Structure-Function Analysis of the Zinc-binding Region of the ClpX Molecular Chaperone*

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The ClpX heat shock protein of Escherichia coli is a member of the universally conserved Hsp100 family of proteins, and possesses a putative zinc finger motif of the C4 type. The ClpX is an ATPase which functions both as a substrate specificity component of the ClpXP protease and as a molecular chaperone. Using an improved purification procedure we show that the ClpX protein is a metalloprotein complexed with Zn(II) cations. Contrary to other Hsp100 family members, ClpXZn(II) exists in an oligomeric form even in the absence of ATP. We show that the single ATP-binding site of ClpX is required for a variety of tasks, namely, the stabilization of the ClpXZn(II) oligomeric structure, binding to ClpP, and the ClpXP-dependent proteolysis of the AO replication protein. Release of Zn(II) from ClpX protein affects the ability of ClpX to bind ATP. ClpX, free of Zn(II), cannot oligomerize, bind to ClpP, or participate in ClpXP-dependent proteolysis. We also show that ClpXACys, a mutant protein whose four cysteine residues at the putative zinc finger motif have been replaced by serine, behaves in similar fashion as wild type ClpX protein whose Zn(II) has been released either by denaturation and renaturation, or chemically by p-hydroxymercuri-phenylsulfonic acid.

The Clp family of ATPases can function both as specificity components for their corresponding proteolytic subunit partners or perform certain chaperone functions on their own. For example, they are able to protect or dissociate other protein aggregates, or dissociate specific tertiary protein structures (1–9). Both the proteolytic and chaperone activities of the Clp ATPases depend on ATP hydrolysis. More recent data demonstrate that chaperone and protease activities can occur concurrently in the ClpAP complex. For example, the ClpP protein is able to restore the chaperone activity of a ClpA mutant carrying an amino acid substitution in its N-terminal ATP-binding site. ClpA(K220V) is unable to activate RepA on its own, but in the presence of ClpP restores its ability to activate RepA (10).

In the presence of ATP, the ClpA or Hsp104 proteins oligomerize to give rise to a hexameric ring structure (10–13).

Electron microscopic studies show that the hexameric ClpA or ClpX ring structure binds to the double ring, barrel-like 7-fold symmetric ClpP component, thus giving rise to a structure that closely resembles the eukaryotic 26 S proteasome (14–17). The Clp ATPase activity is induced in the presence of its corresponding specific protein substrates (4, 18, 19). ClpA, ClpB, and Hsp104 each possess two ATP-binding sites. It has been shown by site-directed mutagenesis that the first ATP-binding site of ClpA, located near the N-terminal end of the protein, is responsible for hexamer formation and chaperone activity, whereas the second ATP-binding site is essential for ATP hydrolysis (10, 12, 20). Interestingly, in the case of yeast Hsp104 the roles are apparently reversed; the first ATP-binding site is responsible for ATPase activity and the second site is essential for oligomerization (11). In contrast to ClpA, ClpB, or Hsp104, the ClpX ATPase possesses only one ATP-binding site, highly homologous to the one responsible for hydrolysis (21, 22).

In this paper we investigate the putative role of Zn(II) in ClpX structure and function. We show that ClpX binds Zn(II) and that such an effect is important for binding of ATP to ClpX, and its proper oligomerization. These events influence binding of ClpX to ClpP and, consequently, the proteolysis reaction catalyzed by the ClpXP protease.

MATERIALS AND METHODS

DNA Cloning and Mutagenesis—Plasmid pBAD24, which contains the clpX wild type gene, was constructed as follows. The coding sequence of clpX was amplified from DNA extracted from Escherichia coli strain B78 by means of two oligonucleotides (synthesized by Life Technologies, Inc.) derived from the published sequence of clpX (21).

The N-terminal primer was 5'-GGTTCTCCTAGGATACCTAAAAAGGCA- AAGATGCC-3', introducing a BspHI restriction site and the C- terminal primer was 5'-CCAAAGTTCTCTCAGATTCCACAGATGCC- TGTGTGCCG-5', carrying a PstI restriction site. Polymerase chain reaction was carried out in a reaction volume of 50 μl. The annealing temperature was 60 °C and 35 cycles were performed. The polymerase chain reaction product was cloned into the Ncol-PstI sites of the pBAD24 expression vector.

