaliana* and *Saccharomyces cerevisiae* require ClpB or Hsp104, respectively (Hong and Vierling, 2000; Sanchez and Lindquist, 1990). In *Bacillus subtilis, Listeria monocytogenes*, and *Streptococcus pneumoniae*, Clp proteins function in stress tolerance and in other processes. In *B. subtilis*, ClpP is important for tolerance to heat, salt, oxidative stress, exposure to ethanol and puromycin, and glucose and oxygen deprivation (Gerth *et al*., 1998; Kruger *et al*., 1994; Msadek *et al*., 1998; Volker *et al*., 1994). Additionally, ClpP is vital for competence development (Turgay *et al*., 1998), sporulation (Nanamiya *et al*., 2000), correct cell morphology and motility (Gerth *et al*., 1998), and ClpX and ClpP are critical for proper developmental regulation of transcription (Nakano *et al*., 2001; Nakano *et al*., 2003). *L. monocytogenes* ClpC is involved in stress tolerance (Rouquette *et al*., 1996) and is important for virulence, as are ClpE and ClpP (Gaillot *et al*., 2000; Nair *et al*., 1999; Rouquette *et al*., 1998). ClpE is also important for cell
division in *L. monocytogenes* (Nair *et al.*, 1999). ClpC in *S. pneumoniae* is involved in heat tolerance, cell division, autolysis, genetic transformability, ability to adhere to host cells (Charpentier *et al.*, 2000), and virulence, as is ClpE (Lau *et al.*, 2001; Polissi *et al.*, 1998). *Streptomyces coelicolor* ClpP is important for the differentiation of mycelium (de Crecy-Lagard *et al.*, 1999), whereas *Synechococcus* sp. PCC 7942 ClpP null cells are filamentous and grow slowly (Clarke *et al.*, 1998). Virulence roles have been ascribed for *Salmonella typhimurium* ClpP (Hensel *et al.*, 1995), *Yersinia enterocolitica* ClpP (Pederson *et al.*, 1997), *Staphylococcus aureus* ClpX (Mei *et al.*, 1997), *Serpulina hydysenteriae* ClpC (ter Huurne *et al.*, 1994), *Mycobacterium leprae* ClpC (Misra *et al.*, 1996), *Mycobacterium tuberculosis* ClpC (Misra *et al.*, 1996), and *Leishmania donovani* ClpB (Hubel *et al.*, 1997; Krobitsch and Clos, 1999). *Bacillus thuringiensis* ClpC and ClpP2 are involved in sporulation, ClpP2 is important for motility, and ClpP1 is critical for cell division and response to low temperatures (Fedhila *et al.*, 2002). Finally, *Caulobacter crescentus* ClpXP is needed for progression through the cell cycle and for viability (Jenal and Fuchs, 1998; Jenal and Shapiro, 1996); *Pseudomonas fluorescens* ClpP is needed for biofilm formation (O'Toole and Kolter, 1998); *Caenorhabditis elegans* OOC-5, a putative Clp protein, is necessary for mitotic spindle orientation and polarity in embryos (Basham and Rose, 1999; Basham and Rose, 2001); and the *Chlamydomonas* plastid ClpP is essential for correct chloroplast function and for resulting algal growth (Huang *et al.*, 1994).

Biochemical studies of Clp/Hsp100 ATPases and proteases have focused most intensively on the *E. coli* enzymes. *E. coli* contains four Clp ATPases (ClpX (Gottesman *et al.*, 1993; Wojtkowiak *et al.*, 1993), HslU (Chuang *et al.*, 1993; Rohrwild *et al.*, 1996), ClpA (Katayama *et al.*, 1988), and ClpB (Kitagawa *et al.*, 1991)) and three of these proteins also form bipartite protease
complexes with either the ClpP (Katayama et al., 1988; Maurizi et al., 1990a) or HslV (Chuang et al., 1993; Rohrwild et al., 1996) peptidases. ClpX or ClpA interact with ClpP to form the ClpXP or ClpAP proteases, respectively, whereas HslU joins HslV to form the HslUV protease. In genetic studies, E. coli Clp proteins often function to combat cellular stress. ClpX-defective cells survive less well after UV irradiation (Neher et al., 2003a) and cannot be lysed by bacteriophage Mu (Mhammedi-Alaoui et al., 1994). Additionally, both ClpX-defective and ClpP-defective cells are less viable during lengthened stationary phase (Weichart et al., 2003), die after UV exposure under amino acid deprivation (Burger et al., 2002), are poorly mutable by UV light (Frank et al., 1996), are defective in acquiring type IA and IB restriction systems (Makovets et al., 1998), and do not support restriction alleviation of the type IA system after DNA damage (Makovets et al., 1999). Cells lacking HslU are inhibited for growth at high temperatures and are smaller in size at low temperatures (Katayama et al., 1996). ClpA-defective cells have growth defects at 46 °C (Thomas and Baneyx, 1998), but not at temperatures between 25 °C and 42 °C (Katayama et al., 1988). ClpB-defective cells also show problems with thermal stress, as they grow slowly at 44 °C and survive poorly at 50 °C (Squires et al., 1991). ClpB-defective and ClpP-defective cells also fail to grow with more than 0.5% SDS, whereas wild-type cells grow in the presence of 20-fold higher detergent concentrations (Rajagopal et al., 2002). Furthermore, ClpP-defective cells show delayed recovery after repeated cycles of carbon starvation and growth (Damerau and St John, 1993), and HslV-minus cells are lethal under conditions where there is an altered amount of signal recognition particle present (Bernstein and Hyndman, 2001). Finally, recent studies suggest that ClpXP may be important in response to oxidative stress and to changes between aerobic and anaerobic conditions (Flynn et al., 2003).
E. coli Clp proteins function as disassembly chaperones and proteases. ClpB is believed to act only as a disassembly chaperone, whereas HslU, ClpA, and ClpX have additional roles as part of HslUV, ClpAP, or ClpXP, three of E. coli’s five known ATP-dependent proteases (Gottesman, 1996; Schirmer et al., 1996; Wickner et al., 1999). ClpB is particularly important for solubilizing large protein aggregates (Diamant et al., 2000; Laskowska et al., 1996; Thomas and Baneyx, 2000), a function which also requires the DnaK chaperone system (Zolkiewski, 1999). ClpB is thought to act first in aggregate disassembly, exposing hydrophobic patches which are then bound by the DnaK system (Goloubinoff et al., 1999; Weibezahn et al., 2003). Aggregated proteins which have small Hsps bound also are disaggregated by ClpB with DnaK/DnaJ/GrpE (Mogk et al., 2003a). This ClpB/DnaK system acts more specifically when it disassembles dimers of TrfA, plasmid RK2’s replication initiation protein, to active monomers (Konieczny and Liberek, 2002).

In contrast to ClpB’s general disassembly of large protein aggregates, known chaperone activities for the remaining Clp proteins are far more substrate specific. HslU disaggregates SulA, an inhibitor of cell division, both in vivo and in vitro (Seong et al., 2000). ClpA activates P1 RepA DNA-binding activity by converting inactive RepA dimers into monomers (Wickner et al., 1994). ClpX disassembles three known substrates. In two cases, TrfA (Konieczny and Helinski, 1997) and λO (Wawrzynow et al., 1995), ClpX disassembles dimers into monomers. In the third case, disassembly of the MuA4-DNA complex, an interesting phenotype results if ClpX is not present to take apart this protein-DNA complex (Kruklitis et al., 1996; Levchenko et al., 1995; Mhammedi-Alaoui et al., 1994). Namely, bacteriophage Mu cannot replicate in or
lyse bacterial cells lacking functional ClpX because failure to disassemble the MuA-DNA complex prevents replication of Mu DNA. The MuA-DNA complex is an intermediate in a recombination reaction catalyzed by MuA (Mu transposase). The timing of ClpX action is controlled exquisitely by MuB, a protein which stimulates MuA’s catalysis of recombination events (Baker et al., 1991). MuB and ClpX bind overlapping parts of MuA’s C-terminus (Levchenko et al., 1997). ClpX commences disassembly only after recombination is completed and MuB has dissociated because ClpX-mediated disassembly requires recognition of MuA’s last ten amino acids (Levchenko et al., 1997).

Like the proteasome, Clp proteases are compartmentalized, barrel-shaped machines with their peptidase active sites sequestered in an internal chamber accessible only by passage through narrow axial portals (Larsen and Finley, 1997). Therefore, native proteins must be denatured and translocated into the proteolytic chamber prior to degradation. The list of substrates for degradation by HslUV, ClpAP, and ClpXP is growing steadily. HslUV degrades SulA in vivo and in vitro (Kanemori et al., 1999; Seong et al., 1999; Wu et al., 1999) and more generally degrades misfolded proteins (Missiakas et al., 1996). ClpAP degrades itself (Gottesman et al., 1990a), ClpA-lacZ fusion proteins (Gottesman et al., 1990a), P1 RepA (Wickner et al., 1994), HemA (glutamyl-tRNA reductase) (Wang et al., 1999), some carbon starvation proteins (Damerau and St John, 1993), and heat-aggregated proteins (Dougan et al., 2002). ClpXP degrades λO (Gottesman et al., 1993; Wojtkowiak et al., 1993), σ^s (RpoS) (Schweder et al., 1996), UmuD’ in a UmuD/UmuD’ heterodimer (Frank et al., 1996), Mu vir repressors (Mhammedi-Alaoui et al., 1994; Welty et al., 1997), P1 Phd (Lehnherr and Yarmolinsky, 1995), the HsdR subunit of EcoKI (Doronina and Murray, 2001), and Dps (Flynn et al., 2003; Stephani...
et al., 2003). Recently, more than 50 new ClpXP substrates were identified (Flynn et al., 2003), including transcription factors, ribosomal proteins, stress response proteins, and metabolic enzymes. Additionally, ClpXP and ClpAP process HlyC in the presence of HlyA (Guzman-Verri et al., 2001) and degrade ssrA-tagged proteins (Gottesman et al., 1998). The latter proteins contain an 11-residue C-terminal ssrA degradation tag that is added to nascent chains on stalled ribosomes via a cotranslational process (Karzai et al., 2000; Keiler et al., 1996). ClpXP and ClpAP degradation of ssrA-tagged proteins is part of a generalized protein quality control system.

**Structural overview of *E. coli* Clp/Hsp100 proteins**

**AAA+ protein family functions.** The Clp/Hsp100 protein family is part of the AAA+ ATPase family, a large group of structurally related proteins which are involved in a diverse variety of cellular activities, including membrane fusion, proteolysis, DNA replication and repair, recombination, and transcription (Neuwald et al., 1999). Most AAA+ ATPases use nucleotide binding or hydrolysis to exert mechanical force, thereby remodeling macromolecular complexes and assisting in their assembly, disassembly, or activation (Neuwald et al., 1999; Ogura and Wilkinson, 2001). An ancient family, AAA+ ATPases are found in all three divisions of life (bacteria, archaeabacteria, and eukaryotes) (Neuwald et al., 1999), underscoring the functional importance of AAA+ proteins. Two subgroups, AAA proteins and Replication Factor C (RFC)-related clamp-loader proteins, are present in all organisms. AAA proteins are a group of related chaperones which function in diverse processes (Confalonieri and Duguet, 1995; Patel and Latterich, 1998; Swaffield et al., 1995). One AAA subfamily, p97/Cdc48/VCP, is found in some bacteria (like *M. tuberculosis*) as well as in all archaeabacteria and eukaryotes (Neuwald et al.,
p97/Cdc48 is important for Golgi, endoplasmic reticulum, and nuclear envelope membrane fusion events (Acharya et al., 1995; Latterich et al., 1995; Rabouille et al., 1995) and for endoplasmic reticulum-associated degradation (Kopito, 1997; Rabinovich et al., 2002). Other important AAA proteins include the 19S regulatory component ATPases of the 26S proteasome (Baumeister and Lupas, 1997; Baumeister et al., 1998) and the N-ethylmaleimide-sensitive fusion protein (NSF) (Hay and Scheller, 1997; Weber et al., 1998), which is critical for SNARE-mediated membrane fusion events (Hanson et al., 1997; May et al., 2001; Sollner et al., 1993).

