Human Mitochondrial ClpP Is a Stable Heptamer That Assembles into a Tetradecamer in the Presence of ClpX*

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The functional form of ClpP, the proteolytic component of ATP-dependent Clp proteases, is a hollow-cored particle composed of two heptameric rings joined face-to-face forming an aqueous chamber containing the proteolytic active sites. We have found that isolated human mitochondrial ClpP (hClpP) is stable as a heptamer and remains a monodisperse species (s20,w 7.0 S; Mapp 169,200) at concentrations ≥3 mg/ml. Heptameric hClpP has no proteolytic activity and very low peptidase activity. In the presence of ATP, hClpP interacts with hClpP forming a complex, which by equilibrium sedimentation measurements has a Mapp of 1 × 106. Electron microscopy confirmed that the complex consisted of a double ring of hClpP with an hClpX ring axially aligned on each end. The hClpXP complex has peptidase activity and greatly increased peptidase activity, indicating that interaction with hClpX affects the conformation of the hClpP catalytic active site. A mutant of hClpP, in which a cysteine residue was introduced into the handle region at the interface between the two rings formed stable tetradecamers under oxidizing conditions but spontaneously dissociated into two heptamers upon reduction. Thus, hClpP rings interact transiently but very weakly in solution, and hClpX must exert an allosteric effect on hClpP to promote a conformation that stabilizes the tetradecamer. These data suggest that hClpX can regulate the appearance of hClpP peptidase activity in mitochondria and might affect the nature of the degradation products released during ATP-dependent proteolytic cycles.

ClpXP is an ATP-dependent protease that catalyzes unfolding and degradation of misfolded proteins as well as specifically tagged native and non-native proteins (1, 2). Both components, ClpX and ClpP, are highly conserved and are found in all euorganisms and within mitochondria or chloroplasts of higher eukaryotes (3–5). In bacterial cells, ClpP performs essential functions as a global regulator by targeting short lived regulatory proteins for degradation (2) and is an important component of protein quality control systems (6). The human homologs, hClpP and hClpP, are imported into mitochondria, but little is known regarding their specific biological functions (7–9). In vitro, hClpP has a structure and enzymatic properties similar to the Escherichia coli enzyme, although it displays an altered specificity toward protein substrates (5).

Active complexes of ClpXP consist of the Clp/Hsp100 chaperone (10), ClpX, and the compartmentalized protease, ClpP (11). In many organisms, ClpP also associates with another chaperones, ClpA. Both ClpP and ClpA are members of the AAA protein superfamily (ATPases associated with various cellular activities) (12). AAA proteins are a functionally diverse group of unfoldases that catalyze restructuring and translocation of macromolecules (12,13). ClpX and ClpA from E. coli function autonomously as chaperones, catalyzing protein unfolding (14–16), protein remodeling (17), and dissociation of protein complexes (18). ClpX and ClpA have binding sites for specific peptide motifs (19, 20), allowing them to target different proteins for degradation by ClpP. Additionally, small adaptor proteins associate with the complexes to facilitate interaction with specific substrates (21–24).

The functional form of ClpP is a tetradecamer assembled from two 7-fold symmetric rings stacked face-to-face to form a stable double ringed structure (25, 26). The rings enclose a large aqueous chamber with the 14 proteolytic active sites inside, sealed off from the surrounding solution except for narrow axial channels through each ring (25, 27). ClpX is a hexameric ring with 6-fold symmetry. ATP binding stabilizes the ClpP rings and the holoenzyme complex; the latter is formed by co-axial stacking of the ClpP hexamers on one or both ends of ClpP (5, 11). During a catalytic cycle, ClpX binds a protein substrate, unfolds it, and translocates it through the narrow axial channels into ClpP.

On its own, ClpP has limited peptidase activity, and this activity can be stimulated >100-fold in the presence of ClpX or ClpA (Kat 10,000 min−1/tetradecamer of eClpP) (28). Peptidase activity does not require ATP hydrolysis, suggesting that ClpA and ClpX exert an allosteric effect on the conformation of ClpP, either improving peptide access to the active sites or enhancing the catalytic efficiency of the active sites. Without ATP hydrolysis, only relatively short peptides <15 residues long are degraded rapidly by ClpXP; longer peptides and proteins are degraded very slowly or not at all. The restriction of proteolytic activity has been thought to reflect the restricted access of larger polypeptides to the active site chamber of ClpP. The current paradigm with respect to ClpP and other proteasome-like proteases is that sequestration of the active sites is required to protect cellular proteins from unwanted proteolytic damage (29). In this paper, we report that purified hClpP occurs as a stable heptamer in solution, in contrast to E. coli ClpP, which is predominantly a stable tetradecamer (30). Binding of ClpX promotes interaction between the two hClpP rings. The occurrence of stable free heptameric forms of ClpP suggests that allosteric control of the catalytically active conformation of ClpP is required to avoid untimely or inappropriate degradation of cellular proteins.

**EXPERIMENTAL PROCEDURES**

Protein Expression and Purification—Native hClpP, hClpP-ΔC, in which the C-terminal domain was deleted, and hClpX proteins were...
purified as described (5). C-terminally His-tagged hClpP and mutants of hClpP were cloned and purified as described (27). Polyclonal antibodies against hClpP were raised in rabbits inoculated with purified recombinant hClpP mixed with incomplete Freund’s adjuvant. Green fluorescent protein carrying a C-terminal degradation tag (GFP*-SsrA) was prepared as described (15). Sequence of SsrA is AANDENYALAA.