The sequence of clpX was confirmed by restriction analysis and automated sequencing using the ABI Prism 310 DNA sequencer (PerkinElmer Life Sciences, Applied Biosystems). The resulting plasmid construct was used to transform E. coli DH15a (F-, adlacZΔM15, lacZYA-argF, U189, deoR, recA1, end A1, phoA, hsdR17, supE44, xthi1, gyrA96, relA1) and CJ236 (dut1, ung1, thi1, relA1/pCJ105, Cm') strains for overproduction and mutagenesis, respectively.

Oligonucleotide-directed Mutagenesis of clpX—The clpX expression plasmid was mobilized with VCSM13 (Stratagene) in strain CJ236 and amino acid substitutions were generated using the site-directed mutagenesis method of Kunkel et al. (23). The cysteine to serine substitutions at residues 15, 18, 37, and 40 were introduced using the following oligonucleotides (synthesized by Interactiva Biotechnologie): C15S, 5'-GGCCGAAAAGGAGATACGCAATT-3'; C18S, 5'-GCTTTGGCGCTAAAAGAGCAATTT-3'; C37S, 5'-ACACATTCGGAGAT-
ATACAGC-3'; C40S, 5'-CATATAATCAACAGATTGTCGGGAT-3'. All residues downstream from codon C40S were as in wild type ClpX. The DNA sequence was verified by automated sequencing using the ABI Prism 310 DNA sequencer (PerkinElmer Life Sciences Inc., Applied Biosystems).

Proteins—In all experiments described in this paper highly purified proteins (95% or greater purity) were used. All steps of purification were carried out at 0–4 °C. Thirty grams of ClpX overexpressing E. coli cells were lysed in 170 ml of X buffer composed of 50 mM Tris (pH 7.8), 200 mM KCl, 5 mM DTT, 10% (v/v) glycerol, 300 mM spermidine-HCl, and 1 mg/ml lysozyme. To ensure complete lysis, following a 45-min incubation at 0 °C, the lysate was transferred to 42 °C for an additional 5 min and the concentration of salt was increased to 1 M. The lysate was centrifuged in a Beckman R35 rotor at 20,000 rpm for 30 min at 0 °C. Proteins in the supernatant (160 ml) were precipitated with ammonium sulfate (0.29 g/ml) and centrifuged at 25,000 rpm for 5 min at 0 °C using the same rotor. The pellet was resuspended and dialyzed for 6 h at 0 °C against buffer X1, composed of 50 mM Tris-HCl (pH 7.6), 100 mM KCl, 5 mM DTT, 0.1 mg/ml phenylmethylsulfonyl fluoride, 10% (v/v) glycerol, and 0.01% Triton X-100. The extract was centrifuged in an R35 Beckman rotor at 25,000 rpm for 4 min at 0 °C. The supernatant was applied onto a Superose 12 (Amersham Pharmacia Biotech) column (2.5 × 20 cm) which had previously been equilibrated with buffer X1. After extensive washing (12 h at 30 ml/min) of the Q-Sepharose column with buffer X2 (40 ml), the bound proteins were eluted with a salt gradient (2 × 300 ml) from 100 to 450 mM KCl. Fractions containing the ClpX protein were pooled (50 ml) and applied onto a hydroxyapatite column (Bio-Rad; 1 × 10 cm) equilibrated with buffer X1. The bound proteins were eluted with potassium phosphate in buffer X1 (2 × 25 ml); from 0 to 300 mM Kπ, (pH 7.2). Pooled fractions containing the ClpX protein (15 ml) were dialyzed against buffer X2, composed of 50 mM Tris-HCl (pH 7.6), 100 mM KCl, 10% (v/v) glycerol, 5 mM DTT, and 0.01% Triton X-100 and applied onto a Resource MonoQ (Amersham Pharmacia Biotech) column (6 ml). A linear gradient of KCl (2 × 30 ml) from 100 to 400 mM (1 ml/min flow rate) was used to elute ClpX from the column using an fast protein liquid chromatography (Amersham Pharmacia Biotech) system. Fractions containing homogenous ClpX were pooled together (7 ml) and Bradford Bio-Rad assay was performed to estimate the protein concentration as 5 mg/ml. SDS-PAGE and size exclusion chromatography on Superdex 200 (Amersham Pharmacia Biotech) equilibrated with the same buffer. The chromatography was carried out at a flow rate of 0.3 ml/min (room temperature) using Gold HPLC system (Beckman) equipped with a diode array detector. In the case where no ATP (or 0.2 mM) was used in the omission buffer, eluting proteins were monitored using absorption at 280 nm. Fractions were collected and the presence of protein was visualized following SDS-PAGE and Coomassie Blue staining. The relative amount of protein was estimated using densitometry (Bio-Rad). The Superdex 200 column was calibrated with the following Bio-Rad molecular weight standards: thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17.5 kDa).