RFC-related clamp-loader proteins are the most conserved AAA+ proteins involved in DNA replication, but other less conserved AAA+ proteins are also involved in replication and repair. The five S. cerevisiae RFC proteins which comprise the heteropentameric clamp-loader complex are AAA+ proteins (Cullmann et al., 1995; Neuwald et al., 1999). PCNA, the trimeric ring-shaped processivity factor, is loaded onto DNA by the RFC clamp loader to initiate DNA synthesis (Kelman and O'Donnell, 1995; Krishna et al., 1994). An analogous system, involving the γ-complex clamp loader and β-clamp processivity factor, exists in E. coli (Jeruzalmi et al., 2001; Kong et al., 1992). Four of the five essential γ-complex components (Pritchard et al., 2000; Stewart et al., 2001; Studwell and O'Donnell, 1990) are AAA+ proteins (Neuwald et al., 1999), though only one type (γ) is capable of interacting with nucleotide (Xiao et al., 1995). Less conserved AAA+ proteins are involved in DNA replication initiation and repair. Initiation of DNA replication is mediated in S. cerevisiae and other eukaryotes by sequential assembly of different protein complexes at origins of replication (Aparicio et al., 1997; Bell and Stillman, 1992; Diffley and Cocker, 1992; Donovan et al., 1997; Tanaka et al., 1997). A hexameric ORC
(Bell et al., 1993), composed of Orc1-Orc6, binds the origin first and then recruits Cdc6 (Liang et al., 1995), which in turn recruits the heterohexameric MCM (Chong et al., 1996) protein complex (Aparicio et al., 1997; Coleman et al., 1996; Tanaka et al., 1997). Subsequent phosphorylation of MCM complex components leads to replication initiation (Bousset and Diffley, 1998; Lei et al., 1997; Santocanale and Diffley, 1998). Orc1, Orc4, Orc5, Cdc6, and all six MCM proteins are AAA+ family members (Neuwald et al., 1999). In E. coli, the AAA+ DnaA protein, which is conserved in all bacteria (Neuwald et al., 1999), binds oriC replication origins in a manner functionally similar to eukaryotic ORC (Baker et al., 1986; Fuller et al., 1984). The AAA+ Rad17 (Neuwald et al., 1999) in Schizosaccharomyces pombe and humans is a DNA-damage checkpoint protein with homology to RFC1 (of the eukaryotic clamp loader) (al-Khodairy et al., 1994; Enoch et al., 1992; Freire et al., 1998; Griffiths et al., 1995). Human Rad17 associates with RFC2-RFC5 and a PCNA-like clamp composed of three checkpoint proteins (Caspari et al., 2000; Lindsey-Boltz et al., 2001), suggesting a role in repair of lesions for this clamp loader and associated clamp (Kelly and Brown, 2000; O'Connell et al., 2000; Venclovas and Thelen, 2000).

AAA+ proteins also have interesting roles in recombination and transcription. The AAA+ RuvB helicase is found in all bacteria except the extreme thermophile, Aquifex aeolicus (Neuwald et al., 1999). RuvB promotes DNA branch migration during recombination at Holliday junctions (Rice et al., 1997; West, 1997). Two hexameric RuvB rings sandwich RuvA (Iwasaki et al., 1989) and a channel passing through the complex is used presumably for DNA threading (Yu et al., 1997) during branch migration. Bacterial NtrC protein is a AAA+ transcriptional enhancer which helps regulate the expression of genes involved in nitrogen metabolism (Magasanik,
Using energy from ATP hydrolysis, NtrC helps convert a closed complex of promoter-bound RpoN(σ^{54})-RNA polymerase to an active open complex (Hwang et al., 1999; Porter et al., 1993; Wedel and Kustu, 1995; Wyman et al., 1997). NtrC is found in all bacterial species which contain RpoN (Neuwald et al., 1999).

**AAA+ family structural characteristics.** The AAA+ ATPase family belongs to a large group, the P-loop-type NTPases, which have nucleotide-binding sites defined by classical Walker-A and Walker-B motifs (Gorbalenya and Koonin, 1989; Koonin, 1993b; Saraste et al., 1990; Walker et al., 1982). AAA+ proteins further belong to a subgroup, the motif C (or sensor I)-containing ATPases (Koonin, 1993a; Neuwald et al., 1999). Like other P-loop-type NTPases, AAA+ proteins contain a conserved core subdomain with an α,β-fold, generally similar to the RecA ATP-binding domain (Hubbard et al., 1997; Neuwald et al., 1999; Story and Steitz, 1992). This subdomain, typically of 200-250 residues, contains the Walker-A and Walker-B motifs in a structure which contains a five-stranded β-sheet with two or three α-helices on either side (Confalonieri and Duguet, 1995; Neuwald et al., 1999; Patel and Latterich, 1998). The AAA+ module, however, is comprised of a larger structural unit, which includes a smaller predominantly α-helical C-terminal subdomain (Neuwald et al., 1999). Nucleotide is bound between the large and small subdomains (Lupas and Martin, 2002). Amino acids from both subdomains of a AAA+ module, and sometimes residues from an adjacent subunit of a multimeric AAA+ protein, generally contact ATP/ADP. Some AAA+ proteins neither bind nor hydrolyze ATP. For instance, δ' of the *E. coli* γ-complex clamp loader has a disrupted P-loop motif (Guenther et al., 1997), and at least one of the AAA+ clamp-loader subunits in most bacteria also have disrupted P-loops (Neuwald et al., 1999).
In addition to the Walker motifs, AAA+ proteins contain a sensor-I motif and often contain other motifs called RFC boxes because they were initially identified in RFC-related proteins (Cullmann et al., 1995; Guenther et al., 1997; Neuwald et al., 1999). For example, the RFC box-II, box-VII, and sensor-II motifs are conserved to different extents in different AAA+ proteins (Neuwald et al., 1999). In contrast, AAA proteins usually do not contain a sensor-II motif, but instead contain a conserved 19-residue motif called the SRH (for second region of homology) (Karata et al., 1999; Karata et al., 2001; Neuwald et al., 1999). AAA proteins thus constitute a distinct subgroup of the AAA+ protein family. In ATPases containing Walker motifs, it appears that ATP is hydrolyzed by an activated water molecule which attacks the $\gamma$-phosphate of the bound ATP (Karata et al., 2001; Ogura and Wilkinson, 2001). Negative charges in the penta-coordinate transition state are stabilized in part by a magnesium ion. Amino acids from different conserved motifs stabilize interactions with phosphate groups or the magnesium ion, or are involved in activating the water by extracting a proton. The Walker-A motif (P loop) is Gly-(X)$_4$-Gly-Lys-Thr, where X is any amino acid (Ogura and Wilkinson, 2001; Saraste et al., 1990; Walker et al., 1982). The P-loop lysine interacts with the $\beta$- and $\gamma$-phosphates of bound ATP, while the threonine helps bind the magnesium. Walker B is (H)$_4$-Asp-Glu-(X)$_2$, where H is a hydrophobic amino acid and X is any residue. The aspartate also helps coordinate the magnesium ion, whereas glutamate is often the residue which activates a water molecule for attack. The sensor-I motif in the large $\alpha,\beta$-subdomain typically contains a conserved asparagine or threonine residue important for sensing whether the ATP $\gamma$-phosphate is present (Guenther et al., 1997; Koonin, 1993a), whereas the RFC box-II motif appears to be involved in recognition of the ATP adenine moiety (Guenther et al., 1997; Lenzen et al., 1998). Both the box-VII and
sensor-II motifs are found in the small C-terminal subdomain and both typically contain a highly conserved arginine. The box-VII arginine, however, is believed to sense nucleotide binding in an adjacent subunit, whereas the sensor-II arginine is important for sensing whether the terminal \( \gamma \)-phosphate of the ATP/ADP bound to the same subunit is present (Lenzen et al., 1998; Neuwald et al., 1999; Yu et al., 1998).

**Domain structure of E. coli Clp proteins.** ClpX (Kim and Kim, 2003), HslU (Bochtler et al., 2000; Sousa et al., 2000; Wang et al., 2001), ClpA (Guo et al., 2002b), and ClpB (Lee et al., 2003) all form hexameric rings with a central axial channel (Beuron et al., 1998; Ortega et al., 2000). ClpX and HslU contain a single AAA+ module, whereas ClpA and ClpB contain two modules (Figure 2). The ClpX and HslU \( \alpha,\beta \)-subdomains are homologous to the \( \alpha,\beta \)-subdomains in the second AAA+ module of ClpA and ClpB (Gottesman et al., 1993; Schirmer et al., 1996). Whereas ClpA’s first \( \alpha,\beta \)-subdomain (NBD1) is important for hexamerization and its second \( \alpha,\beta \)-subdomain (NBD2) is critical for ATP hydrolysis activity (Singh and Maurizi, 1994), the opposite is true for ClpB’s NBD1 and NBD2 (Barnett and Zolkiewski, 2002; Mogk et al., 2003b). In addition, ClpX contains a unique N-terminal domain (Donaldson et al., 2003), HslU contains an intermediate domain (I-domain) between the Walker-A and –B motifs in its AAA+ module (Schirmer et al., 1996), and ClpA and ClpB both have their own conserved N-terminal domains (Guo et al., 2002a; Li and Sha, 2003; Zeth et al., 2002). ClpB also contains a coiled-coil intermediate domain (I-domain) near the junction between its AAA+ modules (Lee et al., 2003; Schirmer et al., 1996). ClpX and ClpA, which both interact with the ClpP peptidase, also share a conserved tripeptide motif required for binding ClpP in a surface loop (ClpP-loop) in their homologous \( \alpha,\beta \)-subdomains (Kim et al., 2001; Singh et al., 2001). Interestingly,
alternative translational initiation sites (Seol et al., 1994; Squires et al., 1991; Woo et al., 1992) in both the clpA and clpB genes result in synthesis of some ClpA and ClpB proteins lacking the conserved N-terminal domain.

The subfamily-specific domains in the Clp/Hsp100 proteins may help mediate recognition of substrate proteins. ClpX and ClpXP bind substrates either directly, usually via N- or C-terminal peptide sequences (Flynn et al., 2003), or indirectly, via interactions with adaptor proteins which themselves bind substrates. Known ClpX adaptors include SspB (Levchenko et al., 2000), RssB (Zhou et al., 2001), and UmuD (Neher et al., 2003b). ClpX’s N-domain is important for processing λO and MuA, but not ssrA-tagged substrates (Ortega et al., 2000; Singh et al., 2001; Wojtyra et al., 2003). Likewise, delivery of ssrA-tagged substrates by SspB requires this N-domain (Dougant et al., 2003; Wojtyra et al., 2003). HslU’s I-domain is involved in recognition of some substrates but not others (Song et al., 2000). Like ClpXP, ClpAP also has an adaptor protein, ClpS (Dougant et al., 2002), which binds its N-domain (Dougant et al., 2002; Guo et al., 2002a; Zeth et al., 2002). In the presence of ClpP, a ClpA protein lacking the N-domain no longer degrades casein (Seol et al., 1994; Singh et al., 2001) or RepA (Lo et al., 2001), but still degrades ssrA-tagged substrates (Dougant et al., 2002; Lo et al., 2001; Singh et al., 2001). ClpB lacking its N-domain is defective for disaggregation of casein (Barnett et al., 2000; Park et al., 1993) and insulin (Park et al., 1993), but still can disaggregate heat-aggregated MDH and luciferase (Mogk et al., 2003b). Interestingly, specific ClpB N-domain point mutants have been constructed which bind but no longer disaggregate heat-treated luciferase (Liu et al., 2002), suggesting a role for this domain beyond substrate binding. Consistent with this model, the I-
Figure 2. Domain structure of *E. coli* Clp proteins

**ClpA:**
- **N-domain**
- ATPase 1
- ATPase 2

**ClpB:**
- **N-domain**
- ATPase 1
- ATPase 2

**ClpX:**
- **N-domain**
- ATPase 2

**HslU/ClpY:**
- ATPase 2
domain of ClpB has been suggested to be critical for substrate binding (Lee et al., 2003; Mogk et al., 2003b).

**Substrate unfolding and degradation by ClpXP and ClpAP**

ClpX and ClpA either unfold proteins or disassemble macromolecular complexes on their own, or act as the unfoldase components within the ClpXP or ClpAP proteases (Gottesman et al., 1997a; Gottesman et al., 1997b; Wickner et al., 1999). Protein unfolding and translocation provides a mechanism for substrate disassembly, as well as a mechanism to degrade substrates in association with a compartmentalized peptidase. In the former case, the substrate is released to the cellular environment, whereas, in the latter case, the substrate is degraded. ClpXP and ClpAP are composed of hexameric ClpX or ClpA rings stacked coaxially against the double-ring ClpP$_{14}$ peptidase (Beuron et al., 1998; Grimaud et al., 1998; Kessel et al., 1995; Ortega et al., 2000). The two heptameric ClpP rings are assembled face-to-face with the peptidase active sites at the internal ClpP ring interface (Wang et al., 1997). A channel or pore passes through the ClpX or ClpA hexamers into the degradation chamber of ClpP. This channel is too narrow to permit the passage of folded proteins (Wang et al., 1997) which must first be denatured and then translocated into ClpP prior to cleavage into peptides. Electron microscopy studies show that the channels in ClpXP and ClpAP are used for substrate translocation (Ishikawa et al., 2001; Ortega et al., 2002; Ortega et al., 2000).