Mutagenesis and Preparation of Mutant Proteins—The hClpP gene cloned behind the T7 promoter in pVEX11 (Clontech) was used for mutagenesis by the overlapping PCR procedure (31). Mutant clones were confirmed by DNA sequencing. To obtain hClpP-h125_131ie, in which residues 125–131 of hClpP were changed to the corresponding ones in eClpP, the PCR primer sequences used were, for the top, 5′-C-AgCCCTTgggAgCTACAgCAAgCAAgACATggCTATCC-AggAgAg and, for the bottom, 5′-TgTggCTTggCCCTTggAgCCTCC-CgTggCTtgTggATCATgATACggAg. The external PCR primers used to amplify the construct were, for the top, 5′-CgACCGCCgCTAT-CgCgCTATCCATCTgTggAgg and, for the bottom, 5′-CCTCC-CgACTCAgCTTCTCgTgAgATggTgATggTgATggTgATggTgCTAgCTgggAg and, and for the bottom, 5′-TgTggCTTggCCCTTggAgCCTCC-CgTggCTtgTggATCATgATACggAg. An NdeI site and a HindIII site in the primers were confirmed by DNA sequencing. To obtain hClpP-h125_131ie, in which residues 125–131 of hClpP were changed to the corresponding ones in eClpP, the PCR primer sequences used were, for the top, 5′-C-AgCCCTTgggAgCTACAgCAAgCAAgACATggCTATCC-AggAgAg and, for the bottom, 5′-TgTggCTTggCCCTTggAgCCTCC-CgTggCTtgTggATCATgATACggAg. The external PCR primers used to amplify the construct were, for the top, 5′-CgACCGCCgCTAT-CgCgCTATCCATCTgTggAgg and, for the bottom, 5′-CCTCC-CgACTCAgCTTCTCgTgAgATggTgATggTgATggTgATggTgCTAgCTgggAg and, and for the bottom, 5′-TgTggCTTggCCCTTggAgCCTCC-CgTggCTtgTggATCATgATACggAg. An NdeI site and a HindIII site in the primers were allowed the PCR product to be cloned into NdeI/HindIII-digested pVEX11.

Enzymic Assays—ATP-dependent protein degradation assays using α-casein or phage Ω protein as a substrate were described previously (5). Peptidase activity was measured using Cleptide (FAPH-MALVPV) as described previously (5, 28). Degradation of GFP-SsrA was monitored by decreased fluorescence in reaction mixtures containing 0.5–1.0 μM GFP-SsrA, 0.5 μM eClpX, and 0.5 μM hClpP or mutant hClpP in 50 mM Tris/HCl, pH 7.5, 0.1 mM KCl, 0.02% Triton X-100, 1 mM DTT, 10 mM MgCl2, and 1 mM ATP (15). Fluorescence of GFP-SsrA was measured at constant temperature with an Amino-Bowman spectrofluorometer (series 2) with excitation at 395 nm (4-nm bandwidth) and emission at 509 nm (4–8-nm bandwidth). Prewarmed solutions were transferred to a microcuvette (12–25 μl) and readings were started within 15–20 s of addition of the initiating reagent, usually eClpX.

Gel Filtration—Assembly of hClpXP complexes was monitored by size exclusion chromatography on a 0.32 × 30 cm Superdex200 (Pharmacia) in 50 mM Tris/HCl, pH 8.0, containing 0.1 mM KCl, 10% (v/v) glycerol, 10 mM MgCl2, and 1 mM ATPγS. DTT was present at 1 mM when indicated. For detection of hClpP in mitochondrial extracts, aliquots of the gel filtration fractions were subjected to SDS-PAGE, and the amounts hClpX and hClpP were allowed the buffer to be cloned into NdeI/HindIII-digested pVEX11.

Analytical Ultracentrifugation—Proteins were equilibrated for ultracentrifugation by gel filtration in the desired buffers or by overnight dialysis against the buffer. Optima Models XL-A and XL-1 analytical ultracentrifuges (Beckman, Inc.) equipped with four-place An-Ti rotors were used for sedimentation velocity and sedimentation equilibrium experiments. The densities (ρ) of buffer A (50 mM Tris/HCl, 200 mM KCl, pH 7.5), buffer B (50 mM Tris/HCl, 1 mM EDTA, and 1 mM DTT, pH 7.5), and buffer C (50 mM Tris/HCl, 200 mM KCl, 1 mM ATPγS, 10 mM MgCl2, and 1% glycerol, pH 7.5) were determined to be 1.0101, 1.0074, and 1.038 g/ml, respectively, at 20.00 ± 0.01 °C, using an Antoun Paar DMA-58 densitometer. Corresponding values of relative viscosity were determined to be 1.015, 1.017, and 1.415 using an in-house viscometer (32). Partial specific volumes of 0.726 ml/g for hClpP and 0.722 ml/g for the hClpXP complex were calculated from the sum of the contributions of the amino acid residues (33). Absorbance coefficients at 280 nm for hClpP (0.673 cm2/mg) and hClpX (0.365 cm2/mg) were calculated from their Trp, Tyr, and Cys contents (34). For interference optics, the calibration value of 3.191 ± 0.005 fringes (mg/ml)−1 (35) was corroborated with a solution of 3.428 mg/ml of bovine serum albumin. For time derivative analysis (36), the procedures of Zolkiewski et al. (37) were used. Observed sedimentation coefficients (sobs) were corrected to the density and viscosity of water at 20 °C, where s20,w = 1.0574 × sobs for hClpP and s20,w = 1.0463 × sobs for eClpP (reported in Svedberg units, S). Frictional coefficient ratios (f/f0) (where f0 is the frictional coefficient of a sphere having the volume equal to that of an ellipsoid) were calculated from the values for Mz, partial specific volume, and s20,w (38).