Protease Assays—The standard protease assay reaction mixture (100 μl) contained H-labeled AO protein (0.5 μg, 40,000 cpm), ClpX (0.2 μg), ClpP (2 μg) in 20 mM Hepes/KOH (pH 7.2), 10 mM MgCl2, 10 mM ATP, 0.5% Brij 58, 4 mg/ml bovine serum albumin. The reaction was assembled on ice, then transferred to 30 °C for the desired time. The reaction was stopped by the addition of ice-cold trichloroacetic acid (final concentration 10% (v/v)). After centrifugation (10 min, 5,000 g, 4 °C) the radioactivity present in the soluble fraction was estimated following the addition of tolune/Triton X-100 scintillation fluid.

Kinetics of ClpXP-mediated Hydrolysis of AO Protein—Protodysates was carried out in a buffer containing 20 mM Hepes/KOH (pH 7.2), 10 mM MgCl2, 10 mM ATP, 0.5% Brij 58. Each assay included 5 μg of ClpX, 10 μg of ClpXZZn(II), or ClpXZn(−), 5 μg of the AO substrate. The reaction mixtures were incubated at 30 °C, and at the desired times, 25-μl portions were withdrawn and processed by 12.5% SDS-PAGE. The relative amount of non-hydrolyzed protein was estimated using densitometry (Bio-Rad). The data are presented in Table II.

**RESULTS**

ClpX Is a Metalloprotease—ClpX is a molecular chaperone which can also work as a specificity factor for the ClpP protease (4, 24). It contains a single ATP-binding site, a substrate-binding domain called “sensor and substrate discrimination” or SSD domain (31), and a putative Zn finger motif, containing cysteines, of unknown function. The metal ion of the releasing Zn(II) ions upon coelution with PMPS, and by atomic absorption showed that one Zn(II) ion is bound to each monomer of ClpX (Table I and Fig. 1). To determine how many Zn(II) molecules bind to ClpX, we incubated a highly purified ClpX protein preparation, fully active in the AO proteolysis assay (24), with PMPS, known to release Zn(II) from zinc-binding proteins (30). The formation of a mercaptide bond between the

| Protein          | Moles Zn/mol ClpX |
|------------------|-------------------|
| ClpX Zn(II)      | 0.95              |
| ClpX Zn(−)       | <0.05             |
| ClpX ΔCys        | 0                 |

mm Tris/HCl (pH 7.8), 10% (v/v) glycerol, 10 mM MgCl2, 150 mM KCl, 25 mM NaCl in a final reaction volume of 50 μl. Samples contained 20 μg of ClpX or ClpX ΔCys protein and 4 mM ATP. The reactions were stopped by addition of 800 μl of malachite green (0.034%) and ammonium molybdate (10.5 g/liter in 1 N HCl) mixture and 100 μl of 34% citric acid. Absorption at 660 nm was measured after 30 min of incubation at room temperature. Results were compared with the calibration curve prepared for the phosphate salt (28).

Protein-Protein Interaction Assays—The sensitive ELISA assay used for monitoring protein-protein interactions has been previously described in detail by Wawrzynow et al. (4).

Size Exclusion Chromatography—The reacting components (100 μl) were incubated at 30 °C for 30 min in buffer B (25 mM Hepes/KOH (pH 7.2), 10 mM MgCl2, 25 mM NaCl, 5 mM MgCl2, 10% (v/v) glycerol, and 5 mM DTT) before loading onto a Superdex 200 HR 10/30 sizing column (Amersham Pharmacia Biotech) equilibrated with the same buffer. The chromatography was carried out at a flow rate of 0.3 ml/min (room temperature) using Gold HPLC system (Beckman) equipped with a diode array detector. In the case where no ATP (or 0.2 mM) was used in the omission buffer, eluting proteins were monitored using absorption at 280 nm. Fractions were collected and the presence of protein was visualized following SDS-PAGE and Coomassie Blue staining. The relative amount of protein was estimated using densitometry (Bio-Rad). The Superdex 200 column was calibrated with the following Bio-Rad molecular weight standards: thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17.5 kDa).