ClpX and ClpA are responsible for substrate recognition by ClpXP and ClpAP. Generally, ClpX and ClpA bind specific peptide sequences at the N- or C-termini of substrate proteins (Flynn et al., 2003; Gonciarz-Swiatek et al., 1999; Gottesman et al., 1990a; Gottesman et al., 1998;
Hoskins et al., 2000a; Levchenko et al., 1997). Sometimes, however, these sequences are hidden and require other events, like proteolysis, in order to be accessible. For instance, intact LexA is not a ClpXP substrate, but its N-terminal domain becomes a substrate after it has been separated from LexA’s C-terminal domain by auto-proteolytic cleavage (Neher et al., 2003a). In other instances, adaptors are required for degradation. RpoS (σ^5) is a substrate for ClpXP when it is presented by a phosphorylated form of RssB, an adaptor protein (Hengge-Aronis, 2002; Pratt and Silhavy, 1996; Zhou et al., 2001). During stationary phase, however, RssB is not phosphorylated and no longer binds σ^5. Stable σ^5 is then free to activate transcription of genes needed in stationary phase. Adaptor proteins also can improve the recognition of substrates which can be bound on their own. For example, SspB improves recognition by ClpX of ssrA-tagged substrates (Levchenko et al., 2000). A further level of control exists in this instance because SspB binding to the ssrA tag prevents ClpA binding, and thus directs ssrA-tagged substrates to ClpX (Flynn et al., 2001; Gottesman et al., 1998). The adaptor, ClpS, acts in yet another way as it both redirects ClpA to act on aggregated proteins and inhibits ClpA from binding and degrading ssrA-tagged substrates (Dougan et al., 2002).

After ClpX or ClpA bind native substrate proteins, they unfold the proteins and either release them or translocate them to ClpP for degradation. Polypeptide cleavage by ClpP and subsequent peptide release occur very quickly (Thompson and Maurizi, 1994; Thompson et al., 1994), so either unfolding or translocation theoretically is rate limiting. Recent studies show that either step can be rate limiting in ClpXP degradation, depending on the substrate (Kenniston et al., 2003). ATP hydrolysis is needed for both unfolding and translocation (Burton et al., 2003; Hoskins et al., 2000b; Kenniston et al., 2003; Kim et al., 2000; Singh et al., 2000). While
ClpXP unfolds proteins, it hydrolyzes ATP at a constant rate but spends more time (and more ATP) when unfolding a more stable substrate (Kenniston et al., 2003). After substrates are bound via recognition tags, they move into the Clp protein channels in a directional way with regions close to the tags entering the channels first (Reid et al., 2001). In one model, Clp ATPases denature substrates by pulling them into the translocation channel by their recognition tags (Kenniston et al., 2003; Lee et al., 2001; Matouschek, 2003). Both ClpX and ClpA globally unfold substrate proteins (Burton et al., 2001a; Kim et al., 2000; Singh et al., 2000; Weber-Ban et al., 1999), if ClpP is not present, and release them in nonnative forms (Fenton et al., 1994; Kim et al., 2000; Singh et al., 2000; Weber-Ban et al., 1999). Only proteins with recognition tags are denatured, however, even when these proteins are in multimers with untagged proteins (Burton et al., 2001a; Burton et al., 2001b; Gonzalez et al., 2000).

Role of communication in substrate unfolding and degradation by ClpXP

Relatively little is known about the extent and importance of inter-subunit communication, either within a ClpX hexamer during substrate processing or between ClpX and ClpP during processing and degradation. To date, studies with E. coli ClpA, S. cerevisiae Hsp104, and Mus musculus p97 suggest that interdomain communication between parts of each protein’s two AAA+ modules is important for protein function. In ClpA, ATP binding or hydrolysis by the first nucleotide-binding domain (NBD1) is needed for communication to NBD2, and this signaling is critical for chaperone activity (Pak et al., 1999). Hsp104 uses interdomain communication in several ways. The six NBD1 (or NBD2) portions communicate with each other, as each domain shows cooperative ATP hydrolysis kinetics, and NBD2 communicates its nucleotidie-binding state to NBD1 within the same Hsp104 monomer (Hattendorf and Lindquist, 2002).
Functionally, communication upon peptide binding between NBD2’s α-helical C-terminal subdomain and NBD1 is needed for chaperone activity (Cashikar et al., 2002). A conformational change in Hsp104’s I-domain propagates the substrate-binding signal to NBD1, and NBD1 hydrolyzes more ATP in response. Like Hsp104, p97 has interdomain communication between its substrate-binding domain and its NBD (NBD2) which is important for ATP hydrolysis (DeLaBarre and Brunger, 2003). Communication also occurs in HslUV and the 26S proteasome between the ATPase and peptidase components. HslV’s channel into its peptidase active sites is open (Bochtler et al., 1997), unlike the channel into the S. cerevisiae 20S proteasome (Groll et al., 1997). In order for HslV to degrade substrates, however, its peptidase sites require activation by HslU, which is mediated by HslV binding HslU’s C-termini (Ramachandran et al., 2002; Seong et al., 2002; Sousa et al., 2002; Sousa et al., 2000). In contrast, the 19S regulatory particle physically opens up the channel into the 20S proteasome, allowing the already active peptidase sites access to translocated substrates (Groll et al., 2000). In both cases, communication between the ATPases and peptidases is needed for proteolysis.

My efforts have been directed toward addressing these questions about communication for ClpX and ClpXP. For ClpX, I have found that intersubunit communication between the α-helical C-terminal subdomain of one subunit and the adjacent α,β-subdomain of another subunit appears to be critical for proper response to substrate binding and for resulting unfolding activity (Chapter two). Amino acids in the conserved sensor-II helix are important for mediating this communication and help to connect nucleotide-binding sites in adjacent subunits of the ClpX hexamer. To test the extent of communication between ClpX and ClpP in ClpXP during substrate processing and degradation, I developed a solution assay to monitor the affinity
between both enzymes in the working protease. These results show that ClpX and ClpP interact more tightly during substrate denaturation than translocation, revealing that ClpX communicates its protein-processing task to ClpP (Chapter three). The apparent affinity between ClpP and ClpX strengthens significantly when ClpP’s active sites are modified and mimic intermediates during peptide-bond hydrolysis. Thus, ClpP also communicates to ClpX. Intersubunit contacts in a ClpX hexamer and some aspect of ATP cycling also affect interactions with ClpP. Overall, these studies demonstrate that communication between subunits within a ClpX hexamer and between enzymes in the ClpXP protease helps modulate activity and can be required for substrate processing activity.
References


CHAPTER TWO

C-terminal domain mutations in ClpX uncouple substrate binding from an engagement step required for unfolding

Summary

ClpX mediates ATP-dependent denaturation of specific target proteins and disassembly of protein complexes. Like other AAA+ family members, ClpX contains an αβ-ATPase domain and an α-helical C-terminal domain. ClpX proteins with mutations in the C-terminal domain were constructed and screened for disassembly activity in vivo. Seven mutant enzymes with defective phenotypes were purified and characterized. Three of these proteins (L381K, D382K & Y385A) had low activity in disassembly or unfolding assays in vitro. In contrast to wild-type ClpX, substrate binding to these mutants inhibited ATP hydrolysis instead of increasing it. These mutants appear to be defective in a reaction step that engages bound substrate proteins and is required both for enhancement of ATP hydrolysis and for unfolding/disassembly. Some of these side chains form part of the interface between the C-terminal domain of one ClpX subunit and the ATPase domain of an adjacent subunit in the hexamer and appear to be required for communication between adjacent nucleotide binding sites.
Introduction

ClpX and related members of the Clp/Hsp100 protein family are AAA+ ATPases (Neuwald et al., 1999) which serve as specialized energy-dependent molecular chaperones to unfold specific target proteins, disassemble multimeric complexes, and solubilize protein aggregates (Kim et al., 2000; Konieczny and Liberek, 2002; Levchenko et al., 1995; Seonga et al., 2000; Wawrzynow et al., 1995; Weber-Ban et al., 1999; Wickner et al., 1994; Zolkiewski, 1999). The minimal AAA+ unit contains an ATPase domain with a mixed $\alpha$$\beta$-structure and a C-terminal domain that is largely $\alpha$-helical, but most family members also contain additional structural domains (Bochtler et al., 2000; Maurizi and Li, 2001; Neuwald et al., 1999; Schirmer et al., 1996). ClpX subfamily members, for example, contain a short N-terminal Cys-cluster domain, an AAA+ ATPase domain, and a C-terminal AAA+ domain (Singh et al., 2001). One of the current challenges in understanding the biological activities of Clp/Hsp100 family members such as ClpX is to determine how the structural domains and interactions between these domains contribute to overall function.

ClpX forms a ring hexamer that unfolds target proteins and dismantles specific protein complexes in an ATP-dependent fashion; the ClpX hexamer can also serve as the substrate recognition and unfolding module of the ClpXP protease (Gottesman et al., 1997b). Different Clp/Hsp100 family members generally have distinct substrate recognition preferences (Gottesman et al., 1997a; Gottesman et al., 1997b; Suzuki et al., 1997). Well-characterized substrates of ClpX and ClpXP include the bacteriophage Mu transposase and proteins with ssrA degradation tags, both of which are recognized via C-terminal peptide sequences (Gonciarz-Swiatek et al., 1999; Gottesman et al., 1998; Kim et al., 2000; Levchenko et al., 1995;
Wawrzynow et al., 1995). Structural information is not available for ClpX, but several structures of the related HslU (ClpY) hexamer have been determined (Bochtler et al., 2000; Song et al., 2000; Sousa et al., 2000; Wang, 2001; Wang et al., 2001). The amino-acid sequences of the AAA+ domains from Escherichia coli ClpX and HslU are 38% identical over 303 common positions.

Potential roles for the α-helical domain in AAA+ family members include structural stabilization of hexameric or multimeric structures (Bochtler et al., 2000; Singh et al., 2001; Sousa et al., 2000), providing an arginine that serves as an active-site residue for ATP hydrolysis (Guenther et al., 1997; Jeruzalmi et al., 2001; Karata et al., 2001; Neuwald et al., 1999), and mediating recognition of specific protein and peptide substrates (Cashikar et al., 2002; Levchenko et al., 1997a; Smith et al., 1999). Some of these functions, however, are controversial (Feng and Gierasch, 1998; Ishikawa et al., 2001; Ortega et al., 2000; Singh et al., 2001; Wickner and Maurizi, 1999) and additional undiscovered roles for the α-helical AAA+ domain may also exist.

In this paper, we present a genetic and biochemical analysis of the functional roles of residues in the C-terminal domain of E. coli ClpX. By screening sequence substitutions in ClpX, we identified mutations in the C-terminal domain that caused reduced disassembly activity in vivo. Three of these proteins had low disassembly activity for one substrate and essentially no unfolding activity for another substrate in vitro. These mutant enzymes bound substrate proteins but displayed reduced ATP hydrolysis in response to binding, instead of increased hydrolysis. We propose that these ClpX mutants are defective in a reaction step that engages bound substrate proteins. This engagement step is required both for enhancement of ATP hydrolysis and for initiation of the unfolding and/or disassembly process.
Results

Mutant construction, screening, and purification. Using multiple sequence alignments and the structure of the homologous HslU protein (Bochtler et al., 2000; Song et al., 2000; Sousa et al., 2000; Wang, 2001; Wang et al., 2001) as guides, mutations were introduced into the C-terminal domain of *E. coli* ClpX by site-directed mutagenesis of a plasmid-borne gene encoding a variant of ClpX. We initially mutated residues that appeared to be solvent exposed based on the HslU structure and that differed between ClpX and other Clp/Hsp100 family members. After screening this initial set of mutations for activity as described below, we also mutated residues that neighbored positions found to be defective. In all, 50 mutations representing 44 different residue positions in ClpX were constructed.

Activity assays *in vivo* were based on the fact that ClpX is required for lytic growth of bacteriophage Mu because ClpX must disassemble DNA-bound tetramers of Mu transposase (MuA) before DNA replication and subsequent phage assembly and cell lysis can occur (Mhammedi-Alaoui et al., 1994; Nakai and Kruklitis, 1995). In this assay, ClpX-defective host cells containing a Mu lysogen with a temperature-sensitive repressor did not lyse and continued to grow normally after a shift to the restrictive temperature (Fig. 1A). When wild-type ClpX was provided on a plasmid, however, the host cells lysed roughly 30-40 minutes after the temperature shift (Fig. 1A). Most of the ClpX mutants (37 of 50) showed lysis kinetics indistinguishable from wild-type ClpX in this assay (Figs. 1A & 2A; data not shown). However, seven variants showed moderate delays in lysis (Figs. 1B & 2A) and an additional six ClpX mutants showed significant delays in lysis kinetics (Figs. 1C & 2A).
Figure 1. ClpX mutants support cell lysis by bacteriophage Mu to different degrees. (A) Kinetics of wild-type ClpX and three mutants (A364D, Q406E, & I412Q) with no lysis delay. (B) Kinetics of ClpX mutants displaying moderate lysis delays. (C) Kinetics of mutants with long lysis delays.
Figure 2. Positions of mutations in the C-terminal domain of ClpX. (A) Mutations of ClpX.

