Enzymatic and Structural Similarities between the *Escherichia coli* ATP-dependent Proteases, ClpXP and ClpAP*

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*Escherichia coli* ClpX, a member of the Clp family of ATPases, has ATP-dependent chaperone activity and is required for specific ATP-dependent proteolytic activities expressed by ClpP. Gel filtration and electron microscopy showed that ClpX subunits (M₉, 46,000) associate to form a six-membered ring (M₉ ~ 280,000) that is stabilized by binding of ATP or nonhydrolyzable analogs of ATP. ClpP, which is composed of two seven-membered rings stacked face-to-face, interacts with the nucleotide-stabilized hexamer of ClpX to form a complex that could be isolated by gel filtration. Electron micrographs of negatively stained ClpXP preparations showed side views of 1:1 and 2:1 ClpXP complexes in which ClpP was flanked on either one or both sides by a ring of ClpX. Thus, as was seen for ClpAP, a symmetry mismatch exists in the bonding interactions between the seven-membered rings of ClpP and the six-membered rings of ClpX. Competition studies showed that ClpA may have a slightly higher affinity (~2-fold) for binding to ClpP. Mixed complexes of ClpA, ClpX, and ClpP with the two ATPases bound simultaneously to opposite faces of a single ClpP molecule were seen by electron microscopy. In the presence of ATP or nonhydrolyzable analogs of ATP, ClpXP had nearly the same activity as ClpAP against oligopeptide substrates (>10,000 min⁻¹/tetradecamer of ClpP). Thus, ClpX and ClpA interactions with ClpP result in structurally analogous complexes and induce similar conformational changes that affect the accessibility and the catalytic efficiency of ClpP active sites.

The Clp family of ATP-dependent chaperone-linked proteases are high molecular weight complexes composed of a protease with limited peptidase and virtually no intrinsic proteolytic activity and an ATPase that activates proteolysis by binding and unfolding protein substrates (1, 2). Clp proteases were first described in *Escherichia coli*, where ClpAP and ClpXP were shown to consist of a common proteolytic core, ClpP, which can be activated by either of two ATPases, ClpA or ClpX (3–6). Recently another branch of the Clp family consisting of a unique proteolytic component, ClpQ (or HslV), and the ATPase ClpY (or HslU) was described (7–9). Despite high degrees of amino acid sequence homology, Clp ATPases appear to fall into two groups, the ClpA/ClpX-like proteins that have intrinsic chaperone activity and also act as part of proteolytic complexes and the ClpB-like proteins that appear to function solely as molecular chaperones independent of proteolytic components (10, 11). Clp ATPases are widespread in eukaryotes and prokaryotes indicating that, at the least, the protein-remodeling activity of Clp ATPases is highly conserved (1, 10).

The two Clp proteolytic components described in *E. coli*, ClpP and ClpQ, are not related to each other, differing in their amino acid sequences and in their catalytic mechanisms of peptide bond cleavage (12–14). ClpP is representative of a family of serine proteases that is unique both in sequence and in the folding domains seen in the recently solved x-ray crystal structure (15). The ClpP subfamily is highly conserved in prokaryotes and is found in plant chloroplasts as well as in mammalian cell mitochondria (16). ClpQ is a member of the proteasome family (13, 17). Proteasomes are multimeric proteases that not only form the proteolytic core of the major ATP-dependent protease in the eukaryotic cytosol but that are also found in eubacteria and in Archaea (18). ClpQ has an amino-terminal catalytically active threonine residue and a tertiary structure similar to that of the proteasomal β-subunits (17). Surprisingly, ClpQ subunits assemble into rings with only six subunits (8, 19), unlike the proteasome, which has seven subunits per ring. For both ClpP and ClpQ, the active sites are buried within an aqueous cavity formed by the joining of the two rings, and access to the active sites is limited to a narrow axial channel through the center of each ring. It has been proposed that binding of the chaperone component and cycles of ATP hydrolysis may alter the size and properties of the channel and increase substrate access (20, 21).

