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Régis Grimaud, M. Kessel, F. Beuron, A.C. Steven, M.R. Maurizi. Enzymatic and structural similarities between the Escherichia coli ATP- dependent proteases, ClpXP and ClpAP. Journal of Biological Chemistry, 1998, 273 (20), pp.12476-12481. 10.1074/jbc.273.20.12476 . hal-01614793

HAL Id: hal-01614793

<https://hal.science/hal-01614793>

Submitted on 11 Jan 2018

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

(Received for publication, January 5, 1998)

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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 (M_r 46,000) associate to form a six-membered ring (M_r ~ 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. λ O protein was a gift from Sue Wickner, Laboratory of Molecular Biology, NCI, NIH, Bethesda, MD. Polyclonal rabbit antibodies against ClpX were described previously (5).

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 construct will be published elsewhere.¹ BL21(DE3) cells containing the plasmids pLysS and pET11a/*clpX* were grown at room temperature (25 °C) for 17 h in Luria broth containing 100 μ g/ml ampicillin, 30 μ g/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 (Aminco) at 20,000 psi. The crude extract was centrifuged at 30,000 \times 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 \times g for 30 min at 4 °C. The supernatant solution was brought to 0.1 M KCl and loaded on a 2.5 \times 20-cm Q-Sepharose (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 \times 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 \times 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 \times 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 μ l 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 malachite green/ammonium molybdate solution, and 16 μ l 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, FAPHMALVPV, degradation were published previously (27). Assays were performed with limiting ClpP (0.2–0.5 μ g) and 2–5 μ g of ClpX. For degradation of λ O protein and other proteins, the substrates (5–10 μ g) were incubated at 37 °C with 0.5 μ g of ClpP and 4 μ g of ClpX in 50 mM 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-containing buffer and heated at 95 °C for 5 min before loading on 12% acrylamide, SDS gels. The protein remaining was estimated by inspection 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 \times 20-cm Superdex 200 column in either 50 mM Tris/HCl or 50 mM Hepes/KOH, pH 7.5, with 0.2 M KCl and 2 mM β -mercaptoethanol 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 γ S² before running the column, and these ligands were included in the running buffer.

Electron Microscopy—A 4- μ l drop of the sample at a concentration of 20 μ g/ml was placed on a glow-discharged, carbon-stabilized, collodion-coated grid for 30 s. The sample droplet was blotted away, and the grid was negatively stained with several drops 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

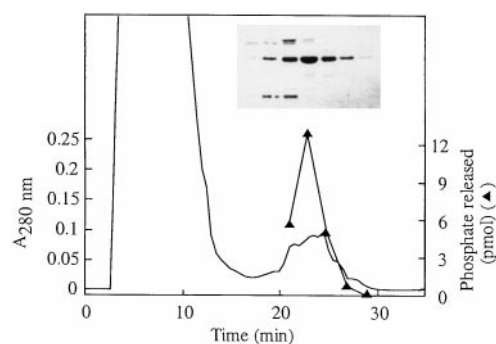


FIG. 1. Purification of ClpX on MonoQ. The ClpX-containing fractions from the TSK gel filtration column were pooled and run over a 10 \times 10-cm MonoQ column (see “Experimental Procedures”). The proteins eluted with a gradient of KCl was monitored by absorbance at 280 nm (solid line), and the 4- μ l 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 μ l of each fraction was applied to the different lanes, which are shown above the appropriate position in the absorbance profile.

the electron micrographs was calibrated using the 40.6-Å spacing of T4 bacteriophage tail striations (28).

Selected images were digitized using a Zeiss-Phodis scanner (Carl Zeiss Inc., Colorado). Sampling corresponded to 0.42 nm/pixel. General image processing and display operations were carried out using the PIC Program (29) implemented on an Alpha Workstation (Digital Equipment Corp., Maynard MA). Particles were interactively selected from digitized fields displayed on the workstation monitor, translationally aligned, analyzed for rotational symmetry, and subjected to correlation averaging as described (30).

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 \geq 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 \sim 0.3 μ mol/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 ClpX in our preparation promoted the degradation of purified λ O 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 -[³H]casein (see Fig. 4, below).

Studies with ClpA and ClpP had shown that ClpAP could degrade a specific oligopeptide, FAPHMALVPV, at a very high rate (turnover number $>$ 10,000/oligomer) in the presence of nonhydrolyzable analogs of ATP (21). Degradation of this peptide required formation of the nucleotide-promoted complex of ClpA and ClpP. ClpX also activated degradation of FAPHMALVPV by ClpP (Table I). Under identical conditions with limiting ClpP, ClpX and ClpA activated cleavage of FAPHMALVPV 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

¹ R. Grimaud and M. R. Maurizi, unpublished observations.