Dataset: ClpX.

colored boxes, yellow shown in moderately defective and severely defective mutants in green above the wild-type sequence. Mutations causing neutral phenotypes in the Mu lysis assay are shown in green above the wild-type sequence.
Figure 2. (B) Mutations were mapped onto the homologous *E. coli* HslU structure\(^{10}\), based on sequence alignments. The ATPase domains of subunits in the ClpX hexamer are colored dark or light gray. Residues in the C-terminal domain are colored dark blue (not mutated), green (neutral), yellow (moderately defective), or red (severely defective) based on their Mu lysis phenotypes.
Most of the defective mutations fell into one of two clusters on a homology model of ClpX (Fig. 2B). The first group, cluster I, included the L381K, D382K, and Y385A mutations and lies near the interface between the C-terminal domain of one subunit and the ATPase domain of the adjacent subunit (Figs. 2A & 2B). The second group, cluster II, included the M390D, V396A, V397T, D399R, and V402D mutations (Figs. 2A & 2B). The mutants that comprise clusters I and II were subjected to a purification protocol that included Ni\(^{++}\)-NTA chromatography and Mono Q anion-exchange chromatography in the presence of ATP. The D399R mutant could not be purified, presumably because of altered stability or solubility properties. The remaining seven mutant proteins purified in a manner similar to wild-type ClpX, except for slightly earlier or later elution from the ion-exchange column for mutants with additional positive/negative charges (data not shown).

**Disassembly of MuA-DNA complexes in vitro.** Based upon their phenotypes *in vivo*, we expected that the purified mutants would be defective in disassembling DNA complexes of the MuA tetramer *in vitro*. Indeed, the cluster-I mutants had substantial defects in disassembly *in vitro*, whereas the cluster-II mutants had disassembly activities that were usually intermediate between those of wild-type ClpX and the cluster-I mutants (Figs. 3A & 4B). There was general agreement between the phage Mu lysis assay and the MuA-DNA disassembly assay. In particular, the cluster-I mutants (L381K, D382K & Y385A) were among the most defective both *in vivo* and *in vitro* (Figs. 4A & 4B). Most cluster-II mutants, by contrast, showed intermediate levels of activity in both assays.
**Unfolding of GFP-ssrA.** The purified ClpX mutants were tested for their ability to denature green-fluorescent protein with a C-terminal ssrA tag (GFP-ssrA). The ssrA tag provides a recognition site for ClpX (Gottesman *et al.*, 1998; Kim *et al.*, 2000) and for SspB (Levchenko *et al.*, 2000), a specificity factor that enhances recognition of ssrA-tagged substrates by ClpX. As assayed by loss of native GFP-ssrA fluorescence, the cluster-I mutants had no detectable unfolding activity either in the presence of SspB (Figs. 3B & 4E; data not shown) or in its absence (data not shown). By contrast, the cluster-II mutants showed robust GFP-ssrA unfolding activities (Figs. 3B & 4E; data not shown). In fact, the M390D, V397T, and V402D mutants, which showed reduced disassembly of MuA-DNA complexes, were slightly more active than wild-type ClpX in denaturing GFP-ssrA.

**ATP hydrolysis and substrate-binding effects.** In ATPase assays, each of the seven purified ClpX mutants showed substantial rates of basal hydrolysis, although usually less than the wild-type rate (Fig. 5). L381K and D382K had the lowest ATPase activities, which were roughly half of the wild-type value. ATP hydrolysis by wild-type ClpX is stimulated in the presence of protein substrates (Wawrzynow *et al.*, 1995). The ATPase activities of the mutants were therefore assayed in the presence of different concentrations of GFP-ssrA and Arc-MuA10. Arc-MuA10 (Arc repressor fused to the 10 C-terminal residues of MuA) was used for these studies because intact MuA was not sufficiently soluble at the concentrations required for these assays, and the C-terminal residues of MuA are sufficient for mediating interactions with ClpX (Levchenko *et al.*, 1997b). The ATPase activity of wild-type ClpX was not stimulated by untagged Arc repressor (data not shown).
Figure 3. Disassembly and unfolding assays.

(A) Disassembly of MuA-DNA complexes in the presence of wild-type ClpX (with and without ATP) or the Y385A, V396A, and M390D mutants. (B) Unfolding kinetics of GFP-ssrA, assayed by the loss of native fluorescence at 511 nm, catalyzed by wild-type or mutant ClpX in the presence of SspB.
Figure 4. Summary of characterized ClpX mutant activities.

(A) Mu lysis activity in vivo.
(B) MuA-DNA disassembly activity in vitro.
(C) $K_{int}$ values for Arc-MuA10.
(D) Effect of substrate (Arc-MuA10 or GFP-ssrA) at saturating substrate concentration on ATP hydrolysis activity.
(E) GFP-ssrA unfolding activity in the presence of SspB.
(F) $K_{int}$ values for GFP-ssrA.
Figure 5. Basal ATPase activity.
**Figure 6. Substrate-dependent changes in ATP hydrolysis.** The panels on the left side show changes in ATP hydrolysis in the absence of SspB as a function of increasing concentrations of the substrate, GFP-ssrA, for wild-type ClpX, a cluster-I mutant (L381K), and a cluster-II mutant (M390D). The right-side panels show parallel experiments with a different substrate, Arc-MuA10.
The ATPase rates of each of the cluster-I and cluster-II mutants changed when GFP-ssrA or Arc-MuA10 was present (Figs. 4D & 6). The four cluster-II mutants, like wild type, showed increased ATP hydrolysis activity in the presence of both protein substrates. Strikingly, however, the ATPase activity of each cluster-I mutant decreased when either GFP-ssrA or Arc-MuA10 was added. Thus, the cluster-I mutants bind protein substrates, but respond to this binding in a manner opposite from the wild-type enzyme.

The changes in ClpX ATPase rates with protein-substrate addition were fit to obtain interaction constants ($K_{int}$) for GFP-ssrA and Arc-MuA10. $K_{int}$ for ClpX interaction with GFP-ssrA was 2.0 ± 0.59 μM, a value indistinguishable from the published $K_m$ for ClpXP degradation of GFP-ssrA (1.9 ± 0.3 μM; Kim et al., 2000). Six of the seven purified ClpX mutants, including all of the cluster-I mutants, had $K_{int}$ values for GFP-ssrA within 50% of the wild-type value (Fig. 4F). Hence, the inability of the cluster-I mutants to unfold GFP-ssrA is not caused by an inability to bind GFP-ssrA. One cluster-I mutant (D382K; $K_{int} = 0.99 ± 0.58$ μM) and one cluster-II mutant (V397T; $K_{int} = 0.34 ± 0.12$ μM) interacted with GFP-ssrA slightly more tightly than the wild-type enzyme (Fig. 4F).

$K_{int}$ for ClpX interaction with Arc-MuA10 was 12.5 ± 4 μM, a value similar to a previously reported interaction constant for Arc-MuA10 with ClpXP (14 ± 3 μM; Kim et al., 2000). The cluster-I mutants had $K_{int}$ values for Arc-MuA10 within 50% of the wild-type value (Fig. 4C). Once again, the poor MuA disassembly activities of these mutants cannot be explained by their inability to bind to substrate. The cluster-II mutants showed a wider range of interaction constants for Arc-MuA10. For example, relative to wild type, $K_{int}$ for Arc-MuA10 was increased.
about 1.5-fold for the M390D mutant and decreased about 3-fold for the V396A and V397T mutants (Fig. 4C).

**Discussion**

We constructed and studied the properties of *E. coli* ClpX mutants with site-directed amino-acid substitutions in the C-terminal domain. In the set of 50 mutants, 37 supported the replication of phage Mu and subsequent cell lysis in a manner indistinguishable from wild type. These positions are marked in green in the homology model of the ClpX hexamer shown in Fig. 2B. In 13 cases, however, we identified mutations that caused delays in Mu lysis. The majority of the defective mutations that caused modest lysis delays (colored yellow in Fig. 2B) or severe delays (colored red in Fig. 2B) formed two clusters on the surface of the C-terminal domain.

Cluster II includes the M390D, V396A, V397T, D399R, and V402D mutations. Most cluster-II mutants had relatively subtle lysis defects *in vivo* and the mutants that were purified from this group generally showed small changes in disassembly or unfolding activities, ATP hydrolysis rates, or substrate interaction constants. Compared to wild-type ClpX, most cluster-II mutants showed reduced activity in MuA-DNA disassembly but had slightly increased activity in GFP-ssrA unfolding. This distinctive substrate-specific behavior of the cluster-II mutants suggests that ClpX interacts with MuA and GFP-ssrA in somewhat different ways during protein unfolding. These differences are not, however, correlated with changes in binding to GFP-ssrA or to an Arc variant (Arc-MuA10) bearing the C-terminal ClpX-recognition residues of MuA. For example, the V396A ClpX mutant showed the poorest MuA-DNA disassembly activity among the cluster-II mutants but bound Arc-MuA10 about 3-fold more strongly than wild type.
The adjacent V397T mutant also bound Arc-MuA10 about 3-fold better than wild type but had the best MuA-DNA disassembly activity among the cluster-II mutants.

Because most of the cluster-II mutants caused relatively small changes in activity and substrate binding, it seems clear that these mutations do not define a substrate interaction site or a site required for ClpX unfolding activity. The cluster-II mutations probably exert their effects indirectly, possibly by destabilizing one enzyme conformation, thereby increasing the population of an alternative conformation.

The purified cluster-I mutants were quite defective in disassembling MuA-DNA complexes in vitro and displayed essentially no ability to catalyze denaturation of another ClpX substrate, GFP-ssrA. These activity deficiencies do not appear to result from major folding or stability defects, as the L381K, D382K, and Y385A mutants had purification and solubility properties similar to wild-type ClpX, and had basal ATPase activities within two-fold of wild type. Moreover, the most severely deficient mutant (Y385A) formed oligomeric complexes indistinguishable from wild-type ClpX in formaldehyde crosslinking experiments in the presence of ATP (data not shown). The dramatic reduction in GFP-ssrA denaturation does not result from an inability of the cluster-I mutants to bind substrates. Each of these mutants have $K_{int}$ values for GFP-ssrA within 50% of the wild-type value. Similarly, all three mutants have $K_{int}$ values for Arc-MuA10 within 2-fold of the wild-type value. One of the most interesting characteristics of the cluster-I mutants is that they display reduced ATPase activities in response to GFP-ssrA or Arc-MuA10 binding. Wild-type ClpX and all of the cluster-II mutants, by contrast, hydrolyze ATP more rapidly in response to substrate binding.
In the structure of the HslU hexamer, each C-terminal domain packs tightly against the ATPase domain of an adjacent subunit, providing a potential avenue of communication between ATP/ADP binding sites in adjacent subunits. The sensor-II helix in the C-terminal AAA+ domain (residues 369-387 in ClpX) plays a central role in this interaction. At the N-terminal end of this helix, the side chain of an invariant Arg residue (Arg370 in *E. coli* ClpX) contacts the nucleotide bound to its own ATPase domain. At the C-terminal end of the sensor-II helix, the side chain of an aromatic residue (Tyr385 in *E. coli* ClpX) intercalates between side chains from \( \alpha \)-helix I and II of the adjacent subunit's ATPase domain, and side chains in the box-II loop connecting helices I and II contact the nucleotide in this domain. This arrangement is illustrated in Fig. 7. Hence, the Y385A ClpX mutation could disrupt subunit-subunit interactions which, in turn, regulate coordinated ATP hydrolysis.

One model that could explain the properties of the cluster-I mutants is as follows. In an initial uncommitted binding step, protein substrates bind to ClpX in the ATP state. This uncommitted complex has decreased ATPase activity relative to the basal rate. Normally, the enzyme then engages the bound protein in a second reaction step that requires communication between adjacent subunits of the ClpX hexamer and some type of conformational change. The ATPase activity of the protein-bound ClpX in this engaged state is higher than the basal rate. The engaged-state could be one in which the native protein is subjected to mechanical forces by the enzyme which increase the probability of substrate denaturation. By this model, progression from the uncommitted binding state to the engaged state would occur significantly more slowly for the cluster-I mutants. We assume that the rate of ATP hydrolysis sets the cycle time for
Figure 7. Y385A could disrupt communication between nucleotide-binding sites in adjacent subunits.