The Clp ATPases not only carry out the energy-dependent steps in protein remodeling and degradation, but they also determine the selection of protein substrates for both activities. Proteins that bind to and are remodeled by ClpX are also degraded by the corresponding holoenzyme complexes with ClpP (5, 6). The same can be said for substrate selection in all three activities carried out by ClpA or ClpAP (22, 23). Thus, it is likely that protein binding and unfolding by Clp ATPases is an integral part of their ability to promote specific protein degradation by ClpP. The structure of the ClpAP complex as revealed by electron microscopy is consistent with protein binding and enzymatic properties of the enzyme. ClpA binds on the planar surface of each ring of ClpP, controlling access to the openings of the axial channels (24). Presumably, substrates must interact with ClpA and pass through or around the rings formed by the two domains of ClpA to gain access to the proteolytic active sites. We have proposed a model in which ATP-dependent protein unfolding is coupled to translocation of segments of the substrate to the interior of ClpP (11, 25). Because of the unequal number of subunits in the respective rings, the subunits in ClpA will not all be in the same register with those in ClpP. Progressive movement of different pairs of

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subunits into alignment during successive rounds of ATP hydrolysis may aid in translocation of protein substrates through the narrow channels.

**EXPERIMENTAL PROCEDURES**

**Materials—**Unless noted, chemicals were purchased from Sigma. Nucleotides were obtained from Sigma and Boehringer Mannheim. Clp proteins were prepared by R. Grimaud and M. R. Maurizi. AO protein was a gift from Sue Wickner, Laboratory of Molecular Biology, NCI, NIH, Bethesda, MD. Polyclonal rabbit antibodies against ClpX were described previously (1).

ClpX Purification—The clpX coding region was amplified by polymerase chain reaction and inserted in plasmid pET11a (Novagen) so that expression was under control of the T7 promoter. Details of the cDNA sequences and expression in E. coli BL21(DE3) cells containing the plasmid pET11a and pET11a/cpX were given at room temperature (25°C) for 17 h in LB medium containing 100 μg/ml ampicillin, 30 μg/ml chloramphenicol, and 0.25 mM isopropyl-1-thio-β-D-galactopyranoside. Cells (6.5 g) were suspended in 26 ml of buffer D (50 mM Tris, pH 7.5, 10 mM MgCl₂, 5 mM dithiothreitol and 10% (v/v) glycerol) and broken by a single pass through an ice-chilled French pressure cell (Amino) at 20,000 psi. The crude extract was centrifuged at 30,000 × g for 30 min at 4°C. Polyethyleneimine was added to the supernatant to a concentration of 0.1%. Precipitated material was removed by centrifugation at 30,000 × g for 30 min at 4°C. The supernatant solution was brought to 0.1 M KCl and loaded on a 2.5 mL MonoQ (Amersham Pharmacia Biotech) column equilibrated with buffer D plus 0.1 M KCl. Proteins were eluted by a 100-ml gradient from 0.1 to 0.4 M KCl, and the ClpX was detected by SDS-polyacrylamide gel electrophoresis and Western blotting. The ClpX-containing fractions, which eluted at about 0.3 M KCl, were pooled, and ClpX was precipitated with 55% saturation of ammonium sulfate. The precipitated protein was collected by centrifugation at 30,000 × g for 30 min at 4°C and dissolved in buffer D plus 0.1 M KCl. The protein solution was clarified by centrifugation at 30,000 × g for 10 min at 4°C and loaded onto a size exclusion TSK 250 column (Bio-Rad) equilibrated with buffer D plus 0.1 M KCl. The ClpX was further purified by chromatography on a 1 × 10 cm MonoQ (Amersham) column using a 40-ml gradient from 0.1 to 0.4 M KCl gradient in Buffer D. ClpX fractions were stored separately at 0–4°C.

**Enzymatic Assays—**ATPase activity was assayed by liberation of inorganic phosphate from ATP after incubating ClpX in 50 μl of 50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, and 1 mM ATP for 25 min at 37°C. The inorganic phosphate was measured by the procedure described by Lanzetta et al. (26), except that the Sterox was omitted from the molybdate/ammonium molybdate solution, and 16 μl of 10% Tween 20 was added to the solution after the addition of the color reagent. Assay conditions and methods for casein and the propeptide, FAPMALVPV, degradation were published previously (27). Assays were performed with limiting ClpP (0.2–0.5 μg) and 2–5 μg of ClpX. For degradation of AO protein and other proteins, the substrates (5–10 μg) were incubated at 37°C with 0.5 μg of ClpP and 4 μg of ClpX in 50 μl Tris/HCl, pH 8, with 10 mM MgCl₂ and 4 mM ATP for 30–60 min. Reactions were quenched with 5% trichloroacetic acid, and the precipitated protein was dissolved in SDS-buffering and heated at 95°C for 5 min before loading on 12% acrylamide, SDS gels. The protein remaining was estimated by estimation after staining with Coomassie Blue.

