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Protein Binding and Disruption by Clp/Hsp100 Chaperones

Review

Michael R. Maurizi* and Di Xia
Laboratory of Cell Biology
National Cancer Institute
National Institutes of Health
Bethesda, Maryland 20892

Clp/Hsp100 chaperones work with other cellular chaperones and proteases to control the quality and amounts of many intracellular proteins. They employ an ATP-dependent protein unfoldase activity to solubilize protein aggregates or to target specific classes of proteins for degradation. The structural complexity of Clp/Hsp100 proteins combined with the complexity of the interactions with their macromolecular substrates presents a considerable challenge to understanding the mechanisms by which they recognize and unfold substrates and deliver them to downstream enzymes. Fortunately, high-resolution structural data is now available for several of the chaperones and their functional partners, which together with mutational data on the chaperones and their substrates has provided a glimmer of light at the end of the Clp/Hsp100 tunnel.

Introduction

Clp/Hsp100 chaperones are ATP-dependent protein unfolding machines (Glover and Tkach, 2001; Horwich et al., 1999; Schirmer et al., 1996). They are important components of the protein quality control system and integral parts of the regulatory arsenal controlling the intracellular levels of global regulatory proteins (Gottesman, 2003; Gottesman et al., 1997). The functions and activities of Clp/Hsp100 proteins are best understood in bacterial cells, but they are well conserved in eukaryotes, where they function in the plant cytosol (Nieto-Sotelo et al., 1999) or chloroplasts (ClpA) (Porankiewicz et al., 1999) and in mammalian mitochondria (Hsp78 and ClpX) (Kang et al., 2002; Krzewska et al., 2001b).

Clp/Hsp100 proteins belong to the AAA^+ superfamily, a diverse family of enzymes that act on other macromolecules and catalyze mechanical processes, such as locomotion, unwinding, disassembly, and unfolding (Neuwald et al., 1999; Ogura and Wilkinson, 2001). The Clp/Hsp100 family itself can be divided into two subfamilies with distinct enzymatic functions. Members of the ClpB/Hsp104 subfamily display a unique protein disaggregating activity that is used in conjunction with the refolding activities of DnaK/Hsp70 chaperone systems to extract and resolubilize proteins from aggregates (Ben-Zvi and Goloubinoff, 2001). Members of the ClpA subfamily, which includes ClpA, ClpC, ClpX, and HslU, have protein unfolding activities and act primarily in conjunction with self-compartmentalized proteases, such as ClpP and HslV (ClpQ), to catalyze ATP-dependent proteolysis (Horwich et al., 1999). The regulatory role of Clp/Hsp100 proteins in most biological pathways entails the targeting of a key regulatory protein for degradation (Gottesman, 2003), although regulatory functions in some cases might require only structure remodeling activity, as in the interconversion of conformers of the yeast transcriptional Psi factor (Serio and Lindquist, 2000).

Research on Clp/Hsp100 proteins has been focused on several questions fundamental to their biological activities: how substrates are recognized, how protein unfolding occurs, how substrates are delivered to the downstream protease or chaperone system, and what role ATP binding and hydrolysis play at each of these stages. The field has had a considerable boost recently from high-resolution structure determinations of full-length of *Thermus thermophilus* ClpB (*T*ClpB) (Lee et al., 2003), the AAA domain of *Helicobacter pylori* ClpX (*H*ClpX-AAA) (Kim and Kim, 2003), N-domain of *Escherichia coli* ClpX (ClpX-N) (Donaldson et al., 2003), and the ClpX-specific adaptor protein, SspB, with a bound peptide substrate (Levchenko et al., 2003; Song and Eck, 2003). This new information complements crystal structural data on full-length *E. coli* ClpA (Guo et al., 2002b), the complex of the ClpA N-domain with its adaptor protein, ClpS (Guo et al., 2002a; Zeth et al., 2002), and intact holoenzyme complexes of HslUV (ClpYQ) from two sources (Bochtler et al., 2000; Sousa et al., 2000; Wang et al., 2001a, 2001b). In this review, we will discuss how the new structural information provides a framework for understanding the basic mechanisms and the biological roles of these chaperones.

Modular Domain Structure of Clp/Hsp100 Subunits

Clp/Hsp100 proteins all have a conserved structural core, called an AAA module, with additional structural domains appended or inserted at specific sites (Figures 1A and 1B). AAA modules function as the ATP-fueled motor at the heart of a variety of molecular chaperones or machines, and structures have now been determined for >15 AAA^+ modules, including those in membrane-fusion ATPases, NSF (Lenzen et al., 1998; Yu et al., 1998), the multifunctional chaperone, p97 (Zhang et al., 2000), the DNA clamp loader complex (Jeruzalmi et al., 2001), and the ATP-dependent proteases, FtsH (Krzywda et al., 2002; Niwa et al., 2002) and Lon (Botos et al., 2004). AAA modules consist of two subdomains, a large α/β domain made up of a five-stranded parallel β sheet flanked by pairs of helices, connected by a mobile linker to a smaller mostly helical C-terminal α domain (Figures 2A and 2B). ATP binds between the two subdomains in a crevice that contains the catalytic residues for hydrolysis (Walker A and B motifs) and one or more functional motifs that respond to the nucleotide state of the site (sensors 1 and 2) (Lupas and Martin, 2002; Neuwald et al., 1999). ClpX and HslU have a single AAA module, while ClpA and ClpB each have two modules in tandem (Figure 1A). The module nearer the N terminus is called nucleotide binding domain 1 (NBD1), and the module nearer the C terminus, which is divergent in sequence, is called NBD2. Superposition of the polypeptide backbones of ClpX, ClpA-NBD2, ClpB-NBD2, and HslU (with the I-domain omitted) in the ADP bound