The position of Tyr385 in the sensor-II helix of ClpX is shown in a homology model based on amino-acid sequence alignments between *E. coli* ClpX and *H. influenzae* HslU. A conserved arginine (Arg370 in *E. coli* ClpX; Arg394 in *H. influenzae* HslU) at the N-terminal end of the sensor-II helix contacts the nucleotide in its own subunit. At the C-terminal end of the sensor-II helix, a conserved aromatic side chain (Tyr385 in *E. coli* ClpX; Phe409 in *H. influenzae* HslU) intercalates between side-chains from $\alpha$-helices I and II in the ATPase domain of the adjacent subunit. In turn, the box-II loop connecting $\alpha$-helices I and II contacts the bound nucleotide in this subunit.
ClpX. Because the overall rate of hydrolysis is similar for the free enzyme, the uncommitted complex, and the engaged complex, mutants that spend substantially more time in uncommitted complex will spend less time in the engaged complex. For substrates like GFP-ssrA, denaturation might not occur in the cluster-I mutants for two reasons. The GFP-ssrA could dissociate from the uncommitted complex before engagement occurs, or the GFP-ssrA could reach the engaged complex slowly but spend too little time in this state to have a significant probability of being denatured.

The model presented above makes several predictions. First, if communication between domains mediated by the sensor-II helix is important, then mutations at the N-terminus of this helix and/or in the parts of the ATPase domain that contact this helix should cause phenotypes similar to the cluster-I mutations. Second, complexes of the cluster-I ClpX mutants with substrates like GFP-ssrA should have kinetic and/or physical properties that differ markedly from complexes of wild-type ClpX with GFP-ssrA. Experimental tests of these predictions are in progress.

**Note added in proof:** The P67H mutation, which alters an ATPase-domain residue that contacts Y385, causes severe defects in Mu lysis and in GFP-ssrA unfolding. This mutant, like the cluster-I mutants, binds GFP-ssrA like wild type and is a substrate-inhibited ATPase, supporting the model that contacts between Y385 and the ATPase domain regulate the coordination of ATP hydrolysis.
Experimental procedures

Strains and Plasmids. To construct *E. coli* strain SJ9, a bacteriophage Mu-cts62 lysogen of strain W3110 (kindly provided by Maurice Fox, MIT) was infected with a P1 lysate prepared from *E. coli* strain SG22177 (Gottesman *et al.*, 1998) and *clpX::kan* transductants were selected. The wild-type ClpX protein with an N-terminal His\(_6\)-tag was encoded in a pET-14b-ClpX vector (Levchenko *et al.*, 1997b). Overlap extension PCR mutagenesis (Ho *et al.*, 1989) was used to introduce each point mutation and a new restriction site into a portion of the *clpX* gene encoding the C-terminal domain, and the resulting DNA fragments were cloned into pET-14b-ClpX containing the rest of the *clpX* gene. Following transformation into *E. coli* strain DH5\(\alpha\), plasmid DNA was isolated from ampicillin-resistant candidates and screened for the new restriction site by digestion with an appropriate restriction enzyme. The plasmid region that was PCR amplified during mutagenesis was sequenced to confirm the presence of the expected mutations.

Solutions. NB buffer contained 50 mM HEPES (pH 7.6), 500 mM NaCl, 40 mM imidazole, 15% glycerol, and 5 mM \(\beta\)-mercaptoethanol. NE buffer was NB buffer with 500 mM imidazole instead of 40 mM imidazole. QB buffer contained 50 mM HEPES (pH 7.6), 15% glycerol, 3 mM dithiothreitol (DTT), 100 \(\mu\)M ZnSO\(_4\), 100 \(\mu\)M ATP, 1 mM MgCl\(_2\), and 0.01% Nonidet P-40 (NP-40). ML buffer contained 43 mM Tris•Cl (pH 8.0), 6% sucrose, 66 mM NaCl, 3.3 mM EDTA, 0.66 mM DTT, 13.3 mM spermidine-HCl, and 19.4 mg mL\(^{-1}\) ammonium sulfate. H buffer contained 25 mM HEPES (pH 7.6), 0.1 mM EDTA, 1 mM DTT, and 10% glycerol. AH buffer contained 42 mM HEPES (pH 7.6), 6.2 mM Tris•Cl (pH 8.0), 133 mM KCl, 5.3 mM MgCl\(_2\), 312 \(\mu\)M EDTA, 1 mM DTT, 34 \(\mu\)M ZnSO\(_4\), 34 \(\mu\)M ATP, 0.035% NP-40, and 15%
glycerol. ATP mix I contained 2.5 mM ATP, 1 mM NADH, 7.5 mM phosphoenolpyruvate, 18.75 U mL\(^{-1}\) pyruvate kinase, and 21.45 U mL\(^{-1}\) lactate dehydrogenase.

**Proteins.** His\(_6\)-tagged ClpX proteins were purified from *E. coli* strain BL21(DE3)/pET-14b-ClpX cells grown at 30 °C in 1.33% tryptone, 2.66% yeast extract, 0.44% glycerol, and 100 μg mL\(^{-1}\) ampicillin. Protein expression was induced with 0.6 mM IPTG, and cells were harvested three hours after induction. Cell pellets were resuspended at 4 °C in 3 mL of NB buffer per gram of cells, and protease-inhibitor cocktail set II (Calbiochem) was added as recommended by the manufacturer. Cells were lysed using a French press, and the lysate was centrifuged for one hour at 15,000 rpm in a Sorvall SA-600 rotor. The supernatant was filtered through a 0.2 μm filter and mixed by rotation with Ni-NTA resin (equilibrated in NB buffer) for 45 minutes at 4 °C. A column was packed by gravity flow at 4 °C and then washed with 100 column volumes of NB buffer. Bound protein was eluted with five column volumes of NE buffer and buffer exchanged into QB buffer plus 150 mM KCl using a PD-10 desalting column (Amersham Pharmacia Biotech). This material was applied to a Mono Q HR 5/5 column (Amersham Pharmacia Biotech), which was developed with a 10-volume linear gradient in QB buffer from 150 to 500 mM KCl. Peak absorbance fractions were analyzed by SDS-polyacrylamide gel electrophoresis, and those containing ClpX protein were pooled, divided into aliquots, and stored at –80 °C.

MuA protein, expressed in *E. coli* strain BL21(DE3)/pMK591 (Baker *et al.*, 1993), was purified using a protocol based on a previously published procedure (Williams *et al.*, 1999). Cells were resuspended in ML buffer and lysed by French press. After precipitation with ammonium sulfate, pellets were collected by centrifugation, resuspended in H buffer plus 100 mM NaCl,
filtered through 0.2 μm filters, and buffer exchanged into H buffer plus 300 mM NaCl using a PD-10 column. This material was applied to a SP Sepharose Fast Flow HR 5/5 column (Amersham Pharmacia Biotech), and a five-volume linear gradient in H buffer from 300 to 1000 mM NaCl was run. Fractions containing MuA were pooled, buffer exchanged into H buffer plus 300 mM NaCl, and loaded onto a Mono S HR 5/5 column (Amersham Pharmacia Biotech), which was developed with a five-volume linear gradient in H buffer from 300 to 1000 mM NaCl. Peak fractions were pooled, divided into aliquots, and stored at –80 °C.

GFP-ssrA was purified using a published protocol (Yakhnin et al., 1998). Purified SspB protein was a gift from David Wah (MIT). Arc-MuA10 (Levchenko et al., 1997b) was purified using a protocol developed for other Arc variants (Burton et al., 2001b). UV absorbance or dye binding (Bradford, 1976) were used to determine final protein concentrations.

**Mu lysis screen.** Overnight cultures of SJ9 host cells containing wild-type or mutant pET-14b-ClpX plasmids were grown in Luria-Bertani broth at 30 °C with 100 μg mL⁻¹ ampicillin. These cultures were diluted 1:100 and grown until the absorbance at 540 nm was between 0.40 and 0.45. The cultures were shifted to 42 °C for 30 minutes and then shifted to 37 °C for 50 minutes. After the initial temperature shift, absorbance readings at 540 nm were taken every 10 minutes.

**Biochemical assays.** MuA-DNA disassembly gel-shift assays were performed as described (Burton et al., 2001a), except 0.1 μM ClpX₆ was used in each reaction. GFP-ssrA unfolding reactions (30 °C) were performed as described (Kim et al., 2000) using 0.26 μM ClpX₆ with or without 0.46 μM SspB protein. ATPase assays (30 °C) were performed with 0.3 μM ClpX₆ in
AH buffer containing ATP mix I but otherwise followed the published protocol (Kim et al., 2001).
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References


CHAPTER THREE

Communication between ClpX and ClpP during substrate processing and degradation


Figure 1: work of G.L.H.
Figures 2-6: work of S.A.J.
Summary

ClpXP is a compartmental protease in which ring hexamers of the AAA+ ClpX ATPase bind, denature, and then translocate protein substrates into the degradation chamber of the double-ring ClpP14 peptidase. A key question is the extent to which functional communication between ClpX and ClpP occurs and is regulated during substrate processing. Here, we show that ClpX-ClpP affinity varies with the protein-processing task in which ClpX is engaged and with the catalytic engagement of the ClpP active sites. Functional communication between the symmetry-mismatched ClpX and ClpP rings depends on the ATPase activity of ClpX and appears to be transmitted through changes in the conformation, flexibility, or accessibility of its IGF loops which contact ClpP. The protein unfolding activity of ClpX mutants with substitutions at an interface between ATPase modules can be rescued by ClpP in a manner that suggests that energy-dependent denaturation of protein substrates generates tension and quaternary distortions at these ClpX interfaces. Communication between ClpX and ClpP appears to stabilize ClpP binding to substrate-engaged ClpX molecules and is probably required for coordination of substrate processing and degradation.
Introduction

AAA+ ATPases function as essential components of energy-dependent compartmental proteases in all biological kingdoms\(^1\). For example, the 19S portion of the eukaryotic proteasome consists predominantly of AAA ATPases, which help recognize and translocate substrates to an associated 20S core protease\(^2\). In bacteria, the ClpXP and ClpAP proteases consist of either the ClpX or ClpA ATPase and the ClpP peptidase, and the HslUV protease consists of the HslU ATPase and the HslV peptidase\(^1,3,4\). In each of these energy-dependent proteases, the active sites used for polypeptide cleavage are sequestered in a degradation chamber formed by a multi-subunit complex with a barrel-like shape\(^5-14\). Entry portals, which are too small to admit native proteins, provide access to this chamber. The ATPases of the compartmentalized proteases from bacteria form ring hexamers that bind appropriate protein substrates and, in reactions dependent upon ATP hydrolysis, unfold these molecules and translocate them through a central protein-processing pore and into the peptidase chamber, where degradation to small peptides occurs\(^1,3,4\).

One key question for all energy-dependent proteases is how interactions between the ATPase and the peptidase allow coordination of substrate processing and degradation. For HslUV, the peptidase and ATPase are both six-fold symmetric and structures of the complex in different nucleotide-bound states have been solved\(^9,11,13,15,16\). These structures show that nucleotide binding modulates the size of both the protein-processing pore and the entry portal and also alters the contacts between HslU and HslV, propagating structural changes to the peptidase active sites. These structural changes undoubtedly regulate the functional communication which has been observed between the hexameric HslU and HslV rings\(^11,15-20\). For ClpXP and ClpAP, docking of the ATPases and peptidase involves a symmetry mismatch between a hexameric ATPase ring and a heptameric ClpP ring\(^7,8,21-23\). Although high-resolution structures of ClpXP or ClpAP have not been solved, flexible surface loops in both ClpX and ClpA, which contain a IGF or IGL motif, have been implicated in ClpP binding\(^24-27\). For both ClpXP and ClpAP, coordination between the activities of the 6-fold symmetric ATPase and 7-fold symmetric
peptidase must occur, as binding of ClpA activates ClpP peptidase activity\textsuperscript{28}, proteins trapped in the degradation chamber of inactive ClpP can be released in a reaction that depends upon ATP hydrolysis by ClpX\textsuperscript{29}, and the ATPase activity of ClpX is depressed upon binding to ClpP\textsuperscript{24}.

To probe functional communication between the ClpX and ClpP enzymes of \textit{Escherichia coli}, we have used changes in ATPase activity to monitor and quantify the strength of the ClpX-ClpP interaction. We find that ClpP binds most tightly to ClpX when the ATPase is denaturing protein substrates, less tightly during translocation of substrates, and least tightly in the absence of substrates. ClpX is also able to detect the catalytic status of the ClpP active sites, as evidenced by a significant increase in affinity when the active-site serines of ClpP are modified. ClpP binding suppresses the protein-unfolding defects of ClpX variants with mutations at an intersubunit interface, but rescue occurs at the expense of binding affinity and reverses the response to substrate processing. This result suggests that subunit-subunit interactions in wild-type ClpX play an important role in the unfolding of protein substrates by preventing quaternary distortions that prevent substrate denaturation and weaken ClpP binding. Finally, we demonstrate linkage between the nucleotide state of ClpX and functional interactions with ClpP.

\textbf{Results}

\textbf{Substrate design.} Protein substrates had C-terminal ssrA tags to target them to ClpX\textsuperscript{30}. Unfolding of GFP-ssrA by ClpX or degradation by ClpXP results in loss of fluorescence\textsuperscript{29,31}. Unlabelled and \textsuperscript{35}S-labelled variants of the human titin-I27-ssrA protein were degraded by ClpXP either as native proteins or as denatured, carboxymethylated (CM) molecules\textsuperscript{32}.