Purified GroEL was also used for the estimation of the molecular weight of ClpP/ClpX complex.

Stoichiometry of Zinc Cations in ClpX—Increasing amounts of PMPS (p-hydroxymercuriphenylsulfonic acid), at a concentration 1 mM, were added to a sample containing 600 μl of 5 μM ClpX. The reactants were mixed and the absorbance at 500 nm was measured. Before addition of the first aliquot of PMPS, the spectrophotometer was adjusted to the zero absorbance value. PAR (4,2-pyridylazopyridine) and PARCys was present in the cuvette at 0.1 mM throughout the measurements. The absorption coefficient for the (PAR)2-Zn(II) complex at 500 nm is $e = 6.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (29).

Removal of Zn2+ Cations from the ClpXZn(II) Protein using a Denaturation and Renaturation Procedure—ClpX was denatured in the buffer containing 50 mM Tris-HCl (pH 7.8), 10% (v/v) glycerol, 150 mM KCl, 25 mM NaCl, 10 mM DTT, and 8 M urea. In order to remove the zinc ions the same buffer supplemented with 10 mM EDTA was exchanged several times. The renaturation was carried out upon slow dialysis in the same buffer but in the absence of urea and EDTA.

CD Spectra—CD spectra measurements were carried out using a Jasco-5000 CD spectrophotometer in 1-mm cuvettes at 25 °C in a buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM KCl, and 25 mM NaCl.

InfraRed Spectra—The IR spectra of ClpX Zn(II), ClpXΔCys, and ClpX Zn(−) were collected as described previously (30).

Atomic Spectroscopy—This was performed using a AAS 30 Carl Zeis Jena spectrometer.

**RESULTS**

The abbreviations used are: DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; PMPS, p-hydroxymercuriphenylsulfonic acid; PAR, 4-(2-pyridylazo)resorcinol; HPLC, high performance liquid chromatography.
An increasing amount of PMPS was added to the ClpX protein (filled squares) or ClpXΔCys (open circles) both at 5 μM. The reactants were mixed and the absorption was measured at 500 nm in the presence of 0.1 mM PAR.

Free Cys residue and PMPS can be monitored by absorbance at 250 nm. The addition of the high affinity metallochrome indicator PAR, which changes color (absorbance at 500 nm) after the formation of the PAR-Zn(II) complex, allowed the monitoring of the amount of Zn(II) released from ClpX after PMPS treatment (Fig. 1). Control experiments, using atomic absorption, show that treatment of ClpX with PMPS (see “Materials and Methods” for details) leads to the complete removal of Zn(II) from the ClpX protein (Table I).