*Correspondence: mmaurizi@helix.nih.gov

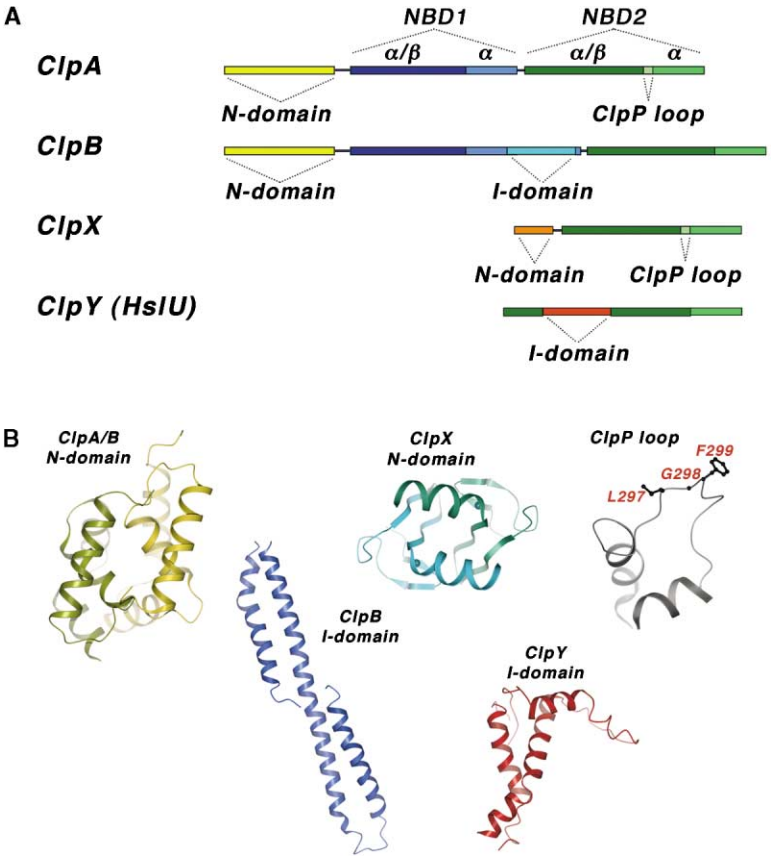

Figure 1. Structure of Clp/Hsp100 Proteins

(A) Clp/Hsp100 proteins are composed of structural domains built onto one or two tandem AAA protein modules. The AAA modules consist of an α/β subdomain, with a RecA nucleotide fold, connected to an α subdomain (see Figure 2). The tandem AAA modules in one protein are divergent, whereas the N- and C-terminal modules are each well conserved within different families. Clp/Hsp100 proteins can have no N-domain (HslU), a helical N-domain (ClpA and ClpB), or a zinc binding N-domain (ClpX). Intermediate domains (I-domains) in HslU and ClpB are spliced between secondary structure elements in different parts of the AAA module 1. Smaller structural motifs, such as the ClpP binding loop in ClpA and ClpX, are inserted within the C-terminal module, and facilitate interaction with functional partners.

(B) Ribbon diagrams of the auxiliary domains in Clp/Hsp100 proteins. The N-domains of ClpA and ClpB are helical pseudo dimers (shown: PDB 1KHY), whereas the smaller N-domain of ClpX (shown: PDB 1OVX) has a treble cleft zinc binding motif, and two domains associate to form a molecular dimer. I-domains are also highly helical and, in the case of ClpB, form a double coiled-coil (shown: PDB 1QVR); the HslU structure is incomplete (shown: PDB 1E94). Helices flanking the ClpP interaction motif cause it to project out from the surface and may regulate its ability to access binding sites on ClpP (shown: PDB 1UM8).

state shows a high degree of structural similarity (Figure 2B). The NBD1 domains of ADP bound ClpA and *T*ClpB (with the I-domain omitted) also overlap quite well with each other (Figure 1A). Although ClpA, *T*ClpB, and *H*ClpX crystallized in a hexagonal spiral rather than in a planar hexagonal ring, as in HslU and most other AAA^+ proteins, the quality of the structural overlaps suggest that the structures of the AAA modules were largely maintained in the crystal despite the breaking of true molecular symmetry by crystal packing forces.