\textbf{ClpP interaction requires more than two IGF loops.} The IGF loop (residues 264-278) of \textit{E. coli} ClpX mediates binding to ClpP\textsuperscript{24,25}. We constructed and purified ClpX\textsuperscript{loopless}, a variant in which the IGF loop was replaced with a short linker. As expected, ClpX\textsuperscript{loopless} did not support degradation of an ssrA-tagged protein substrate in the presence of ClpP (Fig. 1A), and did not
bind His$_6$-tagged ClpP in Ni$^{2+}$-NTA pull-down assays (data not shown). ClpX$^{\text{loopless}}$ was, however, as active as wild-type ClpX in unfolding GFP-ssrA as monitored by loss of native GFP fluorescence (Fig. 1B). Moreover, ClpX$^{\text{loopless}}$ formed stable ternary complexes with GFP-ssrA and the delivery protein SspB as assayed by gel filtration in the presence of ATP$_7$S (Fig. 1C). These mutant complexes chromatographed at the same position as wild-type ternary complexes, suggesting that ClpX$^{\text{loopless}}$, like ClpX, is hexameric under these conditions. We conclude that ClpX$^{\text{loopless}}$ fails to interact with ClpP but otherwise assembles normally and is fully active in binding and denaturing ssrA-tagged substrates.

It is not known how many IGF loops are required for functional interactions between ClpP and a ClpX hexamer, which assembles as a trimer of stable dimers. An IGF-loop peptide bound ClpP very weakly (K$_d$>200 μM; data not shown), suggesting that stable ClpP binding requires interaction with several IGF loops and/or requires the loop to be held in a specific conformation by ClpX. To test the activity of hexamers containing mixtures of wild-type and loopless dimers, we added ClpX$^{\text{loopless}}$ to wild-type ClpX and assayed degradation of an ssrA-tagged substrate in the presence of excess ClpP. As shown in Fig. 1D, a modest excess of ClpX$^{\text{loopless}}$ inhibited degradation, with the data fitting well to a model in which hexamers with only one wild-type dimer have less than 1% activity, those with two wild-type dimers are roughly 60% active, and mixing of wild-type and mutant dimers is relatively unbiased. We confirmed that wild-type and loopless dimers do in fact form mixed multimers by showing that untagged ClpX$^{\text{loopless}}$ subunits bound Ni$^{2+}$-NTA only when mixed with His$_6$-tagged wild-type ClpX (data not shown). Thus, we conclude that functional interactions between ClpX and ClpP require IGF loops in at least two of the dimers that comprise the ClpX hexamer.

**An ATPase assay for ClpX-ClpP affinity.** To determine ClpX-ClpP affinity, we took advantage of the observation that ClpP binding decreases the rate of ATP hydrolysis by ClpX$^{24}$. ATP turnover by 50 nM ClpX$_6$ was assayed in the presence of ClpP$_{14}$ concentrations ranging
from 0 to 2.5 \textmu M. The change in observed rates fit well to a simple binding isotherm, whereas no significant changes were observed for the ClpX\textsuperscript{loopless} control (Fig. 2A). This experiment confirms the importance of the IGF loop in binding ClpP and indicates that the changes in ATP hydrolysis observed when ClpP binds to wild-type ClpX are mediated by the same set of interactions that permit collaboration in protein degradation. Assays performed with 200 nM ClpX\textsubscript{6} gave similar results, with the combined data obtained for the two ClpX concentrations fitting a model in which ClpX and ClpP interact with an apparent equilibrium dissociation constant (K\textsubscript{app}) of 126 ± 15 nM (Fig. 2B). It is important to note that this apparent affinity constant reflects a population-weighted average of the actual K\textsubscript{d} values of ClpP for ClpX in each of its different enzymatic and conformational states as it passes through its cycles of ATP hydrolysis and coupled conformational changes.

**Substrate processing strengthens the ClpX-ClpP interaction.** Recent studies using titin-ssrA substrates with a range of thermodynamic stabilities have shown that ClpX denatures these molecules at different rates but translocates the denatured proteins to ClpP at the same rate\textsuperscript{32}. Hence, we reasoned that by assaying ClpX-ClpP affinity during the degradation of these titin substrates, it should be possible to determine whether ClpX engaged in substrate degradation binds ClpP in a different manner than in the absence of substrate and, if so, to deconvolute the contributions of protein denaturation and translocation to this difference. Changes in the ATPase rate of ClpX were used to monitor ClpP affinity in the presence of titin-ssrA substrates that were roughly 10-fold above the K\textsubscript{m} for denaturation to ensure approximately 90% saturation of the enzyme by substrate. When ClpP was titrated against ClpX in the presence of the most stable native substrate, wild-type titin-ssrA, K\textsubscript{app} was 38 ± 4 nM, a decrease of roughly three-fold compared to the absence of substrate (Figs. 2A & 3A). In the presence of less stable native variants, K\textsubscript{app} values were 54 ± 6 nM (V15P) and 84 ± 14 nM (V13P). When a denatured titin substrate (CM-V13P) was present, K\textsubscript{app} was 105 ± 8 nM. With other denatured substrates (CM-titin; CM-V15P), K\textsubscript{app} values were within error of the value determined for CM-V13P (data not
**Figure 1. Properties of ClpX^{loopless}.** (A) ClpX_{6}^{loopless} (100 nM) did not support degradation of CM-titin-V13P-ssrA (1 mM) in the presence of ClpP_{14} (300 nM), whereas wild-type ClpX mediated efficient degradation under these conditions. (B) GFP-ssrA (1 mM) in the presence of SspB_{2} (1 mM) was unfolded at essentially the same rate by ClpX_{6} or ClpX_{6}^{loopless} (300 nM) as assayed by loss of native GFP-ssrA fluorescence. (C) ClpX^{loopless} forms stable ternary complexes. ClpX_{6} or ClpX_{6}^{loopless} (6 mM), SspB_{2} (3 mM), GFP-ssrA (6 mM), and ATP$_{S}$ (5 mM) were chromatographed on a Superdex 200 column. The elution position of GFP-ssrA was monitored by A$_{500}$. Positions of the ternary complex (X$_{6}$S$_{2}$G$_{2}$), binary complex of SspB and GFP-ssrA (S$_{2}$G$_{2}$), and free GFP-ssrA (G) are shown. (D) Degradation of titin-V4A-ssrA (5 mM) by wild-type ClpXP (100 nM ClpX$_{6}$; 2 mM ClpP$_{14}$) was inhibited by addition of ClpX$_{6}^{loopless}$. The solid line is a fit to eq. 2 (see Methods) with a bias factor of 0.99, an A$_{21}$ value of 0.63, and an A$_{12}$ value of 0.003.

*Work of Greg L. Hersch*
Figure 2. An assay for ClpX-ClpP interaction in solution. (A) Changes in the ATP hydrolysis rate of 50 nM ClpX6 or ClpX6\textsuperscript{loopless} as a function of ClpP concentration. (B) Percent inhibition of ATP turnover as a function of ClpP concentration. The filled symbols are for experiments performed with 200 nM ClpX6 and the open symbols for 50 nM ClpX6. The fitted line is that expected for a one-to-one binding reaction with an apparent affinity of 126 ± 15 nM.
Figure 3. ClpX-ClpP affinity changes in an ATPase-dependent fashion during substrate denaturation and translocation. (A) Binding of ClpP to 50 nM ClpX\textsubscript{6} in the presence of 15 M concentrations of four variants of titin-ssrA. The fits represent $K_{app}$ values of $38 \pm 4$ nM for wild-type titin-I27-ssrA, $54 \pm 6$ nM for the native V15P mutant, $84 \pm 14$ nM for the native V13P variant, and $105 \pm 8$ nM for the denatured CM-V13P protein. (B) $K_{app}$ values for the ClpX-ClpP interaction in the presence of titin-ssrA substrates vary linearly with the rate constant for ClpXP degradation of these substrates\textsuperscript{32}. The line is a fit to eq. 1 (see Methods), with affinities of 38 and 99 nM, respectively, when ClpXP is denaturing or translocating titin-ssrA substrates. (C) $K_{app}$ for the ClpX-ClpP interaction correlates linearly with the ClpXP ATPase rate ($R = 0.99$) and the ClpX ATPase rate ($R = 0.97$) during the processing of different titin-ssrA substrates.
These results show that the apparent affinity for ClpP is stronger when ClpX is processing protein substrates, with the strongest binding observed for native substrates that are denatured most slowly.

$K_{\text{app}}$ for ClpX-ClpP binding in the presence of different titin-ssrA substrates showed a linear correlation (R = 0.99) with the rate constant for degradation of these proteins (Fig. 3B). This relationship is expected if ClpX has one affinity for ClpP while denaturing titin substrates and a different affinity while translocating these substrates (see Methods). Indeed, the linear fit to the data in Fig. 3B represents a model in which $K_{\text{app}}$ is 38 nM when ClpX is denaturing a titin-ssrA substrate and 99 nM when ClpX is translocating these substrates. These results show that the interaction between ClpX and ClpP is stronger during substrate denaturation than during translocation and demonstrate the existence of a molecular mechanism by which ClpP is able to "sense" the protein-processing task in which ClpX is engaged. The apparent ClpX-ClpP affinities were also well correlated with the overall rates of ATP turnover by ClpXP and ClpX in the presence of these titin-ssrA substrates (Fig. 3C). Based on this observation, we suggest that changes in the ATPase rate of ClpX during substrate processing may be an important factor in determining the apparent affinity for ClpP.

**Active-site communication between ClpP and ClpX.** If ClpP can sense whether ClpX is processing protein substrates, then ClpX may be able to detect whether ClpP is degrading substrates. To address this possibility, we used ClpP$_{\text{DFP}}$, a variant in which the active-site serines (S97) were covalently modified by reaction with di-isopropyl-fluorophosphate$^{29}$. Although ClpP$_{\text{DFP}}$ is inactive in protein degradation, the chemical modification mimics the acyl-enzyme intermediate in the normal peptide-bond hydrolysis reaction. Moreover, an oxygen of DFP binds in the oxyanion hole of the ClpP active site, resembling the carbonyl oxygen of a bound peptide substrate$^7$. ClpP$_{\text{DFP}}$ bound ClpX so strongly that the bound and total concentrations of ClpX were essentially the same (Fig. 4). Under these “stoichiometric binding” conditions it was not
possible to determine an affinity constant but this value must be less than 5 nM. Hence, ClpX appears to be capable of sensing whether the ClpP active sites are engaged with substrate. To ensure that the tighter ClpX binding observed for ClpP\textsuperscript{DFP} was caused by modification of the active-site S97, we also assayed binding of a ClpP\textsuperscript{S97A} mutant after treatment with DFP\textsuperscript{35}. DFP-treated ClpP\textsuperscript{S97A} bound ClpX with roughly the same affinity as wild-type ClpP and much more weakly than ClpP\textsuperscript{DFP} (Fig. 4). We conclude that the strong ClpX binding observed for ClpP\textsuperscript{DFP} results directly from acylation of the active-site serines and/or from concomitant substrate-like interactions of the covalent modification.

**ClpP rescues the unfolding defects of ClpX mutants.** In a previous study, we showed that mutations in the C-terminal portion of the ClpX sensor-II helix, which forms part of the interface between the C-domain of one subunit and the ATPase domain of an adjacent subunit, caused defects in substrate unfolding but not in substrate binding\textsuperscript{36}. Because ClpP binds more tightly when ClpX is denaturing substrates, we reasoned that ClpP binding might suppress the unfolding defects of the sensor-II mutants. This result was observed. By themselves, the L381K and D382K mutants of ClpX had undetectable activities in unfolding GFP-ssrA (Fig. 5A). In the presence of ClpP\textsuperscript{DFP}, however, ClpX\textsuperscript{L381K} and ClpX\textsuperscript{D382K} catalyzed efficient unfolding of GFP-ssrA (Fig. 5B). Unmodified ClpP also suppressed the unfolding defects of the sensor-II mutants, albeit less efficiently than ClpP\textsuperscript{DFP} (data not shown). Because ClpP\textsuperscript{DFP} suppression of the unfolding defect of the D382K mutant was most efficient, we selected this ClpX variant for more detailed studies.