Isolation of ClpX Oligomers and the ClpXP Complex—ClpX and various complexes of ClpX were separated on a 0.3 × 20 cm Superdex 200 column in either 50 mM Tris/HCl or 50 mM Hepes/KOH, pH 7.5, with 0.1% (v/v) KCl and 2 mM β-mercaptoethanole with or without 10% (v/v) glycerol. Columns were run at room temperature and at a flow rate of 0.1 ml/min. For stabilization of high molecular weight oligomers, samples of ClpX alone or ClpX and ClpP were mixed with 25 mM MgCl₂ and 1–2 mM ATP at 3°C before running the column, and these ligands were included in the running buffer.

**Electron Microscopy—**A 4-μl drop of the sample at a concentration of 20 μg/ml was placed on a glow-discharged, carbon-stabilized, collagen-coated grid for 30 s. The sample droplet was blotted away, and the grid was negatively stained with several droplets of 1% aqueous uranyl acetate. Specimens were viewed in a Zeiss 902 or a Phillips CM120 transmission electron microscope, and micrographs were recorded on Kodak SO-163 emulsion at a nominal magnification of 50,000. Magnification of

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1. R. Grimaud and M. R. Maurizi, unpublished observations.
2. The abbreviation used is: ATPβS, adenosine 5’-O-(thiotriphosphate).

**FIG. 1. Purification of ClpX on MonoQ.** The ClpX-containing fractions from the TSK gel filtration column were pooled and run over a 10 × 10-cm MonoQ column (see “Experimental Procedures”). The proteins eluted with a gradient of KCl were monitored by absorbance at 280 nm (solid line), and the 4-μl aliquots of the fractions were assayed for ATPase activity (triangles). Proteins in the fractions were observed by staining with Coomassie Blue after SDS/polyacrylamide gel electrophoresis; 5 μl of each fraction was applied to the different lanes, which are shown above the appropriate position in the absorbance profile.

**RESULTS**

ClpX Purification—When ClpX was expressed under the strong T7 promoter, only 10–20% was found in the soluble portion of cell extracts, and the remainder was found in the low speed pellet. The soluble material was used for purification by the procedure outlined under “Experimental Procedures.” After the last purification step, anion exchange chromatography on MonoQ, several fractions contained ClpX that was ≈95% pure, estimated by inspection of Coomassie-stained SDS-polyacrylamide electrophoresis gels (Fig. 1). These fractions were stored separately and used for the studies presented here.

ATPase Activity—During purification the activity of ClpX was monitored by measuring the ATPase activity. The ATPase activity eluted from the MonoQ column coincided with the major protein band (Fig. 1), which also cross-reacted with anti-ClpX antibodies (data not shown). The ATPase activity of purified ClpX was −0.3 pmol/min/mg (turnover number 15 min⁻¹), which is consistent with what was previously reported by Wawrzynow et al. (23).

**Protein and Peptide Degradation in the Presence of ClpX**—In the presence of MgATP and ClpP, the ClpP in our preparation promoted the degradation of purified λO protein (data not shown), which has been shown to be a specific substrate for ClpX and ClpP in vivo and in vitro (5, 6) but, as has been reported (6), had virtually no activity against α-1H]casein (see Fig. 4, below).

Studies with ClpA and ClpP had shown that ClpAP could degrade a specific oligopeptide, FAPMALVPV, at a very high rate (turnover number >10,000/dimer) in the presence of non-hydrolyzable analogs of ATP (21). Degradation of this peptide required formation of the nucleotide-bound complex of ClpA and ClpP. ClpX also activated degradation of FAPMALVPV by ClpP (Table I). Under identical conditions with limiting ClpP, ClpX and ClpA activated cleavage of FAPMALVPV at the same rate. Thus, ClpX induces a conformational change in ClpP comparable to that induced by ClpA, making the active sites more accessible to oligopeptides and
increasing the catalytic efficiency of the proteolytic active sites. It is also interesting to note that both ClpXP and ClpAP cleaved FAPHMALVPV between the Met and the Ala by ClpAP and ClpXP. The peptide (200 μg/ml) was incubated for 30 min at 37 °C with 0.2 mg of ClpP and 2 μg of ClpA or ClpX in the presence of ATPγS. The substrate remaining and the peptide products formed were isolated and quantitated by reverse phase chromatography (21).

| Addition | Activity (min⁻¹) |
|----------|------------------|
| ClpX     | <10              |
| ClpP     | <10              |
| ClpXP    | >10,000          |
| ClpAP    | >10,000          |

*Activity is expressed as peptide bonds cleaved/min/tetradecamer of ClpP.

