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<th>The N-terminal zinc binding domain of ClpX is a dimerization domain that modulates the chaperone function (Main article)</th>
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Protein Structure and Folding:
The N-terminal Zinc Binding Domain of
Cpx Is a Dimerization Domain That
Modulates the Chaperone Function

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Ashleigh Tuite and Walid A. Houry
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The N-terminal Zinc Binding Domain of ClpX Is a Dimerization Domain That Modulates the Chaperone Function*

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Clp ATPases are unique chaperones that promote protein unfolding and subsequent degradation by proteases. The mechanism by which this occurs is poorly understood. Here we demonstrate that the N-terminal domain of ClpX is a C4-type zinc binding domain (ZBD) involved in substrate recognition. ZBD forms a very stable dimer that is essential for promoting the degradation of some typical ClpXP substrates such as λO and MuA but not GFP-SsrA. Furthermore, experiments indicate that ZBD contains a primary binding site for the λO substrate and for the cofactor SspB. Removal of ZBD from the ClpX sequence renders the ATPase activity of ClpX largely insensitive to the presence of ClpP, substrates, or the SspB cofactor. All these results indicate that ZBD plays an important role in the ClpX mechanism of function and that ATP binding and/or hydrolysis drives a conformational change in ClpX involving ZBD.

Clp ATPases are a unique group of ATP-dependent chaperones associated with the disassembly of protein complexes (1). They are members of the AAA family of proteins, which are ATPases associated with a variety of cellular activities. The key feature of this family is a highly conserved AAA module of about 230 amino acids that is present in one or more copies in each protein. Each module can typically be divided into two subdomains: one αβ and one α (2). In the αβ subdomain, there is a conserved Walker A motif, involved in binding the phosphate of ATP, and a Walker B motif, involved in metal binding and ATP catalysis (3). The unifying structural feature of these AAA proteins is the arrangement of the subunits into ring-shaped hexameric or heptameric complexes.

The main Clp ATPases in the Escherichia coli cytoplasm are ClpA, ClpB, ClpX, and HslU. They function to unfold proteins or to disaggregate protein aggregates. They all have N-terminal domains followed by AAA modules, except for HslU, which has an insertion in the AAA module that is thought to correspond to the N-terminal domains in the other Clps (4). Both ClpA and ClpB have two AAA modules, whereas ClpX and HslU have only one, which is homologous to the second AAA module in ClpA and ClpB (3). ClpA and ClpX form hexameric complexes that can associate with ClpP (5), a serine protease consisting of two rings with 7-fold symmetry (6). Several ClpXP and ClpAP substrates are known (reviewed in Ref. 1). ClpA substrates include RepA, HemA, N-end rule proteins, and ClpA itself. Known ClpX substrates include λO, MuA, Mu repressor, UmuD, α5, and Phd. Some of these proteins, such as λO and RepA, are recognized by the chaperones mainly through their N termini, whereas others, like MuA and Mu repressor, seem to be recognized mainly through their C termini. More recently, a proteomics approach revealed more than 50 potential ClpXP substrates in E. coli (7). Analysis of these substrates revealed three C-terminal ClpX recognition consensus sequences and two such N-terminal sequences.

Both ClpXP and ClpAP have been implicated in the degradation of C-terminally SsrA-tagged proteins (8). The SsrA tag, which has a very hydrophobic sequence of 11 amino acids, is cotranslationally added to the C terminus of targeted nascent chains (9). In addition, ClpA and ClpX have been shown to bind to adaptor proteins that affect their function (10–12). SspB enhances the degradation efficiency of C-terminally tagged SsrA proteins by ClpXP (13, 14). It has been proposed that an SspB dimer binds to two GFP-SsrA molecules and that this complex subsequently binds to the ClpX hexamer (12).

A number of elegant studies have demonstrated the ability of ClpX and ClpA to unfold native proteins (15–18). Experiments have suggested that unfolding, translocation, and degradation take place in a unidirectional manner starting from the attachment point of the degradation tag (19, 20). The importance of the N-terminal domain of Clp ATPases in these processes has been rather enigmatic. The N terminus of ClpB from Thermus thermophilus has been shown not to be essential for chaperone activity in vitro (21), and ClpB from the cyanobacterium Synechococcus sp. strain PCC 7942 with the N terminus deleted was able to restore bacterial thermotolerance in vivo (22). On the other hand, E. coli ClpB lacking its N terminus was shown to be either inactive (23) or active (24) as a chaperone in vitro depending on the assay being used. ClpA deleted of the N terminus can mediate the ClpP-dependent degradation of SsrA-tagged substrates (11, 25, 26) but not RepA (25). Similarly, the N terminus of ClpX is not required for the degradation of GFP-SsrA by ClpXP but is required for the degradation of λO (26).