In addition to the AAA modules, ClpA and ClpB have large helical N-domains that fold independently (Figure 1B). The *T*ClpB N-domain is similar to the pseudo-dimer of four-helix bundles seen in the N-domain of ClpA and in the structure of the isolated N-domain of *E. coli* ClpB (Li and Sha, 2003), except that the 2-fold symmetry is broken by an altered secondary structure in helix 3′. In ClpA, the halves of the pseudo-dimer produce a hydrophobic peptide binding site (Xia et al., 2004), which is somewhat smaller in the *T. thermophilus* and *E. coli* ClpB N-domains, possibly explaining a lesser role in unfolded substrate interactions. ClpX N-domain has a sequence and structure completely different from those of ClpA and ClpB. The NMR structure of the isolated *E. coli* ClpX N-domain (Donaldson et al., 2003) showed a four-cysteine Zn^{2+} binding motif found in the treble clef zinc binding family (Grishin, 2001). The isolated ClpX N-domain forms a stable dimer, but whether this form is present in the intact protein is not known. The Zn^{2+} is needed for stability of the ClpX N-domain, but its functional role has not been defined, although this motif facilitates protein-protein interactions in other systems. Curiously, the N-domain of ClpA also has a Zn^{2+} binding site, related however to Zn^{2+} metalloproteases (Xia et al., 2004). Quite possibly the Zn^{2+} sites in both proteins are used for binding peptide extensions in substrates or other interacting proteins.

A defining characteristic of ClpB/Hsp104 proteins is a large intermediate domain (also called the middle region or linker) spliced into its sequence near the junction of NBD1 and NBD2 (Schirmer et al., 1996). In the ClpB structure, the I-domain erupts from the NBD1 α domain just prior to its C-terminal helix (Lee et al., 2003). It is composed of two coiled-coils (Figure 1B) running in opposite directions from the point of attachment, forming a propeller-like appendage that runs along NBD1 (Figure 3). The attachment to the α domain assures that the I-domain will undergo significant displacement in response to nucleotide hydrolysis in NBD1. The I-domain of HslU, a more complex highly helical structure (Figure 1B), is connected through the N terminus of the sensor-1 β strand and projects from the distal surface (defined as the surface opposite the protease interacting surface). It might also undergo conformation change due to changes in nucleotide state, which would affect its interactions with bound substrate proteins.

Oligomeric Assembly: Are All Clp/Hsp100 Proteins Hexamers?

Most AAA^+ modules or proteins, including one Hsp100 protein, HslU, have been crystallized in stable oligomer forms, which have invariably been hexameric with sub-

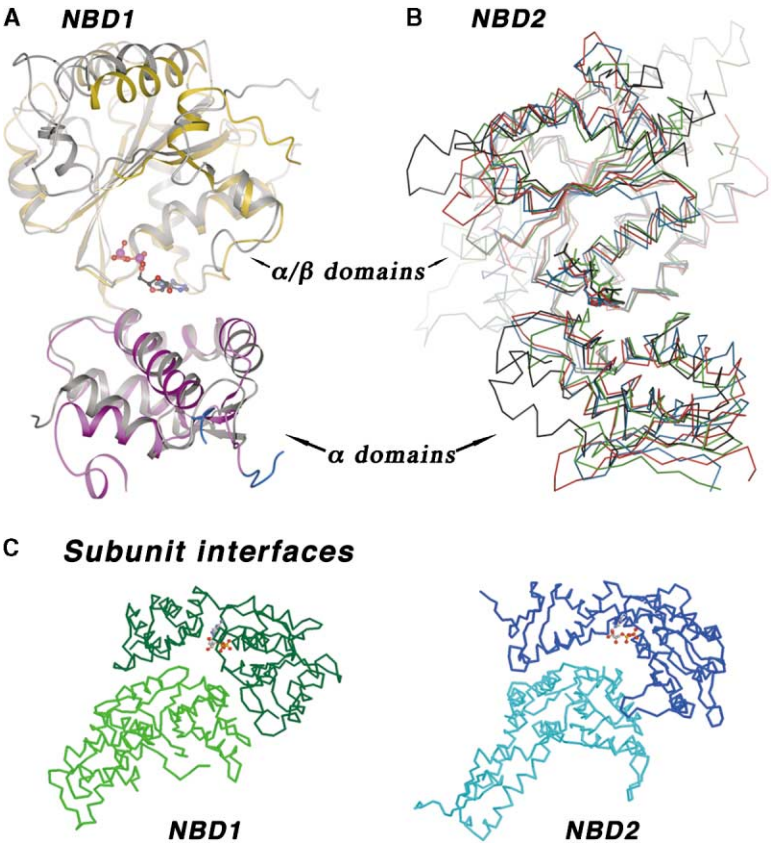

Figure 2. Structural Similarities in Clp/Hsp100 Subunits

(A) NBD1 of *E. coli* ClpA and *T*ClpB have similar folds, but overlap less well with NBD2. The rms deviation is ~2.0 Å for 180 overlapping residues.

(B) *E. coli* HslU, *H*ClpX, *E. coli* ClpA NBD2, and *T*ClpB NBD2 have very similar folds, with an rms deviation of <1.6 Å for >200 residues. Nucleotide binds in the interface between the small α (SSD) and larger α/β domains, and changes in nucleotide state alter orientations of the two domains, affecting interactions and communication between adjacent subunits and more distant parts of the assembled complexes.