Sedimentation equilibrium experiments with hClpP were performed at 4.0 °C. Two 12-mm cells equipped with plane quartz windows and double sector centerpieces were loaded with 0.110 μl of hClpP or hClpP-ΔC in buffer A (with 280 nm absorbance of 0.20); a third cell contained hClpP (with 280 nm absorbance of 0.11). The reference channel of each cell was filled with 0.125 ml of buffer A. When the rotor speed reached 3000 rpm, scans at 280 nm were made to establish the positions of the menisci and the cell bottoms, and the rotor was then accelerated to 5300 rpm. After 24 h, programmed autoscans (9–11 averages, 0.001 cm steps in step-mode) were initiated and made at 2-h intervals. After reaching sedimentation equilibrium (48–56 h) the rotor speed was changed to 11,000 rpm for another 56 h to deplete the meniscus, and scans were recorded as above.

Sedimentation equilibrium of hClpXP complexes was performed at 12 °C. A mixture containing hexameric ClpXP and heptameric ClpP in a 1:1.1:0.1 molar ratio was dialyzed against buffer C, and 0.12 ml (2.9 mg/ml total protein) was loaded into the right channel of a double sector cell (1.2-cm path length) equipped with sapphire windows and a carbon-filled epoxy centerpiece. Dialysate (0.12 ml) was placed in the left reference channel. The rotor was accelerated to 3000 rpm until optical calibrations and initial scans were performed and then maintained at 3500 rpm for the duration of the run. When interference optics was used, scans were made at 2-h intervals until five consecutive scans overlapped. Because the fringe displacement just inside the meniscus must be set to zero at the start of each run, the value of the fringe displacement at the meniscus was added to the final protein concentration gradient after subtraction of the buffer baseline. To obtain a buffer baseline, the protein was removed from the assembled cell at the end of a run, and the right channel was flushed repeatedly before reloading with dialysate; a scan was recorded after the cell came to equilibrium at 3500 rpm and 12 °C. Simulations and reiterative fits of the data took into account the competent loading concentration and the buoyant density of the 2:1 complex. The goodness of the fits were judged on the basis of a random distribution of residuals around zero with either ± 0.01 absorbance or ± 0.003–0.005 fringe displacement. Data analysis was performed with the software provided by Allen P. Minton (NIDDK, National Institutes of Health).

Chemical Modification and Limited Proteolysis—Histidine residues in hClpP were chemically modified by incubating 2 mg hClpP with 0.1

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4 The abbreviations used are: GFP, green fluorescent protein; DTT, dithiothreitol; ATP-γS, adenosine 5′-(o-thiotriphosphate); bis-Tris, 2-(bis-2-hydroxyethyl)amino-2-(hydroxyethyl)propane-1,3-diol.

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Human ClpP Protease Forms a Stable Heptamer

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mm carbobenzoxy-Leu-Tyr-chloromethyl ketone in 50 mm Hepes, pH 7.5, 0.1 M KCl, and 10% (v/v) glycerol at 37 °C for up to 2 h. Samples were withdrawn for assay, and at the end the entire mixture was treated with 1.5 volumes of cold acetone to precipitate the protein, which was then dissolved in 70% formic acid to which 20 mM CNBr had been added. After incubation overnight at 0 °C, the solution was lyophilized, and the cleavage products were dissolved in 6 M guanidine hydrochloride in 0.1% trifluoroacetic acid. Peptides were isolated by reverse phase chromatography using the column and system mentioned above. Control samples, not treated with carbobenzoxy-Leu-Tyr-chloromethyl ketone, were digested and analyzed in parallel. Samples from the reverse phase column as well as aliquots of the original mixture of digestion products were analyzed using a Micromass ToFSpec-2E mass spectrometer.

Chemical cross-linking of subunits was performed by incubating 4 μg of hClpP with 0.1% (w/v) glutaraldehyde in 50 mM Hepes, pH 7.5, 0.1 M KCl, and 10% (v/v) glycerol at room temperature for 30 min. Aliquots of 5 μl were withdrawn and immediately added to 5 μl of 2× SDS sample buffer sitting in a boiling water bath. The entire sample was loaded in each lane, and proteins were separated on a 12% bis-Tris polyacrylamide gel (Invitrogen) and stained with Coomassie Blue.

For limited proteolysis, 10 μg of ClpP was incubated in 30 μl of 50 mM Tris/HCl, pH 8.0, containing 0.1 μl of KCl, and 10% (v/v) glycerol. Aliquots were withdrawn, quenched by adding to an equal volume of hot 2× SDS sample buffer, electrophoresed, and detected by staining, as described above.

**Electron Microscopy and Image Processing**—Electron micrographs of negatively stained complexes were obtained as described previously (39). Micrographs were digitized at 1.55 Å/pixel, using a SCAI scanner (Z/I Imaging, Huntsville, AL). The numbers of complexes with one or two ClpX associated with ClpP were recorded after visual examination of the micrographs. The number of ambiguous particles, not identifiable as ClpXP complexes, was <5% of the total.

**Preparation of Rat Liver Mitochondria**—Frozen rat livers were obtained from Pel-freeze. Livers (20 g) were thawed by suspension in breaking buffer consisting of 50 mM Tris, pH 7.5, 50 mM sucrose, 25 mM KCl, and 5 mM MgCl₂ and then minced. Liver pieces were washed three times by allowing them to settle and decanting excess buffer. Washed pieces were homogenized using an ice-chilled Teflon-glass homogenizer. The homogenate was centrifuged at 1000 g to remove excess lipid and eluted in the position expected for a heptamer (Fig. 1A). This difference in behavior was unexpected because both proteins displayed similar peptidase activity as well as ClpX-mediated protease activity. Light scattering data confirmed a difference in the hydrodynamic radius between the wild type and mutant proteins (data not shown). To determine the basis for the differences in gel filtration behavior, we performed sedimentation velocity and sedimentation equilibrium ultracentrifugation.