The importance of the N-terminal domain of ClpX is evident from its conservation across all sequenced genomes. The domain is present in almost all known clpX sequences (Table I). Here we demonstrate that the N-terminal domain of ClpX is a C4-type zinc binding domain (ZBD)1 that forms a very stable

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1 The abbreviations used are: ZBD, zinc binding domain; GFP, green fluorescent protein; MBP, maltose binding protein; PAR, 4-2-pyridylazo/resorcinol; PMB, p-hydroxymercuribenzoate; ICP-AES, inductively coupled plasma atomic emission spectroscopy; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; Ni-NTA, nickel-nitritoltriacetic acid.
The E. coli ClpP and ClpX protein sequences were used to BLAST against 16 archaeal, 124 bacterial, and 15 eukaryotic completely sequenced genomes in NCBI (July, 2003 version). All ClpP protein sequences were aligned and checked for the presence of the zinc binding motif (CX_4CX_7-HCX_C), where X is any amino acid, as well as for the presence of the ClpP binding motif IGF (37). “No” refers to the absence of the gene if BLAST and tBLASTn programs did not provide a hit (E-value cutoff was e−50).

### EXPERIMENTAL PROCEDURES

**Subcloning and Mutagenesis**—All proteins were expressed from the pProEX HTb vector (Invitrogen) except for MBP-ZBD[1–60], which was expressed from pMAL C2 vector (New England Biolabs). Point mutations were introduced using the QuikChange system (Stratagene) according to manufacturer’s protocol. All constructs were verified by DNA sequencing.

pET9aClpP overexpressing untagged ClpP was a gift from Dr. John Flanagan (Brookhaven National Laboratory). pRLM266 overexpressing MuA (29) was purified according to published protocols. Protein concentrations were determined using either the Bradford assay or the Bio-Rad protein assay according to manufacturer’s protocol. All experiments were carried out in triplicate.

### Measurement of GFP-SsrA Expression and Stability in Vivo—E. coli strains MC4100 and MC4100 clpP::KanR were transformed with pGFP or pGFP-SsrA (Chloramphenicol R) (31), and the ΔclpP strains were subsequently transformed with pProEX HTb (AmpR) plasmid encoding clpP or clpXZBD genes. Cultures were grown at 37 °C in LB medium with appropriate antibiotic selection until A_{600} of ~0.4, and the expression of GFP and GFP-SsrA was induced using 0.2% arabinose. Samples were taken at 20, 40, and 60 min post-induction and diluted to an A_{600} of ~0.3 using Buffer C (25 mM TrisCl, pH 7.5, and 150 mM NaCl). Fluorescence was measured by setting excitation at 405 nm and monitoring emission from 410–600 nm. The emission was normalized to the peak at 456 nm, which is dependent on the density of the cells.

**Coxexpression of H₂O₂-ZBD[1–60] and ClpX**—The plasmids pET28b and pET3a expressing H₂O₂ and ClpX, respectively, were transformed into BL21(DE3) cells, and the coexpression of the two proteins was induced with isopropyl-1-thio-β-D-galactopyranoside. ClpX was also expressed alone as a control. Cells from 50 ml of culture were lysed by sonication in Buffer D (25 mM TrisCl, pH 7.5, 100 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol, and 10 mM imidazole) with 1 mg/ml lysozyme after 20 min of incubation on ice. Purification was carried out according to the manufacturer’s protocol. Bound proteins were eluted with Buffer D plus 250 mM imidazole. Proteins were then separated on SDS-PAGE.

**Cross-linking of ZBD[1–60]**—Cross-linking was carried out in Buffer E (25 mM Hepes, pH 7.9, 150 mM NaCl, 100 μM ZnCl₂, and 1 mM dithiothreitol) using glutaraldehyde at a final concentration of 0.05% and a final protein concentration of 1 μM. Samples were removed at different time points, and the reaction was quenched by adding TrisHCl, pH 8, to a final concentration of 0.5 M.

