Human mitochondrial ClpP (hClpP) and ClpX (hClpX) were separately cloned, and the expressed proteins were purified. Electron microscopy confirmed that hClpP forms heptameric rings and that hClpX forms a hexameric ring. Complexes of a double heptameric ring of hClpP with hexameric hClpX rings bound on each side are stable in the presence of ATP or adenosine 5′-(3-thiotriphosphate) (ATPγS), indicating that a symmetry mismatch is a universal feature of Clp proteases. hClpXP displays both ATP-dependent proteolytic activity and ATP- or ATPγS-dependent peptidase activity. hClpXP cannot degrade α0 protein or GFP-SsrA, specific protein substrates recognized by Escherichia coli (e) ClpP. However, eClpX interacts with hClpP, and, when examined by electron microscopy, the resulting heterologous complexes are indistinguishable from homologous eClpXP complexes. The hybrid eClpX-hClpP complexes de degrade eClpX-specific protein substrates. In contrast, eClpA can neither associate with nor activate hClpP. hClpP has an extra C-terminal extension of 28 amino acids. A mutant lacking this C-terminal extension interacts more tightly with both hClpX and eClpX and shows enhanced enzymatic activities but still does not interact with eClpA. Our results establish that human ClpX and ClpP constitute a bone fide ATP-dependent protease and confirm that substrate selection, which differs between human and E. coli ClpX, is dependent solely on the Clp ATPase. Our data also indicate that human ClpP has conserved sites required for interaction with eClpX but not eClpA, implying that the modes of interaction with ClpP may not be identical for ClpA and ClpX.

Protein remodeling and protein degradation carried out by the Clp family of molecular chaperones and their proteolytic complexes with ClpP have important physiological functions in many organisms. Clp proteases, together with other ATP-dependent proteases, such as the Lon protease and the membrane-associated protease FtsH, are responsible for maintaining proper protein homeostasis, contributing to protein quality control, and modulating the intracellular concentration of important global regulatory proteins (1–3). The ATPase/chaperone components of ATP-dependent proteases target unique substrates for degradation, although some overlap exists in the recognition of misfolded or other abnormal forms of proteins (4–7). In the bacterial Clp system, two separate ATPases, ClpX and ClpA, interact with different protein substrates and confer specific protein degrading ability to ClpP (8).

Clp proteases, Lon, and FtsH are conserved in most eukaryotes, where they are found in organelles, such as mitochondria, peroxisomes, and chloroplasts (2, 9, 10). In yeast, Lon and FtsH homologs have been shown to play important roles in assembly and quality control over membrane protein complexes (11) and in the degradation of misfolded proteins (9, 12). In humans, mutations in paraplegin, an FtsH homolog, have been implicated in the progressive loss of mitochondria function in the hereditary neurodegenerative disease spastic paraplegia (13). The function of mitochondrial Clp proteins has not been identified.

The human genome encodes both ClpP, on chromosome 19 (14), and ClpX, on chromosome 15 (15). mRNAs for both human (h) ClpP and hClpX have been found in greatest abundance in liver, heart, and testes, all of which are mitochondrially rich tissues (14–16). The human CLFP and CLPX sequences encode putative N-terminal mitochondria targeting signals (16, 17). The size of hClpP detected by immunoblotting of human mitochondria suggests that the mature protein has about 56 amino acids removed from the N terminus of the primary gene product (17), whereas maturation of mouse ClpX involves removal of about 65 amino acids from the N terminus (16). The sequences of the human proteins show extensive similarities to their Escherichia coli counterparts (hClpP, 56% identity and 71% similarity in a 192-amino acid overlap; hClpX, 44% identity and 62% similarity in a 415-amino acid overlap), including conservation of all of the known catalytic residues of the E. coli proteins. Although hClpX and hClpP are expected to interact to form a functional proteolytic complex, no protease or peptidase activity has been reported for isolated hClpP or hClpXP complexes.