A time-derivative analysis of the hClpP gradient after ~96 min at 40,000 rpm is shown in Fig. 2. A similar analysis of sedimentation data previously obtained with eClpP (30) indicates that, under similar conditions, eClpP sediments much faster than hClpP (Fig. 2). The hClpP data can be fit by a single Gaussian, indicative of a monodisperse, non-interacting solute with an s_{20,w} of 7.0 S and a D_{20, w} of 3.65 × 10⁻⁷ cm² s⁻¹ from the Svedberg equation, these values provide a calculated M_r of 169,800 (TABLE ONE), corresponding to a heptamer of hClpP (subunit M_r, 24,166). The frictional coefficient ratio (f/f₀) for hClpP obtained from the M_r, the partial specific volume, and the s_{20,w}, was 1.6, indicating an asymmetric shape and a large hydrodynamic radius, consistent with its unusual high mobility during gel filtration. In contrast, eClpP has an s_{20,w} of 12.2 S, M_r of 302,000, and a calculated f/f₀ value of 1.2 (30), consistent with a symmetrical tetradecameric species. Corrected sedimentation coefficients for hClpP obtained at 20 °C were the same in the absence and presence of 10% glycerol (data not shown).

The hClpPΔC mutant had an s_{20,w} value of 8.2 S in sedimentation velocity experiments under the same conditions used for wild type hClpP. The calculated frictional ratio (f/f₀) for the hClpPΔC heptamer was 1.2 (see below), which indicates that removal of the C-terminal 2740 M_r fragment creates a more compact and/or symmetrical heptameric species and explains the more standard behavior of hClpPΔC during gel filtration (Fig. 1).

The molecular weights of hClpP and hClpPΔC were confirmed by sedimentation equilibrium measurements at 4 °C at 5300 or 11,000 rpm for 47–54 h. The heptamers were homogeneous and stable under the

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**RESULTS**

**Recombinant hClpP Is a Heptamer in Solution**—hClpP (subunit M_r, 24,000) displays unusual behavior upon gel filtration. On a Superdex200 column (Fig. 1A), ClpP migrated in approximately the same position as eClpP, which is known to be a tetradecamer, initially leading us to conclude that hClpP is also tetradecameric in solution. However, a mutant of hClpP (hClpPΔC; subunit M_r, 21,000), which lacks the 28-amino acid C-terminal domain, migrated much more slowly during gel filtration and eluted in the position expected for a heptamer (Fig. 1A). This difference in behavior was unexpected because both proteins displayed similar peptidase activity as well as ClpX-mediated protease activity. Light scattering data confirmed a difference in the hydrodynamic radius between the wild type and mutant proteins (data not shown). To determine the basis for the differences in gel filtration behavior, we performed sedimentation velocity and sedimentation equilibrium ultracentrifugation.

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Because ClpX itself was sensitive to proteolysis, both proteins were resistant to limited proteolysis in the presence of ClpX (data not shown), but, proteins at least the size of trypsin (4 kDa). hClpP indicates that hClpP rings do not interact tightly with each other (Fig. 4B). Accessibility of Arg-129 in hClpP was 0.8 at 280 nm. The profile shown represents a time derivative analysis of concentration gradients obtained from four absorbance scans taken late in the cycle. The solid line shows the apparent distribution function of $g(\ast)$ versus the sedimentation coefficient $s$ in Svedberg units (S), and the dotted line shows the fit to a single Gaussian, indicative of a homogeneous non-interacting solute. The observed sedimentation coefficient is given by the $s$* value at the maximum and yielded a solvent corrected $s_{20,w}$ value of 7.0 S for hClpP. For comparison, a similar analysis of data obtained with eClpP ($s_{20,w}$, 12.2 S) under comparable conditions and recorded at harmonic average sedimentation times is shown. Varying the salt concentration between 0.1 and 0.3 M and the presence or absence of glycerol did not significantly alter the sedimentation coefficients of either protein.

Sedimentation equilibrium conditions. At each speed, the concentration gradients were fitted well to heptamer molecular weights for both proteins (Fig. 3 and TABLE ONE), and applying a reversible macromolecular self-association model to any of the data sets showed no evidence of association between heptamers for either protein.

Endogenous Rat Liver ClpP Is a Heptamer—Human ClpP is identical in sequence to rat ClpP in residues 1–193 and has mostly conservative changes in 8 of the 28 residues in the C-terminal domain. The two proteins are expected to have nearly identical physical and hydrodynamic properties. To compare ClpP expressed in vivo to the recombinant human protein purified from E. coli, extracts of rat liver mitochondria were prepared and run over a Superdex200 gel filtration column. ClpP was detected in the fractions using anti-hClpP antibodies. Fig. 1B shows that the rat liver ClpP had the same mobility as the heptameric hClpP protein, indicating that the endogenous mammalian ClpP is also a heptamer. Addition of hClpX and ATPγS to the extract shifted the rClpP to higher molecular weight fractions, suggesting that the heptameric rClpP is capable of forming complexes with hClpX.