**Sedimentation Equilibrium Analysis of ZBD[1–60]**—Sedimentation equilibrium experiments and analysis were performed at the Analytical Centrifugation Facility at the Ontario Cancer Institute (Toronto). ZBD[1–60] was exchanged into Buffer F (25 mM Hepes, pH 7.9, 150 mM NaCl, and 2 mM tris(2-carboxyethyl)phosphine) and diluted to concentrations of 100, 166, and 233 μM. Experiments were performed at four different speeds at 20 °C.

**Circular Dichroism of ZBD**—CD measurements were obtained using an AVIV Circular Dichroism spectrometer Model 62ADS or a Jasco J-810. Proteins were diluted into Buffer G (25 mM TrisCl, pH 8, 150 mM NaCl, 100 μM ZnCl₂, and 1 mM dithiothreitol).

**Fitting the Denaturation Curves**—To analyze the data from thermal and chemical denaturation experiments, a model where the native ZBD dimer (N₂) unfolds directly to two unfolded monomers (U) was used. Analysis was carried out according to Bowie and Sauer (32); however, a global fit to the Gibbs-Helmholtz equation, shown in Equation 1, was used.
ClpX N-Terminus Is a Dimer That Modulates Chaperone Function

Degradation Assays—Assays were typically carried out by preincubating a degradation mixture consisting of 1.5 units of creatine kinase, 16 mM creatine phosphate, 3 mM ATP, 4 mM substrate protein, 1.2 mM ClpP in Buffer H (25 mM Hepes, 5 mM MgCl2, 5 mM KCl, 0.03% Tween 20, and 10% glycerol) at 37 °C for 3 min. Subsequently, 1 mM ClpX or ClpX mutant was added to the reaction, and the incubation was continued at 37 °C. All concentrations are those of monomers. Samples were taken at the indicated time points, and the degradation was stopped by addition of 4× Laemmli buffer and boiling. Proteins were then separated on SDS-PAGE, and the intensities of the protein bands were then quantified using ImageQuant 5.0. The initial rate ($V_0$) was determined from the slope of the plot of ln(IPB) versus time for the first 5 min of the reaction. Degradation of GFP-SsrA was also monitored by fluorescence using a Fluorolog spectrophotometer (Jobin Yvon) with the excitation wavelength set at 395 nm and the emission wavelength set at 509 nm. For the GFP-SsrA competition assays, SsrA peptide, λO, MuA, or SspB were preincubated with the degradation mixture prior to the addition of ClpX.

ATPase Activity Using NADH—ATPase activity of ClpX was measured using a coupled assay (33). Assays were typically carried out by preincubating 1.0 μM ClpX or ClpX mutant, 0.2 mM NADH, 3.0 mM phosphoenolpyruvate, 4.7 units of pyruvate kinase, and 7.4 units of lactate dehydrogenase in Buffer H at 37 °C for 3 min. 1.2 μM ClpX, 4

Fig. 1. Zinc binding and dimerization of ZBD. A, the release of Zn$^{2+}$ from ZBD[1–60] (●) or ZBD[1–60, C36A] (▲) was monitored by absorbance at 500 nm to follow the formation of (PAR)${_3}$Zn$^{2+}$ complex upon addition of PMB. The complete release of Zn$^{2+}$ from ZBD[1–60] required 4 moles of PMB to one mole of ZBD[1–60]. The measured Zn$^{2+}$ content of other ZBD mutants are listed in the table. B, ZBD[1–60] was incubated at 1 μM in Buffer E in the presence of 0.05% glutaraldehyde. At indicated time points, the reaction was quenched by the addition of TrisHCl to 0.5 M, and samples were separated on SDS-PAGE and visualized by silver staining. As control, the protein was either heat-denatured in 1% SDS or denatured in 8 M urea and subjected to cross-linking as above; only the 30-min time point is shown. C. The upper panel shows the sedimentation equilibrium ultracentrifugation data for ZBD[1–60] at 166 μM. Points shown were collected at 33,000 rpm and 20 °C. The solid line is the theoretical fit to the data using a single species function of ZBD[1–60] dimer. The lower panel displays residual deviations from the theoretical fit. D, a mixture of GFP (27 kDa) and ZBD[1–60]-GFP (34 kDa) or of MBP (43 kDa) and MBP-ZBD[1–60] (50 kDa) was loaded onto a calibrated Superdex 200 gel filtration column equilibrated in Buffer G. Molecular mass markers (kDa) are shown on the top x axis. The proteins migrated at the following positions: 35, 100, 40, 120 kDa, respectively. Because the ZBD[1–60] dimer alone migrates as a 28-kDa protein, ZBD[1–60]-GFP and MBP-ZBD[1–60] migrate at the expected molecular masses of dimers (28 + 2 × 35 and 2 × 40 + 28, respectively).
ClpX N-Terminus Is a Dimer That Modulates Chaperone Function