In the absence of protein substrate, ClpP bound ClpX\textsuperscript{D382K} with an apparent affinity roughly two-fold weaker (230 ± 60 nM; Fig. 5C) than for wild-type ClpX. In the presence of protein substrates, however, the observed affinity of ClpP for ClpX\textsuperscript{D382K} was reduced even more. For example, $K_{\text{app}}$ was almost 10-fold weaker (2.4 ± 0.5 μM) in the presence of the V13P variant of titin-ssrA and about 5-fold weaker (1.2 ± 0.4 μM) in the presence of denatured CM-titin-ssrA.
Figure 4. Modification of the ClpP active sites strengthens ClpX binding. ClpP<sub>DFP</sub> bound tightly to 50 nM ClpX<sub>6</sub> as assayed by changes in ATP hydrolysis. ClpP<sub>14</sub> can bind two ClpX hexamers and, at half-maximal binding, the total concentration of ClpP<sub>14DFP</sub> was roughly 15 nM and the concentration of bound ClpX<sub>6</sub> was 25 nM. Although an accurate $K_{\text{app}}$ cannot be determined from these data, the upper limit for this constant is [5 nM. $K_{\text{app}}$ for the interaction of ClpX with DFP-treated ClpP<sub>S97A</sub> (130 nM) was similar to that for wild-type ClpP, confirming that the tight binding of ClpP<sub>DFP</sub> results from modification of the active-site S97 side chain.
Figure 5. ClpP rescues the unfolding defects of ClpX mutants. The sensor-II helix mutants, ClpXD382K and ClpXL381K, fail to unfold GFP-ssrA (310 nM) by themselves (panel A) but unfold this substrate efficiently in the presence of 800 nM ClpP14DFP (panel B). In these experiments, the concentrations of mutant or wild-type ClpX6 were 260 nM, and 460 nM SspB was present. ClpP binds 200 nM ClpX6D382K with an apparent affinity of 230 ± 60 nM in the absence of substrate (panel C) but binds more weakly in the presence of a denatured substrate (13.1 μM CM-titin-ssrA; K_{app} = 1.2 ± 0.3 μM) or a native substrate (15 μM titin-V13P-ssrA; K_{app} = 2.4 ± 0.5 μM) (panel D).
When ClpX\textsuperscript{D382K} engages a protein substrate, it appears to assume a conformation poorly suited both for binding ClpP and substrate unfolding. Although ClpP can stabilize ClpX\textsuperscript{D382K} in a conformation active in substrate processing, binding energy must be used to drive the accompanying conformational change and thus the observed overall affinity for ClpP is weakened significantly. Interestingly, addition of ClpP to ClpX\textsuperscript{D382K} increased rates of ATP hydrolysis in the presence of protein substrates, whereas ATP turnover decreased when ClpP was added in the absence of protein substrates (Figs. 5C and 5D). This pattern is different from that observed for the wild-type interaction, where ClpP binding always reduced ClpX ATPase rates with or without substrate (Figs. 2A & 3A).

**Nucleotide-state of ClpX controls ClpP binding.** The experiments presented above suggest that the ATPase activity of ClpX is linked, in some fashion, to interactions with ClpP. In pull-down assays, untagged ClpP bound to Ni\textsuperscript{2+}-NTA in the presence of His\textsubscript{6}-tagged ClpX and ATP\textsubscript{γS} but did not bind well when ATP or ADP were present (Fig. 6A). ATP is hydrolyzed rapidly by ClpX, raising the possibility that ADP•ClpX may predominate even with excess ATP because ADP release is slow. By contrast, ATP\textsubscript{γS} is hydrolyzed slowly by ClpX\textsuperscript{37}, and thus ATP\textsubscript{γS}•ClpX may be a better mimic of the ATP state.

In the crystal structure of *H. pylori* ClpX, Arg396 in the N-terminal portion of the sensor-II helix is positioned to contact bound nucleotide\textsuperscript{27}. This residue is conserved in the ClpX and HslU families, suggesting an important functional role. We constructed and purified a mutant of *E. coli* ClpX in which the corresponding residue, Arg370, was mutated to lysine. ClpX\textsuperscript{R370K} failed to hydrolyze ATP (Fig. 6B) but bound fluorescent nucleotide analogs with K\textsubscript{d} values of 39 μM for mant-ATP and 18 μM for mant-ADP (Fig. 6C). These nucleotide affinities were similar to
those for wild-type ClpX$^{37}$ (52 µM and 8 µM, respectively; data not shown). Hence, the major defect of ClpX$^{R370K}$ appears to be in ATP hydrolysis rather than ATP binding. Irrespective of the nucleotide present, addition of His$_6$-tagged ClpX$^{R370K}$ did not result in detectable binding of untagged ClpP to Ni$^{++}$-NTA (Fig. 6A). This result further emphasizes that some aspect of the ATP binding/hydrolysis cycle or linked conformational changes plays a role in modulating the binding of ClpX to ClpP.

ClpX$^{R370K}$ did not unfold GFP-ssrA or degrade this substrate when ClpP was present (data not shown). To determine whether hexamers with a mixture of wild-type and R370K dimers were active, we titrated increasing quantities of the ClpX$^{R370K}$ mutant against a fixed quantity of wild-type ClpX and excess ClpP and assayed degradation of GFP-ssrA. As shown in Fig. 6D, addition of the mutant in 12-fold excess caused nearly complete inhibition. We also observed inhibition by ClpX$^{R370K}$ of ClpXP degradation of denatured CM-titin-ssrA and of ClpX unfolding of GFP-ssrA (data not shown). Hence, mixed hexamers with one wild-type dimer and two ClpX$^{R370K}$ dimers must be defective in denaturing native substrates and be unable to engage or translocate denatured substrates to ClpP. The latter defect could reflect an inability to bind ClpP. The best fit of the Fig. 6D inhibition data was obtained from a model in which the 2:1 and 1:2 mixed hexamers were both inactive, and wild-type dimers had a 3-fold preference for assembling with themselves rather than with mutant dimers. Untagged ClpP bound Ni$^{++}$-NTA resin following incubation with His$_6$-ClpX$^{R370K}$, untagged wild-type ClpX, and ATP$\gamma$S (Fig. 6D inset). This result demonstrates that ClpX$^{R370K}$ and wild-type subunits co-assemble and show that at least one species of mixed hexamer can bind ClpP.
Figure 6. ATP binding or hydrolysis is required for productive ClpX-ClpP interactions. (A) Untagged ClpP bound Ni^{++}-NTA in the presence of His_{6}-ClpX and ATP\$; weak binding was observed with ATP or ADP in some experiments but is not visible here. No binding of ClpP to His_{6}-ClpXR^{370K} was detected with any nucleotide. (B) ClpX_{6}^{R370K} (400 nM) hydrolyzed ATP at less than 1% of the rate of wild-type ClpX_{6} (400 nM) as determined in a coupled spectrophotometric assay. (C) Binding of ClpXR^{370K} to 1 \mu M mant-ADP or mant-ATP assayed by changes in fluorescence in the absence of magnesium. The fitted lines are for K_{d}'s of 39 \mu M (mant-ATP) and 18 \mu M (mant-ADP). (D) Degradation of GFP-ssrA (7 \mu M) by wild-type ClpXP (100 nM ClpX_{6}; 2.7 \mu M ClpP_{14}) was inhibited by addition of the ClpXR^{370K} mutant. The solid line is a fit to eq. 3 (see Methods) with a bias factor of 0.33. Wild-type and mutant ClpX were preincubated for 5 min at 30°C, ClpP and ATP mix I were added, and GFP-ssrA was added two min later to start the reaction. Inset—untagged ClpP binds Ni^{++}-NTA in the presence of a mixture of His_{6}-ClpXR^{370K} and untagged wild-type ClpX, but much less in the presence of either single species. All pull-down reactions contained ATP\$. 

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A

B

C

D
Discussion

The studies presented here provide strong evidence for functional communication between ClpX and ClpP during the processing and degradation of protein substrates. For example, ClpP affinity changed as a function of substrate processing by ClpX. In the absence of protein substrates, $K_{\text{app}}$ for the ClpX-ClpP interaction was 126 nM. This value dropped to roughly 100 nM during translocation of denatured substrates and to approximately 40 nM when ClpX was denaturing native substrates. These affinity changes could potentially affect ClpXP function in two ways. First, the processivity of degradation would almost certainly be reduced if complexes dissociated in the midst of degradation of a single substrate molecule. We do not believe that this is a significant problem, however, as the peptide products of degradation were found to be identical for degradation of a titin-ssrA substrate by ClpXP or ClpX$^{D382K}$/ClpP (data not shown), despite the fact that $K_{\text{app}}$ was more than 20-fold higher for the mutant complex. Second, the total ClpX$_6$ concentration in *E. coli* is estimated to be roughly 100 hexamers, which would correspond to an intracellular concentration of 0.3 $\mu$M. This is only about twice $K_{\text{app}}$ for the ClpX-ClpP interaction in the absence of substrate, and thus substrate binding to free ClpX hexamers might help drive assembly of ClpXP complexes by increasing the affinity of this interaction. Because ClpP in the cell can associate with either ClpX or ClpA, this could provide a mechanism to distribute the peptidase based on whether substrates for one ATPase or the other were most prevalent.

Irrespective of the functional consequences, the observed changes in ClpX-ClpP affinity demonstrate the existence of structural changes in ClpX-ClpP interactions that depend upon the physical state of the engaged substrate. Like other molecular machines, the conformation of
ClpX must change during the ATPase cycle. Hence, alterations in the amount of time spent in different stages of this cycle as a function of substrate processing could modulate ClpX-ClpP affinity. In support of this model, apparent ClpX-ClpP affinities were well correlated with the overall rates of ATP turnover by ClpXP and ClpX in the presence of substrates (Fig. 3C). It is possible, of course, that ClpX assumes distinct conformations when it is denaturing or translocating protein substrates and/or that the substrates themselves also contribute in some direct fashion to changes in affinity. During translocation, for example, the denatured protein substrate must be in loose contact with the protein-processing pore of ClpX and the entry portal of ClpP. Indeed, all of these factors—ATPase rate, distinct conformations, and substrate interactions—may contribute to the apparent ClpX-ClpP affinity to varying degrees.

The IGF loop of *E. coli* ClpX appears to be the major determinant of ClpP binding\textsuperscript{24,25} and homologous peptide motifs are found in all of the AAA+ ATPases that collaborate with ClpP family members in protein degradation. In the crystal structure of *H. pylori* ClpX, a homologous LGF tripeptide sits at the tip of a straight but flexible surface loop that extends away from the protease-proximal surface of ClpX\textsuperscript{27}. Alignment of the symmetry axes of the mismatched ClpX and ClpP rings positions these tripeptides from the ClpX hexamer near hydrophobic clefts on the surface of a heptameric ClpP ring\textsuperscript{8,24,27}. In each ClpX subunit, the IGF loop is preceded by a short $\alpha$-helix that connects directly to the sensor-I portion of the ATP/ADP binding site. In ClpA, this loop is disordered\textsuperscript{26} but is also connected to the sensor-I portion of an active site for ATP hydrolysis. It is possible that the length and/or flexibility of the IGF loop changes as a function of nucleotide binding or hydrolysis, thereby mediating communication with ClpP. Alternatively, changes in ClpX hexamer conformation might alter the number of loops that contact ClpP. In this regard, it is interesting that both ClpX and ClpA crystallize not as ring hexamers but in "lock washer" conformations in which subunits are related by a screw axis\textsuperscript{26,27}. 
In a lock-washer conformation, fewer IGF loops would be capable of contacting the relatively flat surface of a ClpP ring because of axial displacements.

The sensor-II helix in AAA+ ATPases provides a communication link between the nucleotide binding sites of adjacent subunits. In *E. coli* ClpX, Arg370 is near the N-terminus of the sensor-II helix, where it should be close to bound nucleotide\(^{27}\). Our results show that this side chain plays an essential role in ATP hydrolysis and also reveal that ClpX hexamers with two R370K dimers and one wild-type dimer are inactive in unfolding GFP-ssrA and in mediating ClpP degradation of unfolded titin-ssrA substrates. The unfolding defect of these mixed hexamers suggests that the ATPase activities of different ClpX subunits must be coordinated in some fashion to drive protein unfolding. Wild-type ClpX is also defective in unfolding GFP-ssrA when ATP\(^\gamma\)S is substituted for ATP\(^37\). Because ATP\(^\gamma\)S is hydrolyzed slowly by ClpX, some type of coordination of NTP hydrolysis rates by different ClpX subunits appears to be required for productive unfolding of native substrates.

Mutations at the C-terminal end of the sensor-II helix disrupt subunit-subunit packing in the hexamer and result in ClpX enzymes that bind but fail to unfold ssrA-tagged substrates\(^{36}\). ClpP binding suppresses the substrate processing defects of several of these sensor-II mutants, including ClpX\(^\text{D382K}\). ClpP binding to ClpX\(^\text{D382K}\) is about 30-fold weaker than to wild type in the presence of native ssrA-tagged substrates, but only 2-fold weaker in the absence of substrate. These results suggest that when ClpX\(^\text{D382K}\) binds to and attempts to unfold a native ssrA-tagged substrate, it becomes trapped in a conformation, perhaps similar to the lock-washer state, that is inactive for protein unfolding and binds ClpP poorly. Hence, maintenance of proper subunit-subunit contacts within ClpX appear to be essential for substrate unfolding and for strong ClpP interactions. We propose that subunit-subunit contacts mediated by D382 and surrounding residues in wild-type *E. coli* ClpX resist tension that is generated when the enzyme applies an unfolding force to a native substrate. Such tension would be a natural consequence of the
unfolding force creating an equal and opposite force. If this tension results in quaternary distortions of the D382K mutant, then binding of ClpP could stabilize the active ATPase conformation, allowing it to resist distortion and thus to use the energy of ATP hydrolysis for productive conformational changes that drive unfolding.