The Aqueous Chamber of hClpP Is Accessible to Trypsin—hClpP was susceptible to cleavage by trypsin, chymotrypsin, V8 protease, and lysyl endopeptidase (data not shown). In contrast, eClpP is more resistant to protease treatment (40). When hClpP was incubated with trypsin in standard buffer at 37 °C, two degradation products appeared after about 1 h (Fig. 4A). N-terminal sequencing revealed that a single cut was made after Arg-129 in hClpP. In the crystal structure, Arg-129 is on strand β9 of the handle and points inward toward the 7-fold symmetry axis (Fig. 4B). The handles interlock to hold the heptameric rings together in the tetradecamer, with Arg-129 pointing into the chamber and sealed off from the surrounding solution (Fig. 4C). Accessibility of Arg-129 in hClpP indicates that hClpP rings do not interact tightly with each other and that parts of the concave surface of the heptamer are accessible to proteins at least the size of trypsin (≈25 kDa), hClpP was more resistant to limited proteolysis in the presence of ClpX (data not shown), but, because ClpX itself was sensitive to proteolysis, both proteins were cleaved upon long incubation.

Glutaraldehyde Cross-linking of hClpP—As a further test of the oligomeric state of hClpP in solution, the protein was cross-linked, and the products were analyzed under native and denaturing conditions. Treatment of hClpP or hClpP–ΔC with glutaraldehyde produced a ladder of seven species separated by SDS-PAGE (Fig. 5A); a small amount of protein migrated as poorly resolved bands of higher molecular weight. The kinetics of appearance of the seven species suggested a pattern of random cross-linking of subunits within a ring with little or no cross-linking of subunits across the rings. In contrast, glutaraldehyde treatment of eClpP resulted only in slow formation of a dimeric species (Fig. 5A), most likely arising from cross-linking of a tetradecamer. When gel filtration was performed under non-denaturing conditions, ~70% of the cross-linked hClpP co-migrated with native hClpP heptamers, and the remainder eluted as apparently higher molecular weight species (Fig. 5B). SDS-PAGE analysis (Fig. 5B) detected only the seven discrete species in the heptamer fractions, while the higher molecular weight fractions produced both the ladder of seven bands and a diffuse array of larger species possibly resulting from residual cross-linking between two heptameric rings. These data allow that hClpP heptamers might transiently associate to form tetradecamers, although the equilibrium highly favors the heptameric species. Further evidence for such transient association between native heptamers is presented below.

The Predominant Form of hClpX Is a Hexamer—To determine the predominant oligomeric state of hClpX (subunit $M_1$, 62,522), hClpX in buffer C with ATPγS was subjected to sedimentation at 40,000 rpm and 20 °C. During the first 20–25 min, hClpX sedimented as a uniform species with an $s_{20,w}$ of 14 S. Based on the previously determined $s_{20,w}$ of 17.2 S and $M_r$ of 505,000 for E. coli ClpP (30), the $M_r$ of the assembled hClpX was calculated (38) to be 375,000, corresponding to a hexamer. At later times, hClpX became somewhat heterogeneous as evidenced by boundary spreading. The instability of hClpX was more marked during sedimentation in buffer without glycerol, which produced two peaks corresponding to different aggregate states (data not shown).

ClpX Promotes hClpP Tetradecamer Formation—The stability of heptameric hClpP in solution raises the question of how complexes between hClpP and hClpX are assembled. Under various conditions, including the dilute solutions used for enzymatic assays (<0.1 μM of either oligomer), assembly of ClpXP complexes occurs very rapidly. When different ratios of hClpP and hClpX were mixed in the presence of ATPγS, the major species by electron microscopy were symmetrical complexes, consisting of a tetradecamer of hClpP and two hClpX hexamers, one on each end (Fig. 6). We refer to these complexes as 2:1 complexes (two ClpX hexamers with one ClpP tetradecamer). The 2:1 complexes predominated even when hClpP was in large molar excess over ClpX. These observations suggested that assembly occurs by binding of an hClpX hexamer to an hClpP heptamer, followed immediately by self-association of the hClpP to form the 2:1 holoenzyme complexes. Particles with one ClpX hexamer bound to a single or double ClpP ring can adopt different orientations on the grids, and it was therefore not possible to perform an exact quantitation of the distribution of the various species. Some 1:1 complexes consisting of a hexamer of ClpX bound to a tetradecamer of ClpP were observed, but these species could well have arisen by dissociation of one of the ClpX hexamers after initial formation of the 2:1 complex.

To confirm the species of ClpXP complexes present in solution, we performed analytical ultracentrifugation experiments on mixtures of hClpX and hClpP. With mixtures in which hexameric ClpX was present in 0.1:1.0 molar ratio to heptameric hClpP, relatively simple concentration gradients were observed when a low speed approach to equilibrium condition was used. After centrifugation for 54 h at 12 °C and a speed of 5000 rpm, analysis of the gradients showed very little [ClpP–], no
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**TABLE ONE**

Summary of hydrodynamic values at pH 7.5

| Clp protein       | $s_{20,w} \times 10^{14}$ (±0.2 S) | $D_{20,w} \times 10^7$ (±0.1 cm²/s) | $f/f_0$ | $M_r (s/D)^a$ (±5%) | $M_r (SedEq)^a$ (±3%) |
|-------------------|-----------------------------------|------------------------------------|---------|---------------------|-----------------------|
| hClpP (wild type) | 7.0                               | 3.65                               | 1.6     | 169,800 (20 °C)     | 169,200 (4 °C)        |
| hClpPΔC           | 8.2                               |                                    | 1.2     | 150,000 (20 °C)     |                       |
| eClpP             | 12.2                              | 3.6                                | 1.2     | 300,000 (20 °C)     |                       |
| hClpX₆P₁₄X₆       | 31                                |                                    | 1.3     | 1,000,000$^a$ (12 °C) |                      |
| eClpA₆P₁₄A₆       | 27                                |                                    | 1.8     | 1,310,000$^a$       |                      |

$^a$ From $s/D$ and the Svedberg equation; determined at 20 °C.

$^b$ The hClpP-ΔC mutant exhibited an instability after centrifugation for long times at 20 °C by excessive boundary spreading.