The N Terminal of ClpX Is a Dimer Containing a C4-type Zinc Binding Motif—Purified ClpX (10 μg in 140 μl) was subjected to limited proteolysis using sub-stoichiometric amounts of trypsin. The resulting peptides were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. At very low trypsin quantities (0.1 ng), a 7-kDa peptide that mapped to the N-terminal 60 amino acids of ClpX was detected (numbering according to Swiss-Prot P33138). This region appeared to be very stable and was not degraded with higher protease quantities. Our results are generally consistent with earlier observations made by Singh et al. (26).

Subsequently, the N terminus of ClpX was cloned and purified, and its ability to bind Zn\(^{2+}\) was characterized using ICP-AES and a colorimetric assay (Fig. 1A). Both techniques clearly demonstrated that the N terminus of ClpX spanning residues 1–60 bound Zn\(^{2+}\) in a ratio of one Zn\(^{2+}\) ion per protein monomer. We call this domain ZBD[1–60] for zinc binding domain of ClpX spanning residues 1 to 60. Only the first four cysteines (Cys-14, Cys-17, Cys-36, and Cys-39) are involved in chelating the Zn\(^{2+}\), because mutants lacking the fifth cysteine (Cys-43) were still able to bind Zn\(^{2+}\) in one to one molar ratio, whereas the C36A mutant resulted in the formation of a destabilized domain that did not contain Zn\(^{2+}\) (Fig. 1A). These results, as well as previous observations (34), clearly suggest that the highly conserved CX\(_2\)CX\(_{18–40}\)CX\(_3\)-C motif in ZBD of ClpX is a C4-type zinc finger.

ZBD[1–60] migrated as a 28-kDa protein on a gel filtration column (not shown). This is much larger than a monomer, because the predicted molecular mass of ZBD[1–60] is 7.046 kDa. Consequently, the oligomeric state of ZBD[1–60] was further investigated using several techniques. In an initial approach, chemical cross-linking was performed using glutaraldehyde. It was found that a cross-linked dimer could be detected in as little as 15 s, and higher oligomers were not detected even after 30 min (Fig. 1B). This oligomerization was judged to be specific at the protein concentration used (1 μM), because denaturation of ZBD[1–60] either by chemical or heat treatment (Fig. 1B) yielded no detectable dimeric species even after 30 min. ZBD[1–60] was further characterized by sedimentation equilibrium analytical ultracentrifugation at higher concentrations (100, 166, and 233 μM). The data was best fit to a single species with an apparent molecular mass of 14.94 kDa (Fig. 1C). This value is very close to the expected molecular mass of a dimer. The dimerization of ZBD[1–60] is not mediated by Cys-43, because ZBD[1–41] also formed a dimer as determined by gel filtration (not shown). ZBD[1–58], which has the domain boundaries identified by Singh et al. (26), also migrated as a dimer.

Furthermore, ZBD was able to force the dimerization of known monomers. Two fusion proteins, ZBD[1–60]-GFP (N-terminal fusion with green fluorescent protein) and MBP-ZBD[1–60] (C-terminal fusion with maltose binding protein), migrated as dimers when examined by size exclusion chromatography (Fig. 1D). Both GFP and MBP migrated as monomers. These experiments, together with the cross-linking and ultracentrifugation data, clearly demonstrate that the N-terminal domain of ClpX is not a monomer as published previously (26), but rather forms a stable dimer.

To demonstrate that this domain exists as a dimer in the context of the full-length chaperone, ClpX and His-tagged ZBD[1–60] were coexpressed and purified on a Ni-NTA column. ClpX co-purified on Ni-NTA only when coexpressed with His-tagged ZBD, whereas it did not bind to the resin nonspecifically in the absence of His\(_6\)-ZBD[1–60] (Fig. 2A). However, when purified, untagged ZBD[1–60], which is already dimeric, was mixed with purified His-tagged ClpX, which forms mixed oligomers, and then passed over the Ni-NTA column, no interaction between the two proteins was observed (Fig. 2B). His\(_6\)-ClpX bound specifically to the column, whereas untagged ZBD[1–60] did not bind to the column. Similarly, Singh et al. (26) had shown that the N-terminal domain of ClpX does not interact with the rest of the chaperone as determined by gel filtration. Hence, in the coexpression experiment, untagged ClpX must be interacting with His-tagged ZBD[1–60] through the dimerization of newly synthesized His\(_6\)-ZBD[1–60] with the N-terminal domains of newly synthesized ClpX. This surprising observation of the dimerization of the ClpX N terminus leads us to propose that the hexameric ClpX could possibly function as a trimer-of-dimers.