(C) The domain responsible for oligomerization, NBD1 of ClpA or NBD2 of ClpB, is more closely packed. In the ClpA crystal (shown), NBD1 residues from the adjacent subunit make numerous contacts with the nucleotide pocket, whereas the NBD2 interface is more open. In the *T*ClpB crystal, the opposite was true.

units in nearly identical orientations around a ring. Solution and electron microscopic studies of ClpA, ClpX, and ClpB also indicate that they are predominantly hexameric (Beuron et al., 1998; Ortega et al., 2000), forming planar hexagonal structures, although some lingering doubt remains because minor populations of heptameric forms are also seen (Grimaud et al., 1998; Rohrwild et al., 1997). Similar uncertainty has plagued ClpB (Kim et al., 2000a). In the case of HslU, the issue was resolved by isolation and crystallization of the assembled HslUV complex, in which both HslU and HslV rings were shown to be hexamers (Sousa et al., 2000). For ClpA and ClpX, which form a complex with a heptameric partner, ClpP, the issue is critically important to understanding the functional interactions between them during ATP-dependent proteolysis.

The crystal structures of ClpB, ClpX, and ClpA have added to the argument in favor of the hexamer, although still do not settle the issue definitively, because the molecules were arranged in a spiral rather than a planar hexagon. NBD1 and NBD2 from the crystal structures of ClpA and ClpB fit reasonably well into their respective cryo-EM densities using six subunits for each tier (Ishikawa et al., 2004; Lee et al., 2003). The EM model of ClpB was missing the N-terminal domains and significant amounts of the I-domains, while the EM model of ClpA showed diffuse density on the apical surface corresponding to mobile N-domains. Hexamer models

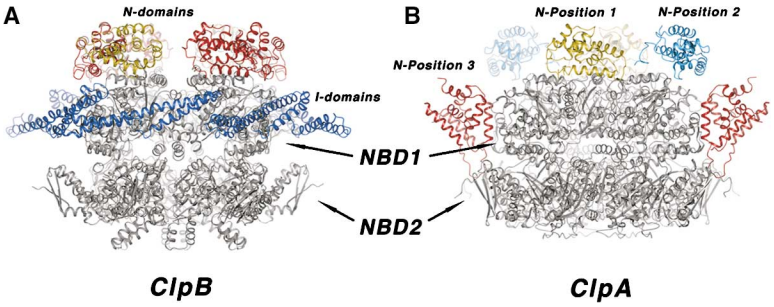

Figure 3. Hexamer Models of *T*ClpB and *E. coli* ClpA

(A) The hexamer of TClpB was made by aligning the crystal structure of the NBD2 onto the hexamer of HslU and the NBD1 onto p97. The orientations of the two rings were set according to a model of ClpA (Guo et al., 2002b). The major features are similar to those seen in the published hexamer model obtained by fitting the crystal structure into the cryo-EM density (Lee et al., 2003). The N-domains localize to the apical surface, and the I-domains, which are essential for disaggregating activity, are positioned on the lateral face of NBD1. One suggestion from this model is the placement of a helix-loop-helix motif arrayed about the axial channel on the NBD2 ring surface.

(B) A hybrid ClpA model is shown in which the N-domain positions observed in the ClpA crystal and the two different N-domain positions observed in the *T*ClpB crystal have been included. In the crystal of the ClpA subunit, the N-domain occupied the lateral position seen for the I-domains of *T*ClpB, but cryo-EM data suggest that they can move to the apical surface, possibly to contribute to substrate binding there.

of ClpA NBD1, using NSF as a template, and of ClpA NBD2 and *H*ClpX, using HslU as a template, have also been constructed. The ClpA model differs in showing a closed configuration of NBD2 and having NBD1 and NBD2 from a single subunit displaced one position around the ring, so that superimposed domains are from adjacent subunits (Guo et al., 2002b). *H*ClpX fits very well into the HslU hexamer (Kim and Kim, 2003), although large loops that lie inside the chamber of the hexamer were not visible.

The assembled rings are bipolar, with the C terminus of the β sheet, where catalytic and regulatory motifs such as the Walker A and B and sensor motifs are located, closer to the ring surface where the protease components bind (proximal surface) and the N terminus and N-domains closer to the distal surface. The *H*ClpX structure revealed the loop containing the ClpP-interaction motif (ClpP-loop; Figure 1B), which in the hexamer model extended out from the proximal surface of the ring. The ClpP-loop is connected to the sensor-1 β strand and is likely to undergo conformational change in response to the presence of nucleotide or to ATP hydrolysis. The loop appears to be mobile, as it was not visible in the ClpA crystal and has relatively high temperature factors in the *H*ClpX structure. ClpB has a structural motif positioned similarly to the ClpP-loop, although it lacks the consensus ClpP binding motif, IG (F/L). When we modeled *T*ClpB NBD2 using the HslU hexamer as a template, a helix-loop-helix motif connected to the sensor-1 β strand appears to project out from the proximal surface of the ClpB hexamer (Figure 3A). It will be interesting to see if this motif mediates interaction with any of ClpB's functional partners or functions similarly to the "second region of homology" motif, which is located at this site in canonical AAA proteins (Neuwald et al., 1999).