$^c$ From Ref. 30.

$^d$ The $M_r$ value was determined from the $d \log (c/d (r^2))$ plot using interference optics (Fig. 7B). The calculated $M_r$ for the 2:1 complex of ClpX₆·ClpP₁₄ is 1,089,000. The predominant species identified was the 2:1 complex.

*Calculated from the component $M_r$ values.

**FIGURE 3.** Sedimentation equilibrium of hClpP and hClpP-ΔC. hClpP (0.35 mg/ml) and hClpPΔC (0.30 mg/ml) in 50 mM Tris/HCl and 200 mM KCl, pH 7.5, were brought to equilibrium at 11,000 rpm and 4 °C. Protein gradients are plotted as 280 nm absorbance vs. radial position at 45, 47, and 49 h (open circles, triangles, and inverted triangles). Fits of the data sets to the calculated gradients (solid curves) for monodisperse heptameric hClpP, $M_r = 169,200$, and heptameric hClpP-ΔC, $M_r = 150,000$, are shown in the lower and middle panels, respectively. The top panel shows the residuals of $<0.01$ absorbance deviation from global fits for the hClpP data; the residuals for hClpP-ΔC (not shown) were identical.

**FIGURE 4.** Trypsin treatment of hClpP. hClpP (10 μg) was incubated with 0.5 μg trypsin at 37 °C. At 0, 30, and 60 min aliquots were removed, subjected to SDS-PAGE, and blotted onto a polyvinylidene difluoride membrane. After staining, the protein bands were cut out, and the proteins were sequenced from the N terminus by automated Edman degradation. A, stained protein bands obtained after trypsin cleavage of hClpP. B, representation of the linear sequences of hClpP and hClpP-ΔC showing the positions of the trypsin cleavage site. C, space-filling model of hClpP heptamer viewed along the 7-fold axis from within the aqueous chamber (front) and the hClpP tetradecamer viewed from the edge (right). The handle region forming the interface between the rings, composed of β-strands 6 and 7 and β-helix 5, is shown as a purple ribbon; Arg-129, the site of trypsin cleavage, is shown in yellow. The structure is from Kang et al. (27).
the mixture prevented precise molecular weight determination, the 21 S species is most likely a complex with one hClpX hexamer bound to the hClpP tetradecamer. A variable amount of a species with an smax of 31 S, corresponding to the 2:1 complex, was also observed. In addition, slower sedimenting species (~5 S) were present. The latter are likely to be dissociated ClpX hexamers because a similar peak was seen with ClpX alone (data not shown). Although peak areas are not reliable in such mixtures (38), the amounts of 2:1 complexes appeared to be less than expected regardless of the ratios of hClpX and hClpP. We infer that the hClpXP complexes are sensitive to the high pressure resulting from sedimentation at >100,000 × g. Given that hClpX itself is unstable during sedimentation experiments (data not shown), it would appear that 2:1 complexes were initially formed but fell apart upon dissociation of hClpX.

Cross-linking between the Handle Regions Stabilizes hClpP Tetradecamers—There are several differences in the residues found in strand β9, which forms the interface between the two rings in hClpP and eClpP (27). Near the start of β9 of hClpP, much smaller Ser and Ala residues replace Leu-125 and Tyr-128 of eClpP, respectively. In addition, Arg-129 of hClpP is found in place of eClpP Gln-129. To test whether the differences in the handle region were responsible for the differences in stability of inter-ring bonding, we replaced Ser-125, Ala-128, and Arg-129 of hClpP with the residues found in eClpP. The mutant hClpP-handle folded and assembled into stable heptamers; however, it did not spontaneously form stable tetradecamers in the way...
The conformation of the handle region might affect enzymatic activity. The handle region is distorted slightly from that in the active tetradecamer. Treatment of the higher molecular weight form with DTT led to rapid conversion to the species moving as a heptamer (Fig. 8). These data show that hClpP rings reversibly associate in solution but are not held together tightly. In the presence of DTT, hClpP-R129C eluted much earlier in fractions consistent with a heptamer (Fig. 8), indicating that other differences between hClpP and eClpP also influence tetradecamer formation. hClpP-ehandle responded to hClpX binding and assembled into 2:1 hClpXP complexes (data not shown). Surprisingly, the complexes were inactive for protein degradation as well as peptidase activity, even though substrates could be translocated into the chamber of the mutant hClpP-ehandle (see Fig. 9 below). The lack of peptide bond cleaving activity suggests that the interaction between the handles has an effect on the conformation of the catalytic site. In the crystal structures of eClpP and hClpP, several residues at the tip of the handle form a hydrogen bonding network with residues within or surrounding the catalytic site.

In the tetradecamer, the side chains of the Arg-129 residues from apposing subunits point toward the center and approach close to each other. We mutated Arg-129 in hClpP to introduce cysteine in its place, purified the mutant protein, and analyzed the assembly in the presence and absence of reducing agents. In the presence of DTT, hClpP-R129C emerged from a Superdex200 column in the same fractions as hClpP, indicating that it is a heptamer (Fig. 8). However, when DTT was omitted, hClpP-R129C eluted much earlier in fractions consistent with a tetradecamer. Treatment of the higher molecular weight form with DTT led to rapid conversion to the species moving as a heptamer (Fig. 8). These data show that hClpP rings reversibly associate in solution but that the heptamer is favored in the absence of the stabilizing effects of disulfide cross-linking. Binding of hClpX, which also stabilizes the tetradecamer, appears to promote a conformational change at the interface that favors association of the hClpP rings or reduces the mobility or flexibility of the handles leading to increased stability of interaction. hClpP-R129C was impaired in activity for both peptide and protein degradation (TABLE TWO), suggesting that the disulfide-cross-linked handle region is distorted slightly from that in the active tetradecamer. The conformation of the handle region might affect enzymatic activity by influencing the orientation of the catalytic His-122 located near the start of β9.