A unique feature of bacterial ClpXP (and the analogous ClpAP) complexes is the symmetry mismatch between the ATPase and the protease. Complexes are formed by the interaction of six-membered rings of ClpX and seven-membered rings of ClpP. Although a similar symmetry mismatch apparently exists in the 26 S proteasomes between the six ATPase subunits of the 19 S regulatory particle and the seven α subunits of the 20 S proteasome, such mismatches are not the rule in ATP-dependent proteases; for example, HslUV (ClpYQ) is a symmetrical complex between six-membered rings of both components. Further, no mismatch is possible in ATP-dependent...
dependent proteases such as Lon and FtsH, which are oligomers of single polypeptide chains with independently folding ATPase and protease domains. The mechanistic consequences of symmetry mismatch are not understood, and until now, it has not been known whether it is a universal feature of ClpXP or ClpAP proteases.

Because Clp proteases play important and even essential regulatory roles in many organisms, we have undertaken an investigation of the activity and substrate specificity of the human mitochondrial ClpXP. Our results confirm that symmetry mismatch between ClpX and ClpP is conserved in human ClpXP complexes. We also show that contacts between the two components are conserved well enough to allow complex formation between heterologous components from E. coli and hu-

MATERIALS AND METHODS

Isolation of cDNA Clones for hClpP and DNA Sequencing—A probe was made with an EcoRI fragment of cDNA clone HCCCGT originally reported by Adams et al. (18) to have a sequence similar to E. coli ClpP. This probe was used to screen by filter hybridization a human hippocampal cDNA library (Stratagene). The cDNA inserts from the positive clones were excised by digestion with HindIII and inserted into pVEX11, creating pVEX11hClpP(his). For the hClpX clone obtained from P. Bross (Aarhus University Hospital, Aarhus, Denmark) and for mClpX, a mouse cDNA clone obtained from Dr. S. Santagata (Mt. Sinai School of Medicine, New York, NY), hClpP, the primers were hclpXD64_1, 5'-ACACGCGCGATATGGCCTAAAAGTGGGTTAAGAGTAGTTG-3' and hclpX_1, 5'-CCGCAATATAAGGTTCCTTTGGCC-3', which were amplified with the primers were mclpXD65_I, 5'-CGCGGTTGACATATGGCCTCAAAAG-3' and mclpX_I, 5'-TCGAGGTCCTAAAGGTTCCTTTGGCC-3'. The primers introduced NdeI and HindIII sites at the 5' and 3' ends, respectively, and were designed to encode the full-length ClpP protein starting from codon 65 of the hClpX or codon 66 of the mClpX reading frame. The NdeI/HindIII-digested PCR fragments were inserted between NdeI and HindIII sites of pVEX11, creating pVEX11/hClpX_NA65p/VEX11/hClpX_NA64 and pVEX11/mClpX_NA65.

Purification of hClpX and mClpX—BL21 (DE3) (3) RP cells (Stratagene) with a null induction were grown in pVEX11 and carrying the plasmid pVEX11/hClpX_NA65 or pVEX11/mClpX_NA65 were grown as described above in the presence of 10 μM chloramphenicol and 50 μg/ml ampicillin. Following the 3-h induction, the cells were harvested and suspended in 50 ml of ice-cold binding buffer (50 mM Tris-HCl, pH 8.0, 0.1 M KCl, 10 mM MgCl2, and 10% glycerol). The cells were then disrupted by French press, and the insoluble material was collected by centrifugation. The insoluble material was suspended in 50 ml of binding buffer containing 10 mM MgCl2, and after 1 h of incubation at 4°C, any residual insoluble material was removed by ultracentrifugation. The supernatant protein was refined by stepwise removal of the urea in the presence of 0.5 mM ATP, 2 mM dithiothreitol, and 10 μM ZnCl2. After refolding the protein, any residual insoluble material was removed by ultracentrifugation.