Stability of the ZBD Dimer—CD spectra of ZBD[1–60] were obtained for the native, denatured, and renatured protein (Fig. 3A). The spectrum of the renatured protein overlays perfectly with that of the native protein, indicating that thermal denaturation is completely reversible. Thermally and chemically induced unfolding of ZBD[1–60], as monitored by CD at 220 nm, was reversible and occurred in a cooperative fashion, with a single transition region (Fig. 3, B and C). The stability of ZBD[1–60] was decreased in the presence of EDTA (Fig. 3B) indicating that Zn\(^{2+}\) is a structural metal necessary for the stability of this small domain. Furthermore, the renaturation profile exhibited a concentration dependence as expected for a protein oligomer (compare 3 to 30 μM in Fig. 3B). This result further establishes ZBD[1–60] as a dimer. The single transition observed upon thermal and chemical denaturation, and the concentration dependence of the renaturation process, all indicate that the dimer is present in the unfolding transition region. The monomer is not populated to a great extent in that region.
The least squares fit to the Gibbs-Helmholtz equation, which yields mol

Solid lines represent the theoretical curves obtained from fitting the data in D to the Gibbs-Helmholtz equation. Circles represent the theoretical fit to the data assuming a linear dependence of ΔG_U to the Gibbs-Helmholtz equation (see Experimental Procedures). The thermodynamic parameters for ZBD[1–60] stability at 25 °C were found to be ΔG_U = 9.9 Kcal mol⁻¹, ΔH_U = 16.5 Kcal mol⁻¹, ΔS_U = 22 cal mol⁻¹ K⁻¹, and ΔCp = 0.41 Kcal mol⁻¹ K⁻¹. These values demonstrate that ZBD[1–60] is a very stable dimer that can withstand thermal stress and is ideally suited to be a domain in a protein involved in heat stress response.

ZBD Is Essential for ClpX Function in Mu Phage Replication—Replicative transposition during the lytic cycle of bacteriophage Mu requires the function of the ClpX chaperone (35). In an E. coli strain lacking the chromosomal clpX gene, the last step in transposition, which is the remodeling of the MuA transposase complexes, is blocked, because MuA remains bound to DNA as a tetramer (36), and, hence, the formation of plaques is prevented. A clpX knockout strain was transformed with plasmids expressing wild-type and mutant versions of the ClpX protein, and the ability to form plaques was assessed (37).

It was found that plaque formation in the wild-type strain was comparable with that of the knockout strain expressing wild-type ClpX from a plasmid (Fig. 4A). Interestingly, ClpX lacking ZBD[1–60] (ClpXΔZBD) could not support the lytic growth of Mu phage even at high concentrations of phage. The inability to complement the knockout phenotype was observed with ZBD[1–60] alone and with an ATPase deficient mutant, ClpX (K124Q); this mutation in the Walker A motif prevents ATP hydrolysis and renders the chaperone inactive. The expression levels and solubility of ClpX and the different mutants were similar in these cells (not shown).

The Role of ZBD in GFP-SsrA Degradation in Vivo—GFP-SsrA was expressed in wild-type cells, as well as in cells deleted of clpX gene expressing ClpX or ClpXΔZBD from a plasmid. After 20, 40, and 60 min of induction of GFP-SsrA, a fluorescence emission scan of the intact cells was obtained. In the clpX knockout cells, the peak because of GFP-SsrA fluorescence is evident with peak maximum at 508 nm (Fig. 4B, top right). This characteristic peak is absent in the wild-type strain or in the clpX knockout strains expressing either ClpX or ClpXΔZBD. In all four strains, untagged GFP was expressed to similar extents, and its levels were not reduced by the expression of ClpX (insets in Fig. 4B). Thus, ClpXΔZBD is active in...
degrading GFP-SsrA in vivo although it cannot function in disassembling MuA-DNA complexes. Hence, ZBD could be required for binding and/or unfolding of some substrates such as MuA but not GFP-SsrA. This possibility is investigated further.