Assembly of two-domain (type 2) AAA^+ proteins such as ClpA and ClpB produces a bilayered structure with two homomeric rings formed by NBD1 and NBD2. In ClpA and ClpB (and in the recently determined structure of intact p97; [DeLaBarre and Brunger, 2003]), the two modules associate head to tail with their β sheets running N-to-C along the axis. With respect to ClpA, the geometry implies that NBD1 and NBD2 could both act in the same direction contributing to vectorial translocation of the unfolded substrate. Whether the same is true for ClpB/Hsp104 is not clear, because there may be significant differences in the functional contributions of NBD1 and NBD2 between these subfamilies.

Roles of Nucleotide Binding Domains in Assembly and Activity

Differences in mechanism of substrate processing between ClpA and ClpB subfamilies are suggested by mutational studies of NBD1 and NBD2. In ClpA, NBD1 has lower ATPase activity than does NBD2 and makes a relatively larger contribution to assembly (Singh and Maurizi, 1994), whereas the opposite is true for Hsp104 (Schirmer et al., 2001) and for several ClpB/Hsp104 homologs from bacteria and other organisms (Barnett and Zolkiewski, 2002; Gallie et al., 2002; Krzewska et al., 2001a; Mogk et al., 2003). Studies with isolated domains also indicate that ClpB/Hsp104 NBD2 domains can oligomerize more efficiently than NBD1 (Mogk et al., 2003), whereas the opposite is true for ClpA (M.R.M., unpublished data). These results are not explained by overall sequence alignments, which indicate that the respective NBD1 domains are more closely related to each other, as are the respective NBD2 domains.

The crystal structures suggest a basis for the difference between ClpA and ClpB/Hsp104. When the individual domains are compared, the backbones of two NBD1 domains of ClpA and *T*ClpB indeed show better alignment, and the same is true for the two NBD2 domains. However, the interactions between adjacent NBD domains is opposite for ClpA and *T*ClpB (Guo et al., 2002b; Lee et al., 2003). In ClpA NBD1 and ClpB NBD2, the surface of the α/β domain opposite the bound nucleotide docks snugly into the nucleotide binding cleft of the neighboring subunit making a number of salt bridges or hydrogen bonding contacts (Figure 2C). However, in ClpA NBD2 and in ClpB NBD1, the domains do not nestle as closely and no residues with long side chains are present on the surface of the docking subunit. *E. coli* ClpB can oligomerize without nucleotide under conditions of low ionic strength (Barnett and Zolkiewski, 2002), confirming that electrostatic interactions contribute to the stability of the oligomer. ClpX also has a number of negative charges that can interact with residues on the adjacent subunit (Kim and Kim, 2003). In ClpB/Hsp104, the reversal of roles for NBD1 and NBD2 with respect to ClpA could signify that protein translocation through NBD2 is very slow to allow time for NBD1 to extract proteins from aggregates, or, alternatively, is abortive, allowing substrates unfolded at NBD1 to be released back in the opposite direction to other chaperones.

Interactions between the α Domain and the Adjacent α/β Domain: Communication within Rings

Hexamer stability is also dependent on contacts between the α domain of one subunit and the α/β domain of its neighbor. Deletion of the α domain blocks assembly of ClpB (Mogk et al., 2003). Crystal structures of HslU with or without nucleotide bound showed that the α domain rotates as a unit with respect to its α/β domain depending on whether nucleotide is present (Bochtler et al., 2000; Wang et al., 2001b). In HslU and ClpX or in ClpA NBD2, this rotation would be expected to influence assembly or stability of subunit contacts in the ring.

Recent studies suggest that nucleotide sites within a ring communicate allosterically with each other and that substrate binding can affect and be affected by these interactions. Mutants in the *E. coli* ClpX α domain in the region of interaction with the adjacent α/β domain had altered responses to substrate binding and defective coupling between ATP hydrolysis and unfolding activity (Joshi et al., 2003). The authors postulated that residues in the sensor 2 helix of the α domain interact with nucleotide interaction motif (box II) of the α/β domain. The mutations apparently affected the geometry or dynamics of interaction between the two domains but did not block it sufficiently to prevent assembly of the rings. Analysis of the kinetics of Hsp104 also point to coopera-

tive interactions between nucleotide binding domains in each ring (Hattendorf and Lindquist, 2002).

Communication between NBD1 and NBD2

Assigning specific functions to the individual domains of ClpA or ClpB is further complicated because biochemical studies suggest that the two domains communicate with each other and that the nucleotide state of one domain influences ATPase activity in the other. Careful kinetic analysis of wild-type Hsp104 identified two catalytic sites, each with a different V_{max} and K_m for ATP hydrolysis (Hattendorf and Lindquist, 2002). Mutations in either NBD1 or NBD2 changed kinetic parameters for both sites. Studies with *E. coli* ClpB lead to similar overall conclusions (Mogk et al., 2003). ClpA NBD1 mutants are affected in a chaperone activity, which can be restored by binding of ClpP at NBD2, indicating that allosteric effects can be transmitted from NBD2 to NBD1 in ClpA as well (Pak et al., 1999).

The physical basis for communication between NBD1 and NBD2 is unclear because details of the interface between them in ClpA and ClpB are missing in the crystal structures. The exact juxtaposition of the domains cannot be determined with the existing models, in which each domain was fit separately to create the two hexameric rings. However, it appears that communication may be different for ClpA and ClpB. The ClpA subunit structure shows only a short, rather inflexible connection between the domains, which would allow conformational changes to be transmitted between them by rigid body movement between the α domain in NBD1 and the α/β domain in NBD2 (Guo et al., 2002b). The linker is connected via the N-terminal helix of NBD2 to the box II nucleotide binding motif in NBD2, providing a mechanism by which the nucleotide state of NBD1 could exert an effect on NBD2 nucleotide sites.