The supernatant was loaded onto a 1.4 × 8-cm hydroxyapatite column (Bio-Rad) equilibrated with 5 mM potassium phosphate buffer (pH 7.5), 10 mM MgCl2, 2 mM dithiothreitol, and 10% glycerol, and eluted with a linear gradient of 0–300 mM potassium phosphate, pH 7.5. The fractions containing hClpX or mClpX were pooled and loaded onto a 0.7 × 2.5-cm Hitrap-Heparin (Amersham Biosciences) equilibrated with 50 mM Tris-HCl buffer, pH 8.0 (50 mM Tris-HCl, pH 8.0, 0.1 M KCl, 10 mM MgCl2, and 10% glycerol). The protein was eluted with a linear gradient of 0–1.0 M NaCl, and the fractions containing mClpX were pooled. The purity was estimated by SDS-PAGE.

Enzymatic and Other Assays—The buffer used for most assays was 50 mM Tris-HCl, pH 8.0, with 0.1 M KCl, 1 mM dithiothreitol, and 0.02% Triton X-100. For nucleotide-dependent reactions, 10 mM MgCl2 and either 1 mM ATP, 4 mM MgATP or 4 mM ATP were included. For the assays for [3H]NTp protein degradation and ATPase activity were described by Grimaud et al. (19) and de Vries et al. (20) and a 4-h incubation at 37°C. The derivatives were described previously (20, 21). The protein concentration was analyzed by the Bradford method (22).

Analytical Gel Filtration—Gel filtration was performed with a 0.32 × 30-cm Superdex 200 column (Amersham Biosciences). The fractions were equilibrated with buffer B with or without 1 mM ATP, 0.1 M Tris-HCl, pH 8.0. The samples were loaded in volumes of 50–100 μl of the same buffer, and the flow rates were 0.08 ml/min. The band was monitored at 280 nm.
near the N terminus of eClpX. just upstream of a sequence of five amino acids identically conserved known. For cloning of hClpX, a start codon was placed in front of Ala-65, eClpX is shown in *italics* a region of homology with eClpX (*eX*), has a putative mitochondrial targeting sequence followed by a continuous sequence of 194 amino acids that overlaps with eClpP (*eP*). The N-terminal sequence of eClpX is shown in *italics*. The N-terminal of mature hClpX is not known. For cloning of hClpX, a start codon was placed in front of Ala-65, just upstream of a sequence of five amino acids identically conserved near the N terminus of eClpX. *mX*, mClpX.

and the fractions were collected at 1-min intervals.

**Electron Microscopy and Image Analysis**—The procedures for specimen sample preparation, recording of electron micrographs, and digital image analysis were previously reported (23). With hClpP protein, concentrations of 50 μM/ml in 50 mM Tris-HCl, pH 7.5, 0.2 mM KCl, 10 mM MgCl₂, and 10% (v/v) glycerol were used. With ClpX, conditions were similar except that 2 mM ATP was added to the buffer. hClpXP complexes were made with 1 mg/ml hClpX and 0.5 mg/ml hClpP in the presence of 2 mM ATP/S, and diluted 1:10 or 1:20 for microscopy. The scanned images were analyzed for symmetry using the rotostat procedure described previously (24). After scanning of the micrograph, correlation averaging of typically 500 particles was performed with PICO-III (25). Images were obtained after further symmetry averaging: 6- or 7-fold rotational symmetry operations for ClpX or ClpP and two 2-fold symmetry operations (top to bottom and left to right) for 2:1 complexes.

**Inactivation of ClpP**—Enzymatically inactive ClpP was prepared by incubating ClpP in 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 10% (v/v) glycerol (v/v). At different times, the reaction was stopped, and the fractions were collected at 1-min intervals.