The ClpXP Complex—When a mixture of ClpP and ClpX was run over Superdex 200 in the presence of ATPγS, both proteins eluted together in a peak with an apparent molecular weight >600,000 (Fig. 2, solid line). In the experiment shown, the amount of ClpP protein in the complex was almost twice the amount of ClpP estimated from the stained bands in Fig. 2 as would be expected if two hexamers of ClpX combined with a tetradecamer of ClpP. In other experiments with ClpP added in excess of ClpX, the new peak had slightly lower molecular weight and an obviously lower ratio of ClpX to ClpP (data not shown). When ClpXP complexes were examined by electron microscopy after negative staining, several different projections were observed. The most informative of these were projections composed of three or four strong parallel striations (Fig. 3b). After selection, the respective projections were rotationally and translationally aligned and then averaged to result in the final reconstructed images (Fig. 3b, inset). The two shorter contiguous striations had the same lengths previously seen for ClpP in the ClpAP complex (24). The third striation, by analogy with previous images of ClpA and ClpAP, was interpreted as a side projection of a ClpX ring. The fourth striation, which flanked the central ClpP molecule, appeared to be a side projection of ClpX that had undergone some flattening or other disruption of structure.

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is reported here for each experiment. 

The rotational symmetry was assessed for each individual particle using the program ROTA STAT (36). A spectral ratio product was calculated for each radii, and the highest statistically significant score using the program ROTA STAT (36). A spectral ratio product was added simultaneously to assay solutions containing all reaction components except ClpP. The reaction was initiated by the addition of ClpP, and the casein degraded was measured after 15 min.

![Electron micrographs and image averaging of ClpX and ClpXP complexes.](image)

**Fig. 3.** Electron micrographs and image averaging of ClpX and ClpXP complexes. *a*, a field of negatively stained ClpX that had been run over a gel filtration column in the presence of ATPγS. *Left inset*, an average top view of ClpX obtained with 79 particles; *right inset*, 6-fold-symmetrized average image of ClpX. *b*, a field of negatively stained particles in the high molecular weight fractions from a gel filtration column run with a mixture of ClpX and ClpP in the presence of MgATPγS. *Insets*, averaged side views of 1:1 (three striations) and 2:1 (four striations) complexes of ClpX and ClpP. The bar equals 10 nm.

**Table II**

Rotational analysis of ClpX particles showing 6-fold symmetry

The rotational symmetry was assessed for each individual particle using the program ROTA STAT (36). A spectral ratio product was calculated for each radii, and the highest statistically significant score is reported here for each experiment.

| Data set | Total number of particles analyzed | Spectral ratio product | Number of particles selected by OMO |
|----------|-----------------------------------|------------------------|-------------------------------------|
| 1        | 200                               | $8.6 \times 10^{10}$   | 178                                 |
| 2        | 100                               | $4.7 \times 10^{9}$    | 76                                  |
| 3        | 100                               | $3.1 \times 10^{9}$    | 84                                  |
| 4        | 100                               | $6.2 \times 10^{8}$    | 77                                  |
| 5        | 100                               | $2.4 \times 10^{8}$    | 73                                  |
| 6        | 100                               | $5.7 \times 10^{8}$    | 79                                  |

* Scans of independent micrographs.

ATP-dependent proteases from a variety of sources and from different evolutionary families have complex, multimeric structures. Modular assemblies (as in ClpAP and ClpXP) or the complex of the proteasome with either the ATP-dependent 19 S or ATP-independent 11 S activators) and alternative subunit compositions (as in the isoforms of eukaryotic proteasomes) are now among the well established features of these proteases (14, 18, 31). The variety of ways in which the different components can be combined allows the specificity, the activity, and the response to regulatory signals to be fine-tuned. One goal of our studies is to distinguish the structural features that underlie the activities common to all the proteases and those that reflect unique functions of particular proteases.