Using site-directed in vitro mutagenesis procedures, we replaced the four cysteine residues at positions 15, 18, 37, 40 with serine. As predicted, the resulting mutant, termed ClpXΔCys, does not bind Zn(II) (Fig. 1 and Table I). Release of Zn(II) ions from ClpX by either PMPS or a denaturation and renaturation procedure (see “Materials and Methods”) does not lead to major changes in the secondary structure of the ClpX protein, as judged from the CD spectra (Fig. 2). Also, in the case of the ClpXΔCys mutant the ClpX secondary structure is largely unaffected (Fig. 2), suggesting that both wild type and mutant proteins fold in an overall similar fashion. If the zinc-binding domain is found predominantly in a β-sheet or loop structure, it could be unfolded in the unfolded state with little effect on the CD spectrum of the full-length protein. The application of a more sensitive approach, namely infrared absorbance spectroscopy, suggests that the differences in secondary structure between ClpX, ClpX treated with PMPS, and ClpXΔCys mutant are indeed minor (Fig. 3). Thus, CD and IR spectroscopy cannot detect the local conformational changes of ClpX, which should occur following the release of Zn(II) from the ClpX protein. However, the release of Zn(II) from ClpX partially inhibits the ATP-dependent hydrolysis of the ClpX protein substrate by the ClpXP protease (Fig. 4). This partial activity of the PMPS-treated ClpX could be due to replacement of the zinc ligand with PMPS, which forms a tight complex with cysteine or/and the formation of S-S bridges between the free Cys residues, which in turn could stabilize the ClpX Zn(II) apo-protein structure, and thus partially compensate for the loss of Zn(II). Consistent with this interpretation, for the ClpXΔCys mutant protein the apo proteolysis reaction is completely blocked (Fig. 4). Since the four cysteines are substituted with serines, the stabilizing effect of PMPS or potential disulfide bond formation should not occur. In a control experiment we showed that the ClpX proteolytic activity is partially inhibited by the presence of increasing concentrations of different divalent cations. It turns out that neither Mg(II) nor Co(II) ions exert an inhibitory effect on ClpXP proteolytic activity, whereas either Ca(II) or Zn(II) ions present in millimolar concentrations partially inhibit ClpXP proteolytic activity (results not shown). Partial inhibition of ClpXP proteolytic activity in the presence of a high concentration of Zn(II) (1 mol of ClpX to 100 or 1000 mol of Zn(II)) is probably due to the partial ClpX precipitation under these conditions (results not shown).

Quantitative analysis of the specific activity of the ClpX protein in the AO proteolytic assay shows that during the various steps of ClpX purification there is a partial loss of ClpX activity (result not shown). This result could be due to a partial loss of the Zn(II) cation. To bypass this potential problem, we elaborated a new method for ClpX purification which ended up with fully active ClpX protein complexed with Zn(II) (see “Materials and Methods” for details). The identity of ClpX was verified by N-terminal sequencing analysis and Western blot analysis (results not shown). Using this new purification procedure, we were able to obtain ~35 mg of 98% pure ClpX Zn(II) protein starting from 30 g of ClpX overproducing bacteria.

The Presence of Zn(II) Affects the Interaction of ClpX with ClpP—Fig. 5A shows that, as judged by the ELISA technique,
ClpX Zn(II) strongly interacts with the ClpP proteolytic subunit only in the presence of ATP. When the ClpX protein was unfolded by 8 M urea and then folded back in the absence of Zn(II), it possessed a substantially reduced affinity for the ClpP protein (Fig. 5B). The subsequent addition of Zn(II) to such a ClpX preparation restored the proteins ability to form a stable complex with ClpP. Moreover, only in the presence of Zn(II) this reaction clearly was ATP-dependent (Fig. 5B).

These ELISA results have been complemented by size exclusion chromatographic studies. The apparent molecular mass of the ClpP/ClpX Zn(II) heterocomplex is estimated to be 700–800 kDa, assuming an overall spherical shape for its structure (Fig. 6 and results not shown). Its Stokes radius is slightly lower than that of the GraEL protein, whose molecular mass is ~800 kDa (results not shown). A similar high molecular weight structure was previously described for the ClpP/ClpA complex (14, 32) and recently for the ClpP/ClpX complex (17). As in the case of the ClpAP protease, the formation of the oligomeric ClpP/ClpX Zn(II) structure requires the continuous presence of ATP, i.e. ATP must be present in both the premixture and mobile phase buffers (Fig. 6 and results not shown).

Zn(II) and ATP Affect the Proper Oligomerization of ClpX—

The data presented in Fig. 6 also show that ClpX Zn(II) alone, in the absence of ATP, or in the presence of low concentrations of ATP (0.2 mM), sufficient for binding of ClpX to ClpP), already behaves as an oligomer. Judging from the shape of its HPLC profile, a mixture of various ClpX Zn(II) oligomeric forms probably exists. The HPLC profiles of ClpX Zn(II) were independent of the presence or absence of exogenous Zn(II) or substitution of ATP by ADP in the running buffer (results not shown). It turns out that in the absence of ATP or ADP the Stokes radius of ClpX Zn(II) (Fig. 6A) is very close to that of the ClpP 14-mer (Fig. 6B), suggesting that ClpXs apparent molecular mass is close to 300 kDa. Therefore, at least a portion of the ClpX Zn(II) structure of ClpX Zn(II) apoprotein and ClpP with and without ATP, respectively. Filled circles and open circles show the complex protein in the presence of 5 μM ZnCl2 and in the absence and presence of ATP, respectively.