**RESULTS**

**Purification of Bacterially Expressed hClpP**—Sequence alignment of hClpP with the amino acid sequence of *E. coli* ClpP showed that hClpP possesses an N-terminal extension of about 42 amino acids, and this extension displays properties of typical mitochondrial targeting sequences (14, 26, 27). Based on alignment with mature *E. coli* ClpP (Fig. 1A), we chose Pro-57 between the first negatively charged residue (Glu-64) and the closest upstream arginine residue (Arg-54) for the start site. Our hClpP start site differs from the hClpPs cloned previously, which were either one amino acid shorter (14) or longer (17). Expression of hClpP in *E. coli* produced a band with an apparent molecular mass of 24 kDa on SDS-PAGE, and the expressed protein was found to be soluble. hClpP was purified to over 95% homogeneity (Fig. 2A). N-terminal sequencing confirmed that the first amino acid was proline, indicating processing of the N-terminal methionine in vivo (data not shown). We also expressed and purified a truncated mutant of hClpP, hClpPΔC, which lacks the C-terminal 28 amino acids of hClpP (Fig. 2A).

**Pepcidase Activity of hClpP**—Earlier studies had indicated that purified hClpP or extracts containing expressed hClpP did not degrade short fluorogenic peptides, such as succinyl-Leu-Tyr aminomethylcoumarin (14). This peptide as well as several other fluorogenic and chromogenic peptides were not degraded by our hClpP (Table I), hClpPΔC, nor by any hClpXP complexes (data not shown). However, we found that when pepcidase activity was monitored by direct observation of peptide products separated by reverse phase chromatography, several other peptides could be degraded by hClpP alone (Table I). hClpP degraded oxidized insulin B chain (Table I) as well as the peptide, FAPHMALVPV (cleptide), which is very rapidly degraded by hClpP (22). Insulin B chain was cut primarily at single sites in an apparently nonprocessive manner (data not shown). Cleptide was cut at the same site cleaved by hClpP, and the cleavage rate was stimulated about 20-fold in the presence of hClpX (Table I). Thus, contrary to the implications of previous reports, hClpP has significant pepcidase activity; however, it might not have good amidase activity, or the bulky aromatic group might interfere with positioning the peptide in the substrate-binding site.

**Expression and Purification of Human ClpX**—hClpPX and mClpX genes have been identified and cloned (15, 16), and the encoded proteins have been shown to be located in mitochondria. mClpP from cell extracts was shown to bind to a glutathione S-transferase-mClpX fusion protein immobilized on a GSH affinity column (16), suggesting that ClpP and ClpX interact in mammalian mitochondria. However, in some eukaryotes, such as *Saccharomyces cerevisiae*, only the ClpX chaperone and not ClpP is present (28). To investigate the structure and enzymatic properties of the hClpXP complex, we expressed and purified hClpX. To maximize the alignment with eClpX (Fig. 1B), a start was initiated from codon 65 of hClpX. hClpX was purified from the soluble fraction (Fig. 2B) or from inclusion bodies; structural and enzymatic properties of both forms were similar. Purified mClpX had a somewhat greater tendency to aggregate but otherwise had properties similar to the human protein.

**Assembly of hClpXP Complexes and Heterologous Complexes** of hClpP with mClpX and eClpX—As initially purified, hClpX was in a mixed oligomeric state, but addition of ATPγS pro-
Table I

| Substrate                   | Amount of peptide cleaved |
|-----------------------------|---------------------------|
|                            | hClpP         | eClpP         |
| Succinyl-Leu-Tyr-AMC        | 0             | 2             |
| cleptide                    | 2.5           | 2.6           |
| Insulin B chain             | 1.7           | 2.4           |

**Table II**

hClpXP and eClpXP recognize different protein substrates

All assays contained 4 mM ATP and 10 mM MgCl₂ in standard assay buffer at 37 °C. The enzymes (E. coli or human form as indicated), and substrate proteins were present as follows: 0.1 μM each of ClpX and ClpP with 0.60 μM [3H]O; 0.5 μM each of ClpX and ClpP with 1 μM GFP-SsrA; 0.4 μM each of ClpX and ClpP with 4 μM α-casein; or 1 mM cleptide with 0.4 μM ClpX and 0.1 mM ClpP. The units of activity for protein degradation are μg/min/μg ClpP and for peptidase activity are nmol/min/nmol ClpP tetradecamer.