**ZBD Modulates Substrate Degradation by ClpXP** — To further investigate the role of ZBD in ClpX function, the degradation reactions of GFP-SsrA, MuA, and λO were carried out at 37 °C in the presence of ClpP, ATP, an ATP regenerating system, and ClpX or ClpXΔZBD (Fig. 5A). At different time points, samples were withdrawn from the degradation assay, boiled in Laemmli buffer, and separated on SDS-PAGE. The degradation of GFP-SsrA was also monitored by fluorescence (Fig. 6). These experiments revealed several unexpected results.

The ClpP-dependent degradation of GFP-SsrA can be mediated by ClpX lacking ZBD as determined by quantifying the bands on the gel or by fluorescence (Fig. 5A, right panel and Fig. 6, E–H, 0 μm curves). This is in agreement with our in vivo observations (Fig. 4B), as well as with the observations of Singh et al. (26), who used enzymatically truncated ClpX. This indicates that the primary binding site for the SsrA tag is in the AAA domain of ClpX and not in the ZBD. However, the degradation of GFP-SsrA by ClpXΔZBD/ClpP was 33% less efficient than by ClpXP; this could reflect that ZBD further enhances the unfoldase activity of ClpX because of the presence of a secondary binding site for GFP-SsrA on ZBD. It should be noted that none of the ClpX constructs were able to degrade GFP-SsrA(DD) or GFP-SsrA in the presence of ADP or absence of ATP (data not shown).

On the other hand, ZBD seems to be absolutely essential for the binding and/or unfolding of λO because ClpXΔZBD/ClpP was not able to degrade λO (Fig. 5A, right panel). This has been observed before with ClpX in which the first four cysteines involved in chelating Zn\(^{2+}\) were mutated to serines (34) and with ClpX treated with limiting amounts of V8 protease to remove the first 54 amino acids (26). Furthermore, ClpXΔZBD was six times less efficient than ClpX in mediating the degradation of MuA (Fig. 5A). This result is consistent with the inability of ClpXΔZBD to promote Mu phage replication (Fig. 4A). Hence, λO and MuA seem to represent a class of ClpX substrates that, unlike GFP-SsrA, requires the full-length chaperone to be efficiently degraded.

To demonstrate that ZBD is directly involved in substrate binding, ELISA analysis was performed (Fig. 5B). GFP-SsrA, MuA, and λO, as well as the nonspecific protein BSA, were bound to the ELISA wells. ZBD[1–60] was added to the wells at
increasing concentrations. The amount of bound ZBD[1–60] was detected by first incubating with rabbit anti-ClpX antibodies, followed by incubating with Protein A conjugated to horse-radish peroxidase. As can be seen in Fig. 5B, ZBD[1–60] bound with similar affinity to λO and MuA but did not interact significantly with GFP-SsrA or BSA. Thus, ClpX contains at least two different binding sites; one is present in the ZBD and is required for binding λO and MuA, and one is within the AAA domain and is essential for binding GFP-SsrA.

To further characterize the substrate binding sites in ClpX, the degradation of GFP-SsrA was performed in the presence of different concentrations of λO, MuA, SsrA peptide, or the cofactor SspB. Each competition assay included ClpP, ATP, an ATP regenerating system, ClpX, or ClpXΔZBD, and the degradation of GFP-SsrA was monitored by fluorescence (Fig. 6). λO, MuA, and the SsrA peptide competed in a concentration-dependent manner for the ClpX-mediated degradation of GFP-SsrA (Fig. 6, A–C), whereas SspB enhanced the degradation of GFP-SsrA as expected (Fig. 6D) (13). However, for ClpXΔZBD, only the SsrA peptide was able to significantly compete for GFP-SsrA degradation (Fig. 6D), further proving that the ZBD is not required for SsrA binding or recognition and that the binding of the SsrA tag occurs in the AAA domain of ClpX. On the other hand, the presence of 50 μM λO, which is at 12.5 times excess over GFP-SsrA, was insufficient to slow down the GFP-SsrA degradation by ClpXΔZBD (Fig. 6E). Similar curves were obtained if λO was added 2 min after the start of the reaction (not shown). Hence, ZBD seems to be essential for binding λO. It should be noted that λO at 2 μM was able to slow the degradation by ClpXP of 4 μM GFP-SsrA (Fig. 6A) indicating that the full-length chaperone has higher affinity for λO than GFP-SsrA. MuA was also able to compete with GFP-SsrA but much more weakly in the case of ClpXΔZBD as compared with ClpX (Fig. 6, F versus B). This suggests that ZBD in ClpX significantly increases the binding affinity of MuA to the chaperone.