² The abbreviation used is: ATP γ S, adenosine 5'-O-(thiotriphosphate).

TABLE I
Oligopeptide degradation by ClpXP

FAPHMALVPV is cleaved once between the Met and the Ala by ClpAP and ClpXP. The peptide (200 $\mu\text{g}/\text{ml}$) 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 ATP γ S. The substrate remaining and the peptide products formed were isolated and quantitated by reverse phase chromatography (21).

Addition	Activity ^a
	min^{-1}
ClpX	<10
ClpP	<10
ClpXP	>10,000
ClpAP	>10,000

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

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, indicating that they do not influence the peptide bonds cleaved by ClpP, even though the Clp ATPases determine the proteins selected for degradation.

Oligomerization State of ClpX—In the absence of nucleotides, ClpX tended to run as a broad peak on gel filtration columns. The fastest migrating fraction had an apparent M_r of $\sim 200,000$, and smaller species were also evident (data not shown). Electron microscopy of this protein showed mostly heterogeneously sized particles with no obvious regularity (data not shown). The addition of ATP γ S to ClpX produced a more uniform, higher molecular weight peak that eluted from a Superdex 200 column with an apparent M_r of $\sim 300 \pm 20 \times 10^3$ (Fig. 2, dotted line).

Electron micrographs of negatively stained ClpX after treatment with ATP γ S showed what appeared to be ring-shaped particles in almost exclusively top or *en face* view (Fig. 3a). It was difficult to assess the symmetry of the molecules from direct observation of the individual images. A quantitative assessment of the symmetry was made using an algorithm developed by Kocsis *et al.* (30) to detect rotational symmetries. In analyses of four independent data sets, 6-fold symmetry was very clearly detected at radii between 40 and 65 Å, and no significant scores were found for other (particularly 5- or 7-fold) symmetry (Table II). An average of 79 top views (Fig. 3a, inset) clearly shows the 6-fold symmetric nature of ClpX. Very similar average images were obtained with three independent preparations of ClpX (e.g. Table I). A close examination of the shape of the subunits shows a symmetrical mass distribution at the outer edge of the hexamer and well toward its middle. Taken together, the molecular weight determined by gel filtration and the appearance of the protein in electron micrographs indicate that ClpX is a hexameric ring and thus resembles ClpA in its oligomeric structure.

The ClpXP Complex—When a mixture of ClpP and ClpX was run over Superdex 200 in the presence of ATP γ S, 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 ClpX 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 re-

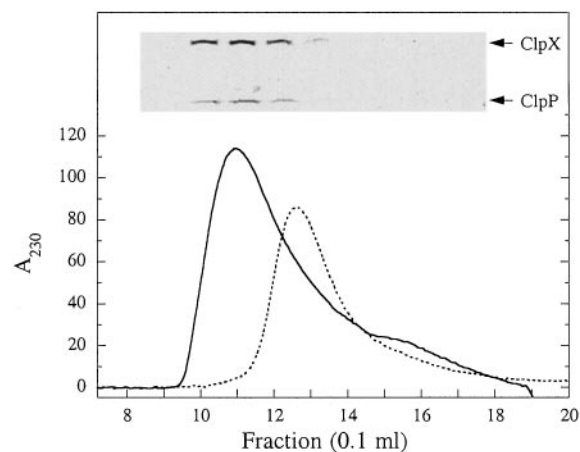


FIG. 2. **Isolation of ClpX and ClpXP by gel filtration.** ClpX ($\sim 70 \mu\text{g}$) (dotted line) or a mixture of ClpX ($\sim 70 \mu\text{g}$) and ClpP ($40 \mu\text{g}$) was run over a Superdex 200 column at 0.1 ml/min in the presence of MgATP γ S (see "Experimental Procedures"). The protein was monitored by absorbance at 230 nm. The presence of ClpX and ClpP in the high molecular weight fractions from the column in which they were run together was confirmed by SDS/polyacrylamide gel electrophoresis and staining with Coomassie Blue (shown above the absorbance profile). Standard proteins, their molecular weights, and their elution times were: glutamine synthetase (600,000) 11.3 min; glutamate dehydrogenase (300,000) 13 min; aldolase (150,000) 15 min.

sult 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.

The 1:1 and 2:1 complexes with the rings of ClpX axially aligned with the ClpP rings are completely analogous to the 1:1 and 2:1 complexes of ClpA and ClpP observed in our earlier study (24). Similar to previous observations with ClpAP, the ring of ClpP not in contact with the ATPase (in this case ClpX) appeared to be less constrained and maintained a concave (inward) shape. The single striation of ClpX is in agreement with the presence of a single ATP binding domain and is analogous to that observed for ClpY (HslU) interacting with ClpQ (HslV) (8, 19).