Reciprocal effects would be expected, and this mechanism might explain the observation that ATPase activity in NBD1 of Hsp104 is allosterically activated by binding of putative model substrates to a region of NBD2 (Cashikar et al., 2002). Substrate binding to NBD2 would have an impact on the box II helix, as shown for ClpX, which in turn could affect the linkage to the α domain of NBD1. Allosteric communication between NBD2 and NBD1 was also influenced by the I-domain, which is attached to the NBD1 α domain. Antibody binding to the I-domain stimulated ATPase activity, possibly by favoring the active conformation of the α domain and making the further conformational change less energetically favorable.

The Positions and Functions of the N-Domains

The N-domains of ClpA and ClpB appear to play an auxiliary role and are required for some but not all activities. Since some ClpB homologs, such as mitochondrial Hsp78 (Krzewska et al., 2001a), are synthesized without an N-domain and yet express chaperone functions, it is clear that some chaperone functions do not require the N-domain. *E. coli* ClpB lacking N-domains has disaggregating activity (Mogk et al., 2003), yet mutations in specific sites in the N-domain of ClpB have defects in chaperone activity (Li and Sha, 2003). Thus, the effects of removing or mutating the ClpB N-domain appear to differ and indicate that the N-domains can influence chaperone activity in complex ways. ClpA lacking the N-domain also expresses activity against specific substrates but has lower affinity for casein (Singh et al., 2001; Xia et al., 2004), suggesting that the N-domains provide weak interaction sites for nonspecific substrates. This binding activity could be used to help recruit substrates to the chaperone, or, alternatively, to help sweep nonspecific proteins away from the apical surface, allowing unhindered access to specific substrates sites.

The N-domains of ClpA and ClpB are mobile and appear as diffuse densities in cryo-EM images (Ishikawa et al., 2004). In the crystal of ClpA, the N-domains were fixed by a contact with the NBD1 α domain and in models of the hexamer appeared on the lateral edge of the NBD1 ring (Guo et al., 2002b). In the *T*ClpB crystal (Lee et al., 2003), the N-domains were in two different orientations, and in the ClpB hexamer model, the N-domains appear in two different conformations on the surface of the ring (Figure 3A). Interestingly, the ClpB N-domains can also be modeled onto the ClpA hexamer without clashing with each other or with other parts of the molecule (Figure 3B), possibly showing the limits of the displacement the N-domains can undergo.

The N-domains of ClpB cannot occupy the equivalent positions as the N-domains of ClpA because those sites are occupied by the ClpB I-domains, which tend to confine the N-domains to the ring surface. In vivo, ClpB would not have six N-domains on the ring surface because about a third of ClpB is synthesized from an internal translational start site that produces an N-domain-deleted form of the protein (Park et al., 1993). The truncated ClpB assembles with full-length ClpB to form mixed hexamers in an average ratio of 2:4 (Zolkiewski et al., 1999). The advantage of the mixed hexamers over homomeric hexamers with six N-domains is not known, but the near universal occurrence of both forms of ClpB (Schirmer et al., 1996) suggest that some difference in activity should be observed if the appropriate substrates are found. Although ClpA is also synthesized from an internal translational start site, this form is produced in much lower amounts. ClpA without its N-domain is resistant to the effect of a small adaptor protein (Dougan et al., 2002), which would imply a difference in activity in vivo, if the truncated form were to accumulate under specific physiological conditions.

The small adaptor protein ClpS binds to the ClpA N-domain and alters the substrate preference of ClpA (Dougan et al., 2002; Guo et al., 2002a; Zeth et al., 2002). ClpS blocks autodegradation of ClpA and the degradation of soluble proteins, while allowing degradation of aggregated proteins. The crystal structure of ClpS bound to the isolated N-domain shows a heterodimeric complex suggesting that ClpA can bind up to six molecules of ClpS. Interestingly, in the model of the ClpA hexamer, the N-domain/ClpS complex binds to the edge of the ClpA rings at the equivalent site occupied by the I-domain of ClpB with its long N-terminal peptide pointing toward the distal ring surface (Guo et al., 2002a). The similarity in the relative positions of the N-domain/ClpS complex and the I-domains of ClpB suggest that aggregate binding sites are maintained on the sides of

the chaperone rings, perhaps to avoid blocking the axial channels on the distal surface. The disaggregating activity of ClpA/ClpS is less robust than that of ClpB (Dougan et al., 2002), possibly reflecting a more limited range of substrates in vivo.

The I-Domain of ClpB

The I-domain is a universal feature of ClpB/Hsp104 proteins and is essential for biological activity. Proteins with the I-domain deleted can fold and assemble into hexamers but are somewhat defective for ATP hydrolysis and completely lack chaperone activity (Mogk et al., 2003). ClpB/Hsp104 works together with the DnaK/DnaJ/GrpE chaperone system to solubilize large protein aggregates (Glover and Lindquist, 1998; Mogk et al., 1999; Zolkiewski, 1999). ClpB/Hsp104 is essential for the overall reaction of resolubilization, indicating that it has an activity lacking in all other chaperones. This activity allows ClpB/Hsp104 to extract proteins from large aggregates and hand them over to the other chaperones. The I-domain appears to be linked to this unique activity, and the crystal structure suggests it functions by a novel mechanism.