The presence of as little as 0.05 μM of the cofactor SspB increased GFP-SsrA degradation by ClpXP (1 μM), and the degradation rate of GFP-SsrA was saturated between 0.33 and 10 μM SspB (Fig. 6D). On the other hand, SspB slowed down, rather than enhanced, the degradation of GFP-SsrA by ClpXΔZBD/ClpP (Fig. 6H). Hence, the ZBD is essential for the enhancing activity of SspB, which is probably because of the direct binding between SspB and ZBD. For the case of ClpXΔZBD, SspB would directly bind the SsrA tag of GFP-SsrA (12, 14) but would not be able to transfer the substrate to the chaperone because of the absence of ZBD. Therefore, the presence of SspB would protect GFP-SsrA from degradation by ClpXΔZBD/ClpP as observed (Fig. 6H). This effect is not seen in vivo in Fig. 4B, probably because the levels of SspB are much lower than those of overexpressed ClpXΔZBD. Because both SspB and λO require the ZBD for binding to ClpX, it is likely that both proteins bind at the same site(s) or at very close site(s) on ZBD considering the small size of this domain.

**ZBD Modulates the ATPase Activity of ClpX—** The ATPase activity of ClpX can be stimulated by substrates (38) but is inhibited by ClpP (39). Under the experimental conditions used, the ATPase activity of ClpX alone was 273 pmol min⁻¹ μg⁻¹ (Fig. 7), which is similar to previously published results (38). The addition of ClpP in the absence of substrates or cofactor lowered the ATPase activity to 218 pmol min⁻¹ μg⁻¹. In the absence of ClpP, the ATPase activity of ClpX increased upon addition of 4 μM λO, GFP-SsrA, or GFP-SsrA with 0.33 μM SspB to 944, 574, and 1160 pmol min⁻¹ μg⁻¹, respectively (Fig.

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**Fig. 5. Role of ZBD in substrate degradation and binding.** A, the degradation of GFP-SsrA, MuA, and λO (each at 4 μM) by ClpXP (left panel) or ClpXΔZBD/ClpP (right panel) (each at 1 μM) was monitored by SDS-PAGE. Aliquots were removed from the degradation mixture at the indicated time points. The chaperone was the last component added to the reaction mixture. b refers to before addition of the chaperone. V₀ is the initial rate of substrate degradation (see “Experimental Procedures”). B, ELISA assays of the binding of ZBD[1–60] to λO, MuA, GFP-SsrA, and BSA. Data points are the average of three experiments.

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**Fig. 6.** A, the degradation of GFP-SsrA, MuA, and λO (each at 4 μM) by ClpXP (left panel) or ClpXΔZBD/ClpP (right panel) (each at 1 μM) was monitored by SDS-PAGE. Aliquots were removed from the degradation mixture at the indicated time points. The chaperone was the last component added to the reaction mixture. b refers to before addition of the chaperone. V₀ is the initial rate of substrate degradation (see “Experimental Procedures”). B, ELISA assays of the binding of ZBD[1–60] to λO, MuA, GFP-SsrA, and BSA. Data points are the average of three experiments.