| Substrate                  | hClpXP | eClpXP | eClpP/hClpP |
|----------------------------|--------|--------|-------------|
| [3H]O protein              | 0      | 0.11   | 0.057 (0.10) |
| GFP-SsrA                   | 0      | 0.050  | 0.044 (0.048) |
| [3H]-Casein                | 0.0017 (0.0026)* | 0 | 0 |
| cleptide                   | 400    | 10,000 | 350         |

* Values in parentheses are activities obtained with hClpPΔC.

a At 1 mM cleptide, activity was ~7000; activity shown was obtained at saturating cleptide (21).

moted formation of a monodisperse species that migrated as a hexamer on a Superdex 200 gel filtration column (Fig. 3A). Electron micrographs of negatively stained hClpX in the presence of ATPγS (Fig. 4A) were analyzed for symmetry, and a strong 6-fold symmetry component was found (Fig. 4H). Averaging of the images showed top views of six-membered rings (Fig. 4C) similar to those observed with eClpX. We also confirmed that our purified hClpP particles had 7-fold symmetry (Fig. 4H) and presented easily discernable seven-membered rings in the averaged images (Fig. 4E). Thus, as reported earlier (17), ClpP assembles into rings of seven subunits. Mixtures of hClpX and hClpP run over a gel filtration column in the presence of ATPγS eluted as a high molecular mass complex (Fig. 3B). Electron micrographs of hClpXP complexes assembled in the presence of ATPγS showed characteristic side views (Fig. 4B), with four parallel striations representing two rings of hClpP in the middle flanked on both sides by a single ring of hClpX (Fig. 4D). Similar complexes were observed between hClpP and mClpP (Fig. 4F), which was expected because the sequence of mClpP is virtually identical (98%) to that of hClpX.

hClpP also formed stable complexes with eClpX, and the complexes were nearly identical in appearance (Fig. 4G). Surprisingly, eClpA did not bind to hClpP by any criteria tested, including gel filtration, electron microscopy, or enzymatic assay (see below). We also could not detect the reverse heterologous complex between hClpX and eClpP by any of these criteria. The formation of hybrid eClpX-hClpP complexes allowed us to compare the activity and specificity of homologous and heterologous complexes.

**Protease Activity of eClpXP:** eClpXP and eClpX Have Different Specificities—When hClpXP complexes were tested for activity against αO protein and GFP-SsrA, protein substrates degraded by eClpXP, no activity was observed (Table II). However, hClpXP was able to degrade α-casein (Fig. 5A), a substrate not degraded by eClpXP. hClpXP also degraded β-casein and κ-casein, neither of which is degraded by eClpXP. κ-Casein degradation shown in Fig. 5A was carried out with hClpXP-hClpPΔC, indicating that the C terminus of hClpP is not required for interaction with hClpX or for enzymatic activity. α-Casein was degraded to acid-soluble products by hClpXP with an S₅₀ of about 0.4 min⁻¹ (calculated as casein monomers degraded per ClpX hexamer). The degradation rates were slightly (20%) faster when hClpPΔC was used.

To test whether ClpXP unfolds or ClpP peptidase activity was rate-limiting, we used a peptide substrate, FAP-MALVPV (cleptide), that is degraded very rapidly by eClpP when activated by either eClpXP (19) or eClpA (21). Cleptide was degraded by hClpXP at about 2% of the rate seen with eClpXP (Table II). With hClpXPΔC, the rate was more than doubled with an estimated turnover number/complex of about 500 min⁻¹. Cleavage of cleptide by hClpXP occurred exclusively between Met and Ala, the same site cleaved by eClpP (22) and hClpP alone. Thus, hClpX can activate both protease and peptidase activity of hClpP. Human and E. coli ClpXs recognize different protein substrates, and hClpX activity is likely to be much higher on its specific substrates.