Many of the projections observed in micrographs of ClpXP complexes were not side views but rather had ring shapes. For several reasons, we believe that a majority of these rings are top views of complexes standing on end. First, gel filtration studies have shown that when mixed in the proper proportions, very little free ClpX or ClpP remain in solution. Second, the uranyl acetate tended to form pools around the particles creating highly contrasted images and suggesting that the particles had greater height than ClpP or ClpX alone. Third, symmetry analysis of the ring-like projections gave very poor symmetry scores and do not show the 6- or 7-fold symmetry expected for separate oligomers of ClpX or ClpP, respectively. Cryoelectron micrographs made with ClpXP also showed predominantly end-on views and very few side views.

ClpX and ClpA Have Similar Affinities for ClpP—The relative affinities of ClpX and ClpA for binding to ClpP were compared by a competition assay using casein degradation as a measure of ClpAP. The addition of ClpX in amounts sufficient to prevent ClpA binding to ClpP should inhibit casein degradation because ClpXP cannot degrade casein. Fig. 4 shows that the addition of increasing amounts of ClpX to assay mixtures with a fixed amount of ClpA resulted in a decrease in casein

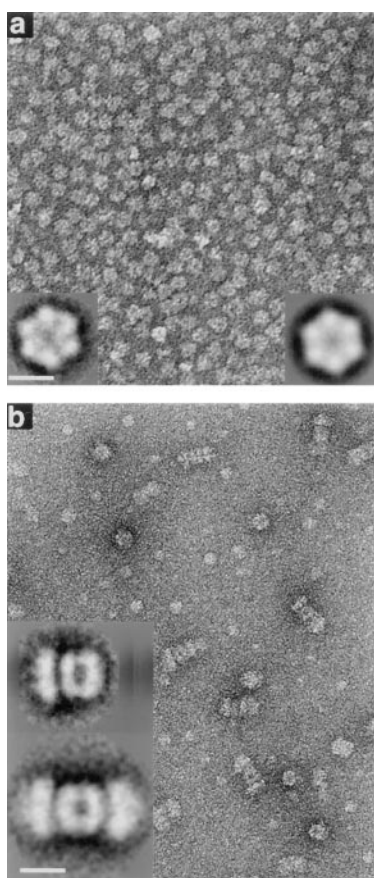


FIG. 3. Electron micrographs and image averaging of ClpX and ClpXP complexes. *a*, a field of negatively stained ClpX that had been run over a gel filtration column in the presence of ATP γ S. *Left inset*, an average top view of ClpX obtained with 79 particles; *right inset*, 6-fold-symmetrized average 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 MgATP γ S. *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 ^a	Total number of particles analyzed	Spectral ratio product ^b	Number of particles selected by OMO ^c
1	200	8.6×10^{10}	178
2	100	4.7×10^{24}	76
3	100	3.1×10^{21}	84
4	100	6.2×10^{25}	77
5	100	2.4×10^{23}	73
6	100	5.7×10^{34}	79

^a Scans of independent micrographs.

^b Product, over all the particles in the data set, of the ratio of the amplitude of a given component of a particle's rotational power spectrum and the average of this component for background areas of the same micrograph (30).

^c OMO scores images based on similarity of density distribution (37).

degradation by ClpP. A 3–5-fold excess of ClpX₆ over ClpA₆ was required to inhibit 50% of the activity measured in the absence of ClpX. The initial aliquots of ClpX produced no inhibition (or even a slight increase in casein degrading activity), suggesting that displacement of a single ClpA₆ by ClpX₆ was not sufficient to block casein degradation. In control experiments, when the ClpAP complex, which dissociates very slowly under assay conditions, was allowed to form before the addition of ClpX, a

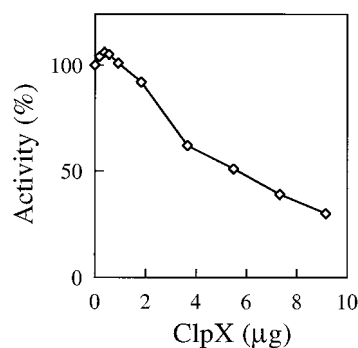


FIG. 4. Competition between ClpX and ClpA for binding to ClpP. Binding of ClpA to ClpP was assayed by ATP-dependent casein degradation in the presence of limiting ClpP (0.5 μ g). ClpA (4 μ g) and different amounts of ClpX (0–10 μ g) were added simultaneously to assay solutions containing all reaction components except ClpP. The reaction was initiated by the addition of ClpP, and the casein degraded was measured after 15 min.

substantial lag in the kinetics of inhibition was observed. Thus, inhibition by ClpX was dependent on its ability to bind to ClpP and not on competition with the substrate, casein, or excessive hydrolysis of the ATP (data not shown). Thus, hexameric ClpX appears to bind ClpP with an affinity similar to that shown by hexameric ClpA.