When the ATP concentration was increased to 6 mM, which is optimal for the ClpXP proteolytic activity (24), an additional oligomerization or stabilization of the specific quaternary structure of ClpX Zn(II) took place (Fig. 6A, and results not shown). The elution profile of ClpX no longer overlapped that of ClpP, suggesting that ClpX is found in a form higher than an hexamer under these conditions. In control experiments we showed that substitution of ATP by ADP in these experiments also leads to the additional oligomerization of ClpX (results not shown). The Stokes radius of ClpP did not change at higher concentrations of ATP or ADP (Fig. 6B, and results not shown). We found that the minimal ATP concentration required for the additional oligomerization/stabilization of ClpX quaternary structure was ~2–3 mM (results not shown). An analogous ATP-promoted oligomerization has been previously reported.

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for the ClpA and Hsp104 family members. In the absence of nucleotide, ClpA or Hsp104 migrate as monomers, dimers, or trimers depending on the particular technique used (11, 12, 20).

Interestingly, increasing the ATP concentration from 0.2 to 6 mM did not change the Stokes radius of the ClpP Cys mutant or ClpX wild type free of Zn(II), ClpX Cys or ClpX Zn(II). As shown in Table II, the ClpX Cys mutant does not possess any detectable ATPase activity in the absence of zinc, the ClpX protein is not able to bind ATP, and as a consequence the ClpX chaperone, and that the oligomeric form is required for binding of ClpX Zn(II) to the ClpP protease.

**Zn(II) Is Required for Binding of ATP to ClpX**—Previously we have shown that ClpX possesses an ATPase activity, stimulatable by its various protein substrates (4). As shown in Table II, the ClpXΔCys mutant does not possess any detectable ATPase activity. Also, when Zn(II) is released from ClpX by PMPs treatment, ATP hydrolysis is strongly inhibited (Table II). This effect is most likely caused by the lack of binding of ATP to either ClpXΔCys or ClpX Zn(–). As shown in Table II, the ClpXΔCys mutant or ClpX wild type free of Zn(II), ClpX Zn(–) do not bind ATP. We conclude that in the absence of zinc, the ClpX protein is not able to bind ATP, and as a consequence not able to oligomerize or bind to ClpP.

**DISCUSSION**

Previous studies have established that zinc fingers, and other metal-binding protein domains, are involved in protein/DNA interactions, protein folding, as well as protein-protein interactions (for a review, see Ref. 34). We had earlier shown that another molecular chaperone, DnaJ, is also a metalloprotein. In that case, the binding of two Zn(II) metal ions per DnaJ monomer stabilized the Zn(II) proteins.

| Protein          | ATP bound to ClpX | ATP hydrolysis by ClpX |
|------------------|-------------------|------------------------|
| ClpX Zn(II)      | 100%              | 100%                   |
| ClpX Zn(–)       | 14%               | 25%                    |
| ClpX ΔCys        | <5%               | <5%                    |

**FIG. 7.** Size exclusion chromatography of ClpXΔCys and ClpX Zn(II) proteins. The ClpXΔCys (lane a) or ClpX Zn(II) (lane b) proteins (40 µg each) were preincubated for 30 min at 25 °C in the presence of 0.2 mM ATP and injected onto a Superdex 200 column equilibrated with buffer B supplemented with 0.2 mM ATP (see “Materials and Methods” for details). Chromatography was performed as described under “Materials and Methods.”

**TABLE II**

Binding and hydrolysis of ATP by ClpX Zn(II), ClpXΔCys, and ClpX Cys.
In the case of ClpX, the putative Zn binding motif probably belongs to the C\(_4\) family and contains the following sequence: C\(_X\)C\(_X\)X\(_2\)C\(_X\)C. However, the data presented in this paper only demonstrate that at least one of the four cysteine residues is required for Zn(II) binding. More detailed analysis of the ClpX zinc-binding motif is necessary to really prove that ClpX contains a C\(_4\) binding motif.

In this work we have shown that dissociation of Zn(II) from the ClpX complex affects all known activities of the ClpX protein, i.e. binding of ATP, protein oligomerization, binding to ClpP, and ClpXP-dependent proteolysis. Although it is difficult to definitely conclude which effect is the primary one, it is very likely that the inability of ClpX lacking Zn(II) to bind ATP, is responsible for eliminating all of its known activities.