**Activity of Heterologous Complexes of hClpP with eClpXP—** During gel filtration in the presence of Mg²⁺ and ATPγS, hClpP and eClpX migrated together in a high molecular mass peak (data not shown), and electron microscopy confirmed that the two proteins formed a 2:1 complex with a eClpX hexamer bound at each end of a double-heptameric ring of hClpP (Fig. 4G). We found that the complex of eClpX-hClpP was active on several E. coli ClpXP substrates. Cleavage of cleptide by hClpP was activated ~20-fold in the presence of eClpXP, and when eClpX was saturating, the rate of cleptide cleavage by hClpP was 2–5% that observed with eClpXP (Table II). Thus, the heterologous interaction of eClpX with hClpP, as with eClpP, induces a conformational change that makes the active site accessible to oligopeptides and increases the catalytic efficiency of peptide bond hydrolysis. Interestingly, eClpA was not able to promote cleptide cleavage by hClpP (data not shown), even though cleptide is cleaved by eClpP in the presence of either ClpX or ClpA (20, 22). The lack of activation by eClpA was consistent with our inability to see complexes between eClpA and hClpP under any circumstances (data not shown).

eClpX targets several specific proteins for degradation by...
eClpP in vivo and in vitro, including λO protein and proteins with a C-terminal extension referred to as SsrA (7). The hybrid eClpX-hClpP complex was tested on λO protein and GFP-SsrA in the presence of ATP. The complex of eClpX and hClpP degraded both proteins in the presence of ATP (Fig. 6A and Table II). Degradation was much faster in the presence of ATP, although, as seen with eClpXP, slow degradation was seen when ATPγS was used in place of ATP. hClpP did not show any proteolytic activity in the presence of eClpA (data not shown). Translocation of GFP-SsrA to hClpP was measured with eClpX and proteolytically inactive hClpP(S97A) (see below). The rate of translocation was similar to that observed with the eClpX-eClpP(S97A) complex (Fig. 6B). Thus, the heterologous complex has unfolding, translocating, and peptide cleavage activities similar to those of the homologous E. coli complex.

hClpX Does Not Bind to or Activate eClpP—We tested the ability of hClpX to interact with and activate eClpP. Gel filtration and electron microscopy studies failed to show stable interaction between these proteins. As expected, hClpX did not promote eClpP proteolytic activity against the λO protein or GFP-SsrA, but it also did not promote proteolysis of the caseins.
Insulin B chain was degraded with hClpXP, and the major peptide products (representing about 90% of the original material) were isolated by reverse phase chromatography and sequenced. The arrows above the sequence indicate the sites cleaved. For comparison, the sites cleaved by eClpP (31) are also shown.

Relative Affinities of hClpX and eClpX for hClpP—Cleptide cleavage requires association of ClpX with ClpP but not ATP hydrolysis. By holding the ClpX concentration fixed and varying ClpP, we obtained relative binding affinities between different combinations of the human and E. coli components. eClpX and eClpP had the highest relative affinity (Fig. 7).

Surprisingly, eClpX and hClpX showed similar affinities for hClpP, but both displayed about 1 order of magnitude weaker binding than seen with eClpXP (Fig. 7). Both eClpX and hClpX had higher affinity for hClpPAC than for the intact hClpP protein. The lower activity of hClpP observed in assays is partly due to incomplete saturation with ClpX. As indicated above, no interaction was evident between hClpX and eClpP.