The I-domain is a long coiled-coil made of two shorter coiled-coil regions joined at the middle, where they are connected to the NBD1 α domain (Lee et al., 2003). When modeled onto the hexamer, the I-domains are distributed around the edge of the NBD1 ring like a set of knitting needles. One end of the coiled-coil projects toward the distal ring surface, while the other projects like a tangent line away from the structure. Insertion of cysteine residues to allow disulfide crosslinks between the I-domain and adjoining regions of NBD1 resulted in severe impairment of activity in the disulfide form, which was reversed upon reduction of the disulfide bonds, suggesting that mobility of the I-domain is needed for disaggregation activity.

The attachment to the α domain provides a means of manipulating the ends of the needles through conformational changes mediated by nucleotide binding and hydrolysis. An indication of the range of motion of the I-domains was obtained in the crystal itself, which contained three molecules of ClpB with different orientations of the NBD1 with respect to NBD2 α/β domain. Assuming that similar displacements can occur upon nucleotide binding or hydrolysis, the end of the coiled-coil can undergo rotation by $\sim$15° and translation by $\sim$17 Å in the different conformations. These motions suggest a mechanism by which the ends of the coiled-coils engage substrate and move in opposite directions, prying tangled sections apart (Lee et al., 2003). Whether the adjacent coiled-coils can engage the same region of the substrate to work in concert is not clear from the structure. Such a mechanism presumably frees large regions of the aggregate, which are then bound by the DnaK chaperone system and further unfolded and refolded. Another possible mechanism is that the ends of the coiled-coil could interact with smaller aggregates bound at the distal surface and tease tangled portions of the polypeptides out of the aggregates. The disaggregating activity of ClpB does not require the N-domains (Mogk et al., 2003), so the mechanism of binding to the distal surface in this case might require other sites comparable to those present on other Clp/Hsp100 proteins.

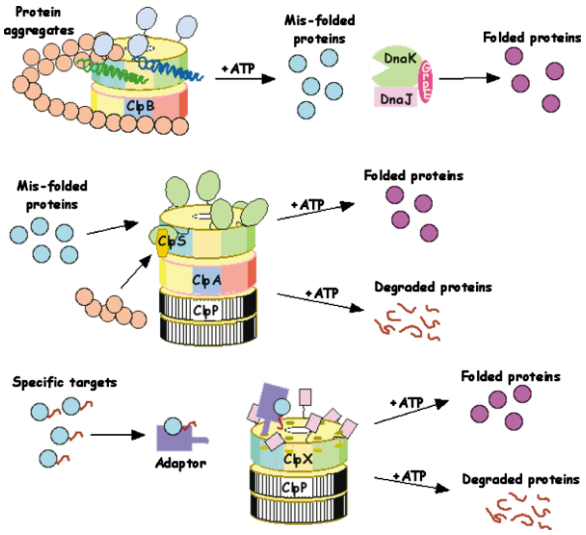

Figure 4. Activities of Clp/Hsp100 Proteins

ClpB/Hsp104 disaggregates proteins and hands them to other chaperones for refolding. ClpB probably needs to anchor to an aggregated substrate in order to apply leverage for disentangled other regions of the aggregate. ClpA can recognize specific motifs for selective targeting of proteins but also can bind unfolded proteins without specific motifs and can disaggregate proteins in the presence of the adaptor, ClpS. ClpX requires specific sequence motifs to recognize its substrates and is helped in doing so by adaptor proteins that bind to separate sites on the same substrates and form ternary complexes with ClpX for efficient delivery of the protein cargo.

Effective application of mechanical forces on large immobile substrates requires that ClpB be anchored to the substrate by some mechanism that does not involve the force-applying I-domains. Because it activates ATPase activity of ClpB/Hsp104, polylysine has been suggested to be a model substrate (Cashikar et al., 2002), but it has also been suggested to be an allosteric effector because it is not competitive with some protein substrates (Strub et al., 2003). We suggest another possibility, that polylysine mimics sites in protein aggregates to which ClpB/Hsp104 anchors in order to exert its disaggregating affect. Polylysine binds to NBD2 (Cashikar et al., 2002), which would leave the I-domains and the distal surface free to interact with other substrates. ClpB/Hsp104 anchored to positively charged patches in aggregated proteins could begin to disentangle surrounding regions of the aggregate using the I-domains and other protein binding and enzymatic sites on the distal surface of the molecule. Allosteric activation of the chaperone activity by such mooring sites would increase the efficiency of the reaction.