We had shown previously that ClpA formed a complex with ClpP in which a hexameric ring of ClpA was bound to each of the two heptameric rings of ClpP (24). One surprising aspect of the ClpAP complex was the symmetry mismatch between the
six-membered and seven-membered rings of the two components. Although intriguing speculations regarding a function of the symmetry mismatch in allowing ratcheting or rotation of the ClpA and ClpP rings about each other during catalysis were attractive, it remained possible that this structural feature was peculiar to ClpAP. Such reservations were of heightened concern because much more was known from genetic and biochemical studies about the physiological activities of a putative complex between ClpA and ClpP, two proteins that are encoded in an operon and subject to co-regulation in vivo.

A unique finding of this study is the formation of a mixed complex between ClpA, ClpX, and ClpP. Because so few side views were available, we were prevented from obtaining accurate quantitation of the relative numbers of the three complexes, ClpAP, ClpXP, and ClpAPX. Experiments are under way to determine the frequency with which the mixed complexes occur. It would appear, however, that there is no bias against such complexes, i.e., no negative cooperativity between ClpA and ClpX, and thus we think it highly probable that mixed complexes exist in vivo as well. Activity measurements with ClpAP had suggested that 1:1 and 2:1 complexes of ClpAP had nearly the same specific activity for casein degradation, indicating that ClpA might not translocate substrates from both sides of ClpP simultaneously (32). Whether ClpA and ClpX can activate degradation of different substrates simultaneously cannot be determined from our data. It is interesting, however, that the inhibition of ClpAP activity by ClpX appeared to be cooperative, suggesting that displacing a single ClpA failed to inhibit casein degrading activity and implying that ClpAPX has casein degrading activity comparable to that of the 1:1 and 2:1 ClpAP complexes.

The 6-fold symmetry of ClpX has been observed in all micrographs of native ClpX that we have studied. In contrast, the homologous ATPase, ClpY (HslU), formed rings with 6- or 7-fold symmetry (8, 19). Sequence homology apparently does not dictate that proteins will form rings with the same numbers of subunits. Another example of such structural deviation is the difference in symmetry between E. coli ClpQ (HslV) (6-fold) and the homologous β-subunits of proteasomes from Archaea and eukaryotic cells (7-fold) (14, 18). The circular alignment of active sites or binding sites produced by the ring-like structure appears to be the critical structural element rather than the exact number of such sites. In the case of ClpYQ, it is interesting to note that, if the predominant form of ClpY has seven subunits per ring (8, 19), there would be an inside out (with respect to ClpAP or ClpXP) symmetry mismatch between the ATPase and the proteolytic component (ClpQ).

It is not yet possible to generalize about the occurrence of symmetry mismatches in other ATP-dependent proteases. Symmetry mismatch cannot be essential, because homooligomeric proteases such as Lon and FtsH, which have the ATPase and proteolytic sites in the same polypeptide, are perforce symmetrical. The structure of the ATPases in the 26 S proteasome has not been defined, but in yeast there appears to be only six such ATPases (33), which suggests at least a nonstochiometric interaction between ATPase and proteasome subunits. A symmetry mismatch between the 11 S activator (PA28) and the 20 S proteosome may exist, although there is controversy regarding the number of subunits in the rings of PA28 (six or seven) (34, 35). With the uncertainty regarding the numbers of subunits in ClpY and in the 11 S, it is reasonable to consider the possibility that some of these proteins may exist in both forms and that the degradative activities of the resulting complexes may vary. We would like to note that preliminary studies of ClpX structure using a His-tagged ClpX protein (kindly supplied by T. Baker, MIT) had shown rings with 7-fold symmetry. Structural or chemical perturbations may have significant effects on the assembly of Clp ATPases.

Fig. 5. Electron micrographs and average images of a ClpAPX complex. ClpA (10 µg) and ClpX (6 µg) were added to buffer containing 25 mM MgCl₂ and 2 mM ATPγS. After 5 min at room temperature, ClpP (3 µg) was added, and after an additional 10 min, samples were diluted in the buffer with MgATPγS and prepared for electron microscopy (see “Experimental Procedures”). a, a field of negatively stained particles is shown. ClpAPX complexes are indicated with white arrows; a ClpAPA complex is indicated by the black arrowhead. b, About 1 in 30 particles (1 in 8 side views) had five striations. These were aligned and averaged to produce the average image shown. c and d, average images of side views of ClpXP (from Fig. 3) and ClpAP (from Ref. 24) are shown for comparison.

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