Specificity of Peptide Bond Cleavage by hClpP—As mentioned above, hClpP cleaved cleptide, FAPHMALVPV, only between Met and Ala, the same site cleaved by eClpP (21). This site was cleaved with hClpP alone or when activated by either hClpX or eClpX. To further test the cleavage specificity of hClpP, several variants of cleptide with substitutions in the P-1, P-2, and P-3 positions were used (Table III). These variants are all cleaved exclusively after the methionine by both eClpP and hClpP (data not shown). Although most cleptide variants were cleaved at comparable rates by hClpP and eClpP, hClpP was more sensitive to the presence of a tryptophan residue in either the P-1 or the P-2 position. Also, hClpP did not cleave cleptide with a glycine residue at P-3. The ATPase does not affect the cleavage specificity of ClpP. The rates of cleavage by ClpXP (Table III) are very similar to those previously obtained with ClpAP (21). Also, cleavage rates by hClpP were the same whether hClpX or eClpX was used for activation (data not shown).

With oxidized insulin B chain as a substrate, the peptide products generated by hClpXP and eClpXP were quite similar, but a few significant differences were observed (Fig. 8). hClpP did not cut after Leu-6 but instead gave quantitative cleavage after Gly-8. Thus, hClpP and eClpP have similar specificities in peptide bond cleavage. Cleavage after Gly by both eClpP and hClpP indicates that occupancy of the P-1 binding pocket is not essential and that the peptide binding groove surrounding the active site triad plays the major role in positioning the scissile bond for cleavage.

**Mutation of the Catalytic Active Site Ser-97 of hClpP—**

Alignment of the hClpP and eClpP protein sequences indicates that the active site triad typical of many serine proteases (Ser-97, His-122, and Asp-171) was conserved in hClpP. (For consistency with the mature form of eClpP, numbering of hClpP starts with the N-terminal Ala-1 after processing of the initiator methionine.) Although eClpP is sensitive to reagents that typically target catalytic residues of serine proteases, hClpP is resistant to such inhibitors or is affected in noncanonical residues. No loss of activity of hClpP was observed after incubation with diisopropylfluorophosphosphate either alone or in the presence of hClpX, and no significant radioactivity was incorporated into hClpP after incubation with [3H]diisopropylfluorophosphate for 3 h (data not shown). hClpP was also resistant to phenylmethylsulfonyl fluoride, 4-(2-aminoethyl) benzene sulfonyl fluoride, N-tosyl-l-phenylalanine chloromethyl ketone, N-tosyl-l-lysine chloromethyl ketone, and 3,4-dichloroisocoumarin. The active site-directed inhibitor, N-carbobenzoxy-Leu-Tyr chloromethyl ketone, which inactivates eClpP, did inhibit hClpP; however, the peptide was found linked to residues expected to be outside of the proteolytic active site and not to the putative active site histidine of hClpP.

To confirm that Ser-97 is the catalytic residue of hClpP, site-specific mutagenesis was used to replace this residue with Ala or Cys. To facilitate isolation of mutant ClpPs, a His6 tag was attached to the C terminus of hClpP, and the expressed proteins were purified by metal chelate chromatography.

![Fig. 7. Peptide bonds cleaved by hClpP in model substrates.](image)

**Fig. 8.** Concentration dependence of ClpP activation by hClpX and eClpX. Peptide degradation was measured in the presence of ATPγS and a fixed concentration (0.1 μM) of either hClpX or eClpX. hClpP and eClpP were added at the concentrations shown, and the initial rates of propeptide cleavage were determined (see "Methods and Materials").

| Table III | Cleavage of cleptide variants by hClpXP |
|-----------|--------------------------------------|
| Cleptide derivative | Relative peptidase activitya | Relative peptidase activityb |
| FAPHMALVPV | 1.0 | 0.9 |
| FAPHMALVPV | 0.9 | 0.9 |
| FAPGALVPV | 0.3 | 0.3 |
| FVAPXMALVPV | 0.9 | 1.0 |
| FAPXWMALVPV | 0.6 | 0.04 |
| FAPKXMALVPV | 0.7 | 0.4 |
| FAPXSMALVPV | 0.6 | 0.7 |
| FAPXMALVPV | 0.5 | 0.6 |
| FAPXHLAVPV | 0.1 | 0.1 |
| FAPXHALVPV | 0.08 | 0.0 |
| FAPXHALVPV | 0.0 | 0.0 |

* The reported rates were normalized to the maximum rate observed (see Table II).