**TABLE TWO**

| Limiting component | Peptidase Activity (μM) | Proteolysis Activity (μM) |
|--------------------|-------------------------|--------------------------|
| Wild type ClpP     | 1.0                     | 1.0                      |
| ClpP-R129C         | 0.3 ± 0.1               | 0.4 ± 0.1                |
| ClpP-h125-131e     | <0.01                   | <0.01                    |

*Activities are normalized to wild type ClpP (5).

**FIGURE 9.** Release of translocated substrates from the ClpP chamber. For these experiments, eClpX, which specifically targets proteins bearing the C-terminal motif, SsrA, was used with hClpP-S97A or other mutant forms of hClpP. Translocation was followed by loss of fluorescence of GFP-SsrA at 37 °C in assay mixtures containing 50 mM Tris, pH 8.0, 0.1 mM KCl, 10 mM MgCl2, and varying concentrations of ATP. The protein concentrations were 0.5 μM GFP-SsrA, 0.4 μM eClpX, and 1.0 μM hClpP heptamer. A, hClpP-ehandle mutant slowly releases translocated substrates. The reaction was initiated by adding eClpX to prewarmed solutions containing all other materials. Solid line, control with wild type hClpP; dotted line, hClpP-S97A; dashed line, hClpP-ehandle; light solid line, no ClpXP. B, GFP-SsrA translocation was carried out with the same amounts of eClpX, hClpP-S97A, and GFP-SsrA as above. The ATP concentration was varied: solid line, 5 mM ATP; dashed line, 2.5 mM ATP; dotted line, 1.25 mM ATP; broken line, ClpX alone with 5 mM ATP. Opening of the hClpP Rings in hClpXP Complexes under Catalytic Conditions—The dependence on hClpX for assembly of the hClpP tetradecamer and the influence of the handle region on activity prompted us to examine the stability of the hClpP tetradecamer under catalytic conditions. hClpP, like the proteasome and HslV, are thought to create a stable degradation chamber in which protein substrates are degraded extensively before the peptide products are released. The mechanism of release of peptide products is still not understood, although a current hypothesis is that they exit through the axial channel opposite to the one through which the substrate entered, possibly assisted by the chaperone component or other regulatory factors. The structural integrity of the ClpP chamber is indicated by the ability to trap translocated proteins inside ClpP (20, 39), although it is possible that the translocated proteins themselves might help stabilize the chamber. Several mutants behaved as though the ClpP tetradecamer was less stable. For example, the hClpP-ehandle mutant was able to unfold and trap GFP-SsrA in a manner similar to wild type hClpP (Fig. 9A), but the trapped GFP-SsrA was slowly released from the complexes. In contrast, GFP-SsrA remained unfolded and was retained by complexes made with a wild type handle region (Fig. 9A). These data suggested that proteins might be released from the degradation chamber under conditions in which the rings of hClpP are not held together tightly.

Because the stability of the double ring of wild type hClpP is dependent on the continuous presence of hClpX and ATP, we reasoned that the hClpP rings might dissociate and release trapped protein substrates, as
the ATP was hydrolyzed and the ratio of ATP to ADP no longer favored association between hClpX and hClpP. We carried out unfolding/trapping assays with wild type hClpXP complexes and varying amounts of ATP (Fig. 9B). GFP-SsrA was unfolded in all cases, but the fluorescence did not decrease to the same level in all cases. Especially with low ATP concentrations, the fluorescence reached a plateau and then began to increase again as the trapped GFP-SsrA was slowly released from the complex and refolded in solution. These data show that, in response to the nucleotide state of the hClpX in the complex, hClpP rings can separate sufficiently to allow proteins to escape from the chamber. The results further suggest that similar or slightly smaller changes in allosteric interaction of hClpX could allow partial separation of hClpP rings, which would provide a mechanism for release of peptide products during catalytic turnover. This mechanism of altering the routes into and out of the hClpP chamber could also explain the enormous increase in rates of degradation of some peptides in ClpXP and ClpAP complexes.

**DISCUSSION**

In this paper we have shown that hClpP expressed in *E. coli* is a stable heptamer. Upon interaction with ClpX in the presence of ATP, ClpP rings join face-to-face forming a stable tetradecamer with a ClpX heptamer stacked on both sides. An important aspect of the current paradigm describing the relationship between the compartmentalized structure of proteases such as ClpP, ClpQ, or the proteasome and their activities in vivo is that sequesterment of the proteolytic sites is required to protect cellular proteins from unwanted damage. Our finding is that heptameric hClpP lacks activity against protein substrates and has very low activity against peptides, lower even than eClpP, which is a stable tetradecamer. Thus, compartmentalization of the active sites in this protease is not required to protect cellular proteins from unwanted degradation by hClpP. Quite possibly, other consequences of the sequestered proteolytic environment, for example, improved processivity of degradation, control over the size distribution of peptide products, and other regulatory or structural constraints, could provide advantages as important, or more so, as protection of cellular proteins *per se*.