ClpX and ClpA Can Bind Simultaneously to ClpP—Efforts to observe the relative amounts of ClpX and ClpA bound to ClpP by electron microscopy were hampered by the tendency of complexes to stand on end on the grid. Nevertheless, these experiments did yield the novel observation of a mixed complex of ClpA and ClpX with ClpP. Fig. 5*a* shows a field of negatively stained particles in which projections having five striations are clearly distinguishable along with the expected ClpAP and ClpXP complexes. The particles with five striations were averaged to give the image shown in the Fig. 5*b*. The dimensions of the subdomains strongly suggest that the particles are composed of a double ring of ClpP flanked on one side by the doubly striated ClpA and on the other by the single-striated ClpX. The average side views for ClpXP and ClpAP are shown in Figs. 5, *c* and *d* for comparison. These data are the first to demonstrate that a mixed ClpAPX complex can be formed and indicate that binding of ClpX or ClpA to one end of a ClpP tetradecamer does not preclude the other ATPase from binding at the other end. Whether such hybrid complexes form *in vivo* (for example, under circumstances in which the supply of ClpP is limiting) and their possible physiological significance remain to be determined.

DISCUSSION

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

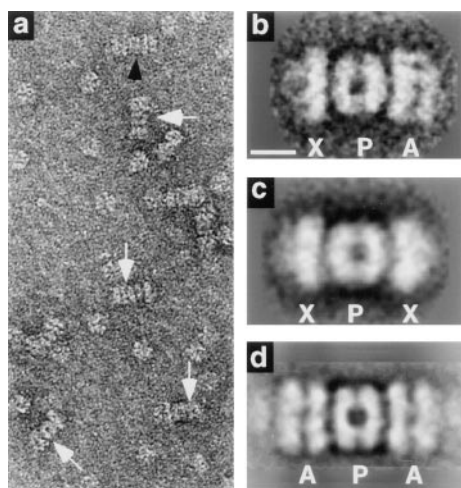


FIG. 5. Electron micrographs and average images of a ClpAPX complex. ClpA (10 μ g) and ClpX (6 μ g) were added to buffer containing 25 mM MgCl₂ and 2 mM ATP γ S. After 5 min at room temperature, ClpP (3 μ g) was added, and after an additional 10 min, samples were diluted in the buffer with MgATP γ S and prepared for electron microscopy (see "Experimental Procedures"). *a*, a field of negatively stained particles is shown. Possible 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.

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 ClpX and ClpP, two proteins that are encoded in an operon and subject to co-regulation *in vivo* (5). It was thus imperative to determine the oligomeric structure of ClpX and to establish the nature of the ClpX and ClpP interaction in forming the active proteolytic complex.

Our results demonstrate that ClpX, like ClpA, is composed of six subunits arranged in a symmetrical ring. Like ClpA, ClpX binds tightly to ClpP only in the oligomeric state stabilized by nucleotide binding. ClpX can form 1:1 and 2:1 complexes with ClpP, and the ClpXP complexes, like ClpAP, have a symmetry mismatch between the ATPase and proteolytic components. The ability to generalize this structural feature of the ATP-dependent Clp proteases suggests that, although the details are still not understood, the symmetry mismatch must be a fundamental determinant of the mechanism of chaperone-mediated proteolysis by ClpAP and ClpXP.

The competition assays (Fig. 4) indicate that the affinities of ClpA and ClpX for ClpP are within a factor of three of each other, and thus, ClpA and ClpX should compete for ClpP *in vivo*. Estimates of the intracellular concentrations of ClpA, ClpX, and ClpP indicate that, in exponentially growing cells on rich media, ClpA is in slight excess of ClpX, and ClpP is limiting compared with ClpA and ClpX combined.¹ Thus, the distribution of ClpP between ClpA and ClpX could affect the selection of substrates under different physiological conditions. ClpA is degraded in cells with a half-life of 1 h, but the degradation appears to be autocatalytic (3) and is not dependent on ClpX.³ Further studies are required to understand the relative

amounts of ClpAP and ClpXP and their significance *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 nonstoichiometric interaction between ATPase and proteasome subunits. A symmetry mismatch between the 11 S activator (PA28) and the 20 S proteasome 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.¹ Structural or chemical perturbations may have significant effects on the assembly of Clp ATPases.

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