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**Fig. 7.** The presence of as little as 0.05 μM of the cofactor SspB increased GFP-SsrA degradation by ClpXP (1 μM), and the degradation rate of GFP-SsrA was saturated between 0.33 and 10 μM SspB (Fig. 6D). On the other hand, SspB slowed down, rather than enhanced, the degradation of GFP-SsrA by ClpXΔZBD/ClpP (Fig. 6H). Hence, the ZBD is essential for the enhancing activity of SspB, which is probably because of the direct binding between SspB and ZBD. For the case of ClpXΔZBD, SspB would directly bind the SsrA tag of GFP-SsrA (12, 14) but would not be able to transfer the substrate to the chaperone because of the absence of ZBD. Therefore, the presence of SspB would protect GFP-SsrA from degradation by ClpXΔZBD/ClpP as observed (Fig. 6H). This effect is not seen in vivo in Fig. 4B, probably because the levels of SspB are much lower than those of overexpressed ClpXΔZBD. Because both SspB and λO require the ZBD for binding to ClpX, it is likely that both proteins bind at the same site(s) or at very close site(s) on ZBD considering the small size of this domain.
The presence of ClpP lowered those ATPase levels to 835, 328, and 932 pmol min$^{-1}$ g$^{-1}$, respectively (Fig. 7). Because the $K_d$ for GFP-SsrA binding to ClpX hexamer is estimated to be around 3 $\mu$M (18), and because $\lambda O$ binds ClpX tighter than GFP-SsrA (see above), the concentrations of $\lambda O$ and GFP-SsrA were increased to 20 $\mu$M (5x) to saturate the binding between the substrates and ClpX in the presence of ClpP. The ATPase activity of ClpX did not change significantly in the presence of increased $\lambda O$ concentration (759 pmol min$^{-1}$ g$^{-1}$) possibly indicating that the lower $\lambda O$ concentration was already satu-
ClpX N-Terminal Is a Dimer That Modulates Chaperone Function

ClpX is a constitutive dimer that contains a C4-type zinc binding region. Cross-linking, unidirectional filter, gel filtration, and thermal melts all support the conclusion that ClpX is a very stable dimer. The ClpX dimer is reminiscent of the pseudo-2-fold symmetry observed in the ClpA and ClpB N termini (2, 40–42), which can be considered a pseudo-dimer. The N-terminal domain of ClpA has also been observed to contain a Zn^2+_ binding site in its first half consisting of two histidines, a glutamine, and a water molecule; however, there is only one Zn^2+_ bound per pseudo-dimer (41).

Pull-down experiments of coexpressed ClpX and ZBD suggest that ZBD is also a dimer in the context of the full-length ClpX chaperone. Hence, ClpX possibly functions as a trimer-of-dimers. On the other hand, the N termini of ClpA and ClpB have been shown to form monomeric domains (25, 42). Based on the ClpA crystal structure, as well as on electron microscopy studies, the N-terminal domains seem to be very flexible and not fixed in one orientation (2). Because the pseudo-dimer domains of ClpA and ClpB are ~150 amino acids, whereas the ZBD monomer of ClpX is less than half that size (60 amino acids), the protein density above the AAA hexamer of ClpA or ClpB is twice as much as that of ClpX. This could indicate that the N-terminal domain movements in ClpA and ClpB are different from those in ClpX; consequently, the ATP-dependent conformational changes that occur in ClpX might be different from those that occur in ClpA or ClpB implying a different mechanism of function.

In vitro and in vivo experiments indicate that ZBD is required for ClpX chaperone function for some substrates such as λO and MuA but not GFP-SsrA (Figs. 4–6). λO is a highly positively charged protein, with arginines or lysines constituting 16% of all its residues, resulting in a pI of 9.4. MuA has a similar character; 14.7% of its residues are lysines and arginines, and it has a pI of 8.9. On the other hand, SsrA (AANDEE- NYALAA) is a very hydrophobic tag with a pI of 3.7. In E. coli proteins, the average R+K content is about 10% (refer to EBI Proteome analysis). Hence, λO and MuA may be characteristic of a typical substrate for which ClpX requires the function of the ZBD.

Substrates, cofactors, and interacting partners modulate the ATPase activity of ClpX (Fig. 7). Removal of the ZBD essentially abolishes this modulation. It is reasonable to speculate that the ATP-dependent movement of the ZBD causes the stimulation in the ATPase activity of ClpX. This could be either because of the direct binding of substrates and cofactors to ZBD such as that observed for λO and SspB or as a result of an as yet unknown secondary effect because of the binding of the AAA domain, as for GFP-SsrA. The ATPase activity of ClpA and ClpB is also affected by the removal of their N-terminal domains (24, 26). In the case of ClpB, the ATPase activity is higher for the chaperone deleted of the N-domain as compared with the full-length chaperone, but the stimulation by substrates is decreased (24). On the other hand, ClpA lacking the N-domain has lower ATPase activity compared with the full-length protein (26). Thus, it appears that the N-terminal domains of the Clp chaperones play an essential role in regulating ATP hydrolysis in the presence of substrates and cofactors, reflecting an ATP-dependent conformational change involving these domains. Mapping out those conformational changes will be critical to understanding the unfoldase activity of this group of proteins.

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REFERENCES

ClpX N-Terminus Is a Dimer That Modulates Chaperone Function