The Zn(II)-dependent binding of nucleotides to proteins was previously shown to occur in the case of the E. coli MukB and SlyD proteins. The homodimeric MukB, which is required for the correct partitioning of the bacterial chromosome into daughter cells, binds GTP or ATP in the presence of Zn(II) but not of Mg(II) (36). In the case of SlyD, the peptidyl prolyl cis-trans isomerase, the binding of nucleotide was also Zn(II)-dependent (37). It is possible that the positively charged Zn(II) is required for the stabilization of the complex between protein and the negatively charged phosphate groups of ATP.

We have shown that ClpX Zn(II) is present in an oligomeric state even in the absence of ATP. Such an oligomeric structure in the absence of ATP was also observed for ClpY(HslU), a daughter cells, binds GTP or ATP in the presence of Zn(II) but not of Mg(II) (36). In the case of SlyD, the peptidyl prolyl cis-trans isomerase, the binding of nucleotide was also Zn(II)-dependent (37). It is possible that the positively charged Zn(II) is required for the stabilization of the complex between protein and the negatively charged phosphate groups of ATP.

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We have shown that ClpX Zn(II) is present in an oligomeric state even in the absence of ATP. Such an oligomeric structure in the absence of ATP was also observed for ClpY(HslU), a protein highly homologous to ClpX (15). Interestingly, in the case of ClpX, the tendency for oligomerization decreases in the absence of Zn(II). In the case of the mutant ClpXΔCys, in which binding of Zn(II) is completely blocked, the protein behaves exclusively as a monomer. The involvement of Zn(II) in ClpX oligomerization could be due to direct protein-protein interaction, or to Zn(II)-dependent exposure of hydrophobic patches on the ClpX protein surface. It has been shown before for the chaperone GroEL that the presence of Zn(II) increases the amount of hydrophobic surface exposed (38). It has also been postulated that zinc-binding finger motifs are directly involved in protein-protein interactions (39).

The Zn(II)-dependent oligomerization of ClpX in the absence of exogenous ATP is not due to the presence of tight ClpX-ATP or ATP complexes in the purified preparations of ClpX protein. Preliminary experiments show that the oligomerization of ClpX is reduced in the case of ClpX point mutants located in the ATP-binding site. In this case, the ClpX mutant protein does not bind ATP but still interacts with Zn(II). The results presented in this paper suggest that two factors influence the oligomerization of ClpX, namely Zn(II) and ATP. It is possible that in the absence of ATP, the ClpX Zn(II) protein possesses a tendency to form different oligomeric structures and that binding of ATP stabilizes one of these structures, very likely the hexameric ring.

Here we have also shown that at physiological concentrations of ATP (2–6 mM), at which the single ClpX ATP-binding site should be saturated, even further protein oligomerization can take place. Such phase transition at high ATP concentrations suggests that ClpX can adopt an unique quaternary structure in the absence of ClpP. One possibility, suggested by EM studies (17), is that under such conditions a double hexameric ring is formed. Interestingly, such a ClpX quaternary structure is formed also in the presence of ADP, suggesting that nucleotide binding but not hydrolysis is required for this event. Moreover, this finding suggests that following ATP hydrolysis this putative ClpX structure can persist.

Finally, we have shown that low concentrations of ATP (0.2 mM), under which ClpX alone is not present in the putative double hexameric ring state, are, nevertheless, sufficient for ClpX-ClpP complex formation. Surprisingly, the Stokes radius of the ClpP-ClpX complex formed at 0.2 mM ATP is similar to that formed at 6 mM ATP, suggesting that both complexes contain the same number of ClpX subunits. Estimation of the molecular mass of the ClpP-ClpX heterocomplex (700–800 kDa) leads to the conclusion, assuming an overall spherical shape for this structure, that ClpX is present in a double hexameric ring state in this complex. This suggests that at 0.2 mM ATP the presence of ClpP helps the ClpX protein to oligomerize further or that two hexameric ClpX structures can bind simultaneously to opposite sides of the ClpP barrel-like structure (17).

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\(^3\) J. Puzewicz, B. Banecki, and M. Zylcic, unpublished results.
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