ClpX-ClpP affinity becomes at least 10-fold stronger when the active-site serines of ClpP are acylated by reaction with DFP, revealing the existence of mechanisms that allow the peptidase active sites to communicate with ClpX. Because the DFP-modified residues are located within the degradation chamber of ClpP, they cannot affect the ClpX-ClpP interaction directly. Instead, modification of the sequestered ClpP active sites must result in a conformational change that is propagated to the external surface of ClpP, where the number or quality of contacts with ClpX could be influenced. Despite the fact that the ClpP and HslV peptidases have unrelated structures and completely different active-site architectures, it is notable that ClpXP and HslUV both have mechanisms that allow communication between the ATPase and the peptidase active sites. This functional conservation in the absence of structural conservation supports an important role for communication in the degradation of protein substrates by these energy-dependent proteases.

Other than the affinity changes discussed above, what role could be served by communication between ClpX and ClpP or HslU and HslV? One possibility is that translocation of denatured substrates from the ATPase to the peptidase requires coordinated changes in the diameters of the ATPase processing pore and the peptidase entry portal. This could allow efficient transfer during the power stroke associated with each cycle of ATP hydrolysis and prevent slippage or dissociation during the recovery phase. It is also possible that coordination is required to allow efficient release of cleaved peptides from the degradation chamber at the same time that uncleaved polypeptide chains are entering the chamber. Finally, when ClpX hexamers are docked with both peptidase rings of ClpP, translocation appears to occur exclusively from one
ClpX hexamer rather than simultaneously from both hexamers\textsuperscript{38}. Communication between ClpX and ClpP would obviously be critical for regulating substrate traffic under these circumstances.

**Methods**

**Solutions.** Buffer A contains 43 mM Hepes-KOH (pH 7.6), 8.5 mM Tris-HCl (pH 8.0), 142 mM KCl, 15% glycerol, 1.1 mM DTT, 5.4 mM MgCl\textsubscript{2}, 420 μM EDTA, 36 μM ZnSO\textsubscript{4}, 36 μM ATP, 0.032% NP-40, and 0.004% Triton X-100. Buffer B contains 35 mM Hepes-KOH (pH 7.6), 4.4 mM Tris-HCl (pH 7.6), 1.5 mM Tris-HCl (pH 8.0), 95 mM KCl, 14% glycerol, 660 μM DTT, 7.4 mM MgCl\textsubscript{2}, 85 μM EDTA, 19 μM ZnSO\textsubscript{4}, 19 μM ATP, 0.16% NP-40, and 0.002% Triton X-100. Buffer L contains 50 mM Tris-HCl (pH 7.6), 10% glycerol, 1 mM DTT, and 0.5 mM EDTA. Buffer M contains 47 mM Tris-HCl (pH 7.6), 284 mM KCl, 9.5% glycerol, 4.7 mM DTT, 95 μM MgCl\textsubscript{2}, and 10 mM EDTA. Buffer N contains 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, and 250 mM imidazole. Buffer S contains 50 mM Tris-HCl (pH 8.0), 300 mM KCl, 10% glycerol, 3 mM DTT, and 10 mM MgCl\textsubscript{2}. ATP mix I contains 5 mM ATP, 16 mM creatine phosphate, and 0.32 mg ml\textsuperscript{-1} creatine phosphokinase. ATP mix III contains 2.5 mM ATP, 1 mM NADH, 7.5 mM phosphoenolpyruvate, 0.05 mg ml\textsuperscript{-1} pyruvate kinase, and 0.025 mg mL\textsuperscript{-1} lactate dehydrogenase.

**Strains, Plasmids, and Proteins.** *E. coli* strain CF150 is an X90 derivative, in which the adjacent *clpP*, *clpX*, and *lon* genes are replaced by a gene for chloramphenicol acetyltransferase, and was a gift from Chris Farrell (MIT). A plasmid expressing His\textsubscript{6}-tagged R370K (pET-14b-C1pX\textsuperscript{R370K}) was produced using overlap extension mutagenesis as described\textsuperscript{36}. A plasmid expressing ClpX\textsuperscript{loopless} (pGH003) was constructed from pET-3a-ClpX\textsuperscript{39} by polymerase chain reaction. In ClpX\textsuperscript{loopless}, a GSGSG sequence replaces wild-type residues 264-278. The sequence of the synthetic peptide containing the ClpX IGF loop was fluorescein-NH-KGRRYTGSGIFGATVKAK-CONH\textsubscript{2}. 127
ClpX<sup>loopless</sup> and wild-type ClpX were purified as described.<sup>37</sup> GFP-ssrA and His<sub>6</sub>-tagged variants of ClpP, ClpX, and titin-I27-ssrA were purified as described.<sup>29,32,36</sup> <sup>35</sup>S-titin-ssrA variants were gifts from Jon Kenniston (MIT), and SspB was a gift from David Wah (MIT). His<sub>6</sub>-tagged ClpP<sup>S97A</sup> was purified from <i>E. coli</i> strain CF150 containing pYK162<sup>35</sup> using a published protocol<sup>29</sup> with modifications. After Mono Q chromatography, fractions containing ClpP<sup>S97A</sup> were applied to a HiPrep 16/60 Sephacryl S-300HR column (Amersham Biosciences) equilibrated in buffer S. Fractions containing purified protein were pooled, divided into small aliquots, and stored at -80 °C. Carboxymethylation of titin-ssrA variants and acylation of ClpP variants with DFP were performed as described.<sup>29,32</sup> Chemically modified proteins were dialyzed extensively prior to use.

<i>E. coli</i> ClpP was purified based on a published protocol<sup>40</sup> with modifications. Cells were resuspended in 3 mL of buffer L plus 150 mM KCl for each gram of cells, lysed by French press, and centrifuged at 15,000 rpm in a SA-600 rotor for 60 min. The supernatant was passed through a 0.2 μm filter, ammonium sulfate was added to 30% saturation, and the supernatant containing ClpP was retained after centrifugation. Ammonium sulfate was added to this supernatant to 60% saturation, and the pellet containing ClpP was recovered by centrifugation. The pellet was resuspended, desalted into buffer L plus 150 mM KCl using a PD-10 column (Amersham Biosciences), and loaded onto a HiLoad 16/10 Q Sepharose HP column (Amersham Biosciences) equilibrated in buffer L with 150 mM KCl. The column was developed with a 200 mL linear gradient from 150 mM to 400 mM KCl in buffer L. Fractions containing ClpP were concentrated by ammonium sulfate precipitation (60% saturation), and the pellet was resuspended, desalted into buffer L plus 100 mM KCl, and loaded onto a HiPrep 16/60 Sephacryl S-300HR column equilibrated in this buffer. Fractions containing purified ClpP were pooled, concentrated by chromatography on a HiLoad 16/10 Q Sepharose HP column, and stored in small aliquots at -80 °C.
Nucleotide hydrolysis and binding assays. Hydrolysis of ATP by ClpX in buffer A was measured at 30 °C using a coupled assay\textsuperscript{24}. ClpX and ClpP were incubated for two min before addition of substrate and/or ATP mix III. Except where noted, 50 nM ClpX\textsubscript{6} was used for all assays. Binding of mant-ADP or mant-ATP to ClpX\textsuperscript{R370K} was assayed at 4 °C in buffer M\textsuperscript{37}.

Unfolding and degradation assays. GFP-ssrA unfolding or degradation\textsuperscript{29} was performed in buffer B plus ATP mix I at 30 °C. Degradation of \textsuperscript{35}S-titin-ssrA substrates was assayed by release of TCA-soluble peptides as described\textsuperscript{30,32}. For ClpXP degradation of titin-ssrA substrates at saturating concentrations, the slow steps are denaturation (k\textsubscript{den}) and translocation (k\textsubscript{trans}), with 1/k\textsubscript{deg} = 1/k\textsubscript{den} + 1/k\textsubscript{trans} and τ\textsubscript{deg} = τ\textsubscript{den} + τ\textsubscript{trans}\textsuperscript{32}. K\textsubscript{app} for the ClpX-ClpP interaction during substrate processing can be expressed as (τ\textsubscript{den}/τ\textsubscript{deg})•K\textsubscript{app}\textsuperscript{den} + (τ\textsubscript{trans}/τ\textsubscript{deg})•K\textsubscript{app}\textsuperscript{trans}. The value of k\textsubscript{trans} for different titin-ssrA substrates is essentially constant (4.3 min\textsuperscript{-1}). Substitution of τ\textsubscript{deg} – τ\textsubscript{trans} for τ\textsubscript{den} and rearrangement of terms, yields the linear equation K\textsubscript{app} = τ\textsubscript{trans}•(K\textsubscript{app}\textsuperscript{trans} – K\textsubscript{app}\textsuperscript{den})•k\textsubscript{deg} + K\textsubscript{app}\textsuperscript{den} (eq. 1). The general equation for inhibition by mixed hexamer formation between active and inactive dimers of ClpX is A = (1 + 3•A\textsubscript{21}•B•R + 3•A\textsubscript{12}•B\textsuperscript{2}•R\textsuperscript{2})/(1 + B•R\textsuperscript{2})\textsuperscript{2} (eq. 2) where A is the fractional activity relative to fully active hexamers, A\textsubscript{21} is the fractional activity of a hexamer with two active and one inactive dimer, A\textsubscript{12} is the activity of a hexamer with one active dimer and two inactive dimers, R is the ratio of total inactive to total active subunits, and B is the mixing bias. B=1 indicates unbiased mixing of active and inactive dimers; B<1 indicates a preference of active dimers to associate with other active dimers rather than inactive dimers. If three active dimers are required for activity, eq. 2 simplifies to A = (1 + B•R\textsuperscript{-1})\textsuperscript{2} (eq. 3).

Pull-down and ternary complex assays. ClpXP pull-down assays were performed by a modification of a published protocol\textsuperscript{24}. Each reaction (30 μL) contained 300 nM His\textsubscript{6}-tagged ClpX\textsubscript{6} (wild-type or R370K) and 300 nM untagged ClpP\textsubscript{14}. Some reactions also contained 300
nM untagged wild-type ClpX<sub>6</sub>. Nucleotide (ATPγS, ATP, or ADP) at a concentration of 3 mM was present during initial complex formation, and in washes after binding to Ni<sup>2+</sup>-NTA agarose. Protein was eluted in 30 μL buffer N and subjected to SDS-PAGE. Gels were stained with Sypro Orange (Molecular Probes) and visualized using a FluorImager 595 (Molecular Dynamics). Assays for ternary complexes of ClpX, SspB, and GFP-ssrA were performed by gel-filtration chromatography on a Superdex 200 column (Amersham Biosciences) as described<sup>33</sup>. 


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References


Biographical note

Shilpa Joshi grew up in Kennewick, Washington, the daughter of Vandana and Arun and the younger sister of Sampada and Suraj. In Maharashtra, India, her maternal grandfather, Gopal Ranade, was the founder of the G.A. Ranade school for uneducated laborers and disadvantaged children in Mumbai, and her paternal grandfather, Nilkanth Joshi, was a physicist and inventor. Before immigrating to the U.S. in 1973, her mother worked as a teacher after being the gold medallist from Elphinstone College, University of Mumbai, in 1963. Her father worked as a nuclear engineer and continued this work after immigrating.

After graduating second in her high school class and being named a National Merit Commended Student, Shilpa went to college at the University of California at Berkeley as a Robert C. Byrd Scholar from Washington state in August 1993. Her first work in a laboratory was with Michael A. Kennedy at Pacific Northwest National Laboratory as an Associated Western Universities-Northwest Fellow in the summer of 1995. During that summer and the summer of 1997, she helped optimize the purification of a protein useful in bioremediation efforts. Back at college, she worked in Bing K. Jap’s laboratory at Lawrence Berkeley National Laboratory and identified the sequence for an aquaporin from bovine bone marrow. She also volunteered at the Berkeley Adult School and tutored homeless adults in reading and mathematics during her last two years of college. After graduating in May 1997 with an A.B. in Molecular and Cell Biology with a minor in American Literature, Shilpa began graduate school at MIT in September 1997. While there, she taught an XL seminar for inorganic chemistry through the Office of Minority Education, and was a teaching assistant for undergraduate biochemistry and experimental microbial genetics courses. Shilpa will now head to Eric Brown’s laboratory at the University of California at San Francisco, where she has accepted a postdoctoral position to study *Mycobacterium marinum*. 