The low activity of hClpP heptamers does not appear to result from limited access to the active sites, because trypsin (*M*, 27,000) is able to cleave a site on the internal surface of the heptamer only 15 Å from the catalytic triad. Also, chemical regents react much faster with a cysteine residue at the active site in the heptameric form than in the assembled hClpP tetradecamer (data not shown). We conclude that, in the heptamer, the catalytic triad is not in an active configuration and that assembly of the tetradecamer is accompanied by conformational changes in the active site leading to an alignment necessary for catalysis. The crystal structures of hClpP and eClpP suggest ways in which assembly of the tetradecamer could affect the configuration of active site residues. The catalytic triad of Ser-97, His-122, and Asp-171 is located in a crevice at the base of the major β sheet. Asp-171 interacts through hydrogen bonding water molecules with Gln-138 and Asp-134 in the handle region of the adjacent subunit. These residues and others in the arm, such as Gln-131 and Thr-133, are in turn held in place through a network of hydrogen bonds with residues from the apposing ring, especially Gln-123, Lys-146, Glu-169, and Arg-170. These residues are all highly conserved in the ClpP protein family and are replaced by other hydrogen-bonding residues when not identical. The greater tendency of hClpP-S97C to form tetradecamers also points to a reciprocal influence of active site residues on association between the rings.

In the ClpP tetradecamer, the heptamer rings are joined by interdigitation of the α/β handle regions of apposing subunits. The primary stabilizing interactions are produced by extensive hydrogen bonding between backbone atoms of the anti-parallel β9 strands from apposing subunits. The space between the pairs of β strands is filled with the side chains of residues from α5, which make additional contacts with the side chains of α5 and β9 from the next subunit in the apposing ring. The inability to restore spontaneous tetradecamer formation to hClpP by changing the handle region to match that eClpP suggests that other residues or structural features that differ between hClpP and eClpP also contribute to assembly. These other structural features may affect the proper orientation of the handles needed to engage each other, or they may affect the mobility of the handle region and its ability to maintain stable contacts. Some of the same conformational changes apparently also influence catalytic activity.

Because both the recognition function and the energy-dependent protein unfolding and translocation functions are carried out by the chaperone components, earlier models of ATP-dependent proteolysis assign relatively passive roles to the proteolytic component. However, studies have now shown that the α subunit of the proteasome performs a dynamic gating function (42), and the crystal structure of the proteasome complexed with the activator protein, PA26, showed a dramatic conformational change undergone by the N-terminal peptide of the α subunits (43). Based on the original crystal structure of eClpP (25), it was predicted that ClpP was unlikely to undergo significant conformational changes during the reaction cycle; however, our observations suggest a more dynamic structure for ClpP. In both eClpP and hClpP (27) as well as in ClpP from *Streptococcus pneumoniae* (45) the N-terminal eight amino acids of all seven subunits are bound within the axial channel, whereas residues 9–16 loop out at the entrance to the channel and are highly mobile. Mutagenic studies indicate further that the N-terminal peptide is essential for activity and may participate in binding and possibly translocation of substrates (27). Further evidence for conformational flexibility comes from our data showing cooperative formation of stable ClpP tetradecamers upon binding to ClpX. The crystal structure of *Helicobacter pylori* ClpX (46) places the ClpP-interaction loop in a position where it should contact the outer edge of the ClpP ring surface. Thus, conformational changes induced by ClpX binding to ClpP must be transmitted allosterically to other affected regions, the N-terminal peptide, the active site, and the handle. Although eClpP tetradecamers appear to be stable at neutral pH and at moderate ionic strength, they undergo reversible dissociation into two heptameric rings upon treatment in the cold with high concentrations of divalent anions (30). Peptidase activity is restored upon dilution into assay buffer, indicating that the tetradecamer interface is readily reformed. This sensitivity of the interface to environmental conditions and to the presence of bound ClpX suggests that limited conformational changes at the interface, not leading to dissociation, could affect the activity of all ClpP molecules.

The apparently tight interfaces between the ClpP rings and between the catalytic rings of the proteasome have posed a difficulty in understanding the complete reaction cycle of proteolysis by these complexes, in particular, how peptide products exit from the degradation chamber. No channels large enough to allow passage of peptide out of the chamber appear at the ring interfaces, and thus most models of activity propose that degradation products exit through the axial channels, possibly on the end opposite from the one where proteins are entering. For the proteasome, the non-ATP-dependent activator PA28 or PA26 appears to activate peptidase activity, possibly by facilitating release of peptides through the axial channel. This model is supported by structural data showing an open-gate configuration of the proteasome when bound to PA26 (43). Our finding that the ring interface in hClpP is allosterically affected by ClpX interaction suggests that other modes of peptide release might be possible.
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The interaction between ClpX and ClpP is dependent on the nucleotide state of ClpX. In the absence of ATP, ClpX does not interact with ClpP, and, in the case of hClpP, the result is destabilization of the tetradecamer itself. Partial occupancy of hClpX by nucleotide could lead to destabilization of the ClpP ring interface. Thus, during the normal cycle of ATP hydrolysis, transient disruption of contacts between pairs of apposing handles could result in formation of temporary gaps in the chamber wall. This effect would provide a mechanism by which peptide products could be released from ClpP. ClpX ATPase activity is activated by the binding of substrates and is sensitive to the thermodynamic properties of the substrates. Slowly unfolded proteins have lower stimulatory effects on ClpX ATPase activity, whereas unfolded or rapidly unfolded substrates have greater activating effects (47). Because lower ATPase activity would favor more stable ClpP tetradecamers, this mechanism would ensure that the chamber remained intact until the substrate was unfolded, translocated, and degraded. More unfolded substrates or faster rates of unfolding would lead to higher rates of ATPase activity and translocation, resulting in more frequent opening of exit ports to accommodate the faster rate of peptide bond cleavage. Coordination between release of peptide, ATP hydrolysis, and degradation would avoid accumulation of products in the chamber but would also avoid premature release of larger undegraded peptides. Such a model suggests an integrated mechanism of action for the chaperone and proteolytic partners in all phases of the unfolding/degradation process.

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