Other Modes of Substrate Interaction: Adaptor Proteins

Clp/Hsp100 proteins also target many specific proteins for degradation with important regulatory consequences for the cell (Figure 4). The mechanism of specific sub-

strate recognition is beginning to be understood and reveals elements of simplicity and complexity. Most good substrates have sequence motifs near their N or C terminus that can be recognized by ClpX, ClpA, or HslU (Gonciarz-Swiatek et al., 1999; Hoskins et al., 2000; Kwon et al., 2004; Levchenko et al., 1997). This rule actually applies to other ATP-dependent proteases, such as Lon and FtsH as well (Ishii et al., 2000; Kobiler et al., 2002). Motifs recognized by ClpXP might not all have been identified, but a ClpXP-specific pulldown of cellular proteins, in which >50 potential substrates for ClpX were identified, suggests that there is a limited number of good motifs (Flynn et al., 2003). One of the most frequently found motifs mimics a sequence, called the SsrA tag, which has evolved specifically to target certain classes of abnormal proteins for degradation by ClpX or ClpA (Karzai et al., 2000). A second motif based on the portion of this tag specifically recognized by ClpX itself was found in >20 of the proteins isolated in the pulldown experiment. A motif that resembled one found at the N terminus of another known substrate was found in other proteins isolated in the same screen.

Other factors also affect the targeting of proteins to Clp/Hsp100 proteins. Accessibility of the motif will depend on the folding near the N or C terminus of the protein or on functional interactions that mask the motif. For example, the protection of an antitoxin protein when it is complexed with its cognate toxin is a common mechanism controlling cell death pathways in bacteria (Van Melderen et al., 1996). A novel masking mechanism was shown for a protein that undergoes limited proteolysis in response to a specific cellular signal. The LexA repressor is cleaved in two following DNA damage (Little, 1984). The surviving N-terminal fragment retains residual activity; however, the exposed C terminus of this fragment bears an SsrA tag and is targeted to ClpXP for degradation (Neher et al., 2003).

While Clp/Hsp100 proteins can interact directly with many motifs or portions of the motif, affinity is enhanced by additional proteins called adaptors. Adaptor proteins interact with the protein target and with the Clp/Hsp100 protein and make presentation of the motif more efficient. The adaptor can be regulated, making presentation of the protein target dependent on specific cellular signals. For example, the stationary phase sigma factor RpoS is presented to ClpXP in a complex with the phosphorylated form of RssB, which is in turn phosphorylated by a two-component signal transduction system (Pratt and Silhavy, 1996; Zhou et al., 2001). As cells enter stationary phase, decreased posphorylation of RssB blocks presentation and leads to stabilization of RpoS (Hengge-Aronis, 2002).

The presence of an adaptor can force the degradation to proceed by a specific pathway. SsrA tagged proteins can be recognized directly by ClpXP and degraded (Kim et al., 2000b; Singh et al., 2000). A small adaptor, SspB, binds to a portion of the SsrA tag that is not bound by ClpX and facilitates presentation of SsrA-tagged proteins to ClpX (Flynn et al., 2001). The adaptor itself can bind to ClpX, but degradation of the tagged protein still requires binding between ClpX and the terminal motif on the protein target (Levchenko et al., 2003; Song and Eck, 2003; Wah et al., 2002). However, when the target protein is bound to the adaptor, access of the terminal motif to its site on ClpX appears to be restricted and can only occur when the adaptor is also docked at its site on ClpX (Wah et al., 2003). This mechanism adds an additional level of specificity to the interactions and additional kinetic steps to the process providing additional levels of control over degradation.

A different phenomenon is observed with ClpS, which inhibits ClpA activity toward soluble proteins (Dougan et al., 2002). ClpS acts through the ClpA N-domains, even though the N-domains are not required for degradation of these substrates in vitro. Thus, ClpS/N-domain complex creates an inhibitory domain, which might act by sterically blocking access to specific docking sites for soluble proteins. Because aggregated proteins are targeted by ClpAP with ClpS bound, this model suggests that soluble proteins and aggregated proteins might interact at different sites or that the ClpS/N-domain complex creates a new site for interaction with aggregated proteins. The activity of *B. subtilis* ClpC is similarly directed toward aggregated proteins by an adaptor protein, MecA, which appears to stimulate both disaggregating activity of ClpC and the targeting of aggregated proteins for degradation by ClpCP (Schlothauer et al., 2002). MecA is also required for degradation of a specific regulatory protein, the competence factor, ComK, indicating that adaptor proteins may affect substrate selection by Clp/Hsp100 proteins and might act by blocking or creating binding sites for both specific and general recognition motifs.

Concluding Remarks

The Clp/Hsp100 chaperones have developed a multitude of mechanisms for attracting and holding on to substrate proteins. Once they encounter substrates, these efficient machines process them rapidly, turning over as many as 30 molecules a minute and hydrolyzing ATP at rates approaching 1000 per minute. ClpXP and ClpAP complexes are present in the range of 200 copies per cell, which, if the seemingly large number of substrates were in fact available, would keep them fully occupied. Although the number of ClpB complexes is perhaps ten times higher, its activity is intrinsically more demanding and proceeds at a slower rate. Anchoring of ClpB to aggregated proteins is an attractive mechanism of keeping it constantly saturated with substrate. Similar methods of allowing ClpX and ClpA equally good access might take advantage of some of the binding sites that are now assigned to substrate interactions. Given the rate of progress in this field, it won't be long before we learn more about the true functions of the N-domains or the SSD domains or other as yet unrecognized sites and the interactive networks in which they are engaged.

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