* With ClpAP, FAIHMALVPV was cleaved faster (relative rate of 1.6), and FAPEMALVPV was cleaved slower (relative rate of 0.2) (21). Cleptides with X = Glu, Tyr, Lys, Ser, or Ile.

S. G. Kang and M. R. Maurizi, manuscript in preparation.
radiation of cleptide and AO by purified wild type hClpP-His$_6$ was similar to hClpP (Table III), and stable interaction of hClpP-His$_6$ with eClpX was demonstrated by gel filtration (data not shown). Purified hClpP-S97A and hClpP-S97C were assayed in the presence of hClpX with i-aceasine as substrate and in the presence of eClpX with i-AO and propeptide as substrates. The mutant hClpP$_s$ were inactive on all substrates (data not shown). Proteolytically inactive eClpP acts as a trap for GFP-SsrA, which is unfolded and translocated into the aqueous chamber of eClpP by eClpX (29). In the presence of eClpX, hClpP-S97A also trapped but did not degrade GFP-SsrA (Fig. 6B), indicating that the mutant protein forms a functional complex with eClpX by lacks proteolytic activity. These data suggest that hClpP is a serine protease and that Ser-97 is the catalytic residue. An explanation for the low reactivity of the active site residues in hClpP must await further studies.

**DISCUSSION**

The chaperone and proteolytic functions of Clp proteases are highly conserved and, in many organisms, perform essential functions necessary for cell growth or for adaptation to environmental stress or changes in nutritional conditions. Single genes have been identified for human ClpX on chromosome 15q22.1 (15) and for human ClpP on chromosome 19 (30). Both proteins appear to be targeted exclusively to mitochondria. Our data establish that mammalian ClpX and ClpP combine to form a functional ATP-dependent protease that has structural and enzymatic properties similar to the well characterized bacterial ClpXP protease. ClpX appears to be the only Clp ATPase present in mammals. Coding regions with homology to subdomains of ClpA are present, but the human genome does not have a homolog containing all of the functional domains of ClpA.

Two differences in enzymatic properties stand out between hClpXP and eClpXP. First, the peptidase and protease activities of purified hClpXP are only 1–5% of those of eClpXP. We do not have a complete explanation for the low activity. The low activity is also reflected in the poor reactivity with active site-directed inhibitors. Inhibitors such as diisopropylfluorophosphate and peptide chloromethyl ketones generally require an activated nucleophile for rapid covalent modification, so it appears that even in a complex with hClpX, the active site residues of hClpP are not all in an activated state. It is possible that hClpX blocks access of these reagents to the active site of hClpP, but given the number of inhibitors tried, this seems unlikely. Moreover, eClpP also has unusual kinetics of reaction with diisopropylfluorophosphate (31), despite the indication from the crystal structure that all of the active sites of eClpP are in the same “active” conformation (32). Coordinated activity of all 14 active sites of ClpP might depend on the state of the complex with ClpX or on the presence of substrate or other effectors. These effects could be less pronounced in the bacterial enzyme where the peptide products are of less consequence but may play a role in determining the nature of the peptide products produced by the mammalian ClpXP. Although physical properties suggest that our purified hClpP and hClpX are both well folded, we are also planning to isolate hClpXP expressed in eukaryotic cells to see whether maturation and folding under different conditions affect the enzymatic properties.

The second major difference in enzymatic properties is that hClpX and eClpX recognize different substrates. Substrate selection by Clp proteases is dependent on the Clp ATPase. In *E. coli*, ClpA and ClpX target different proteins for degradation both in vivo and in vitro, although ClpA also has a weak ability to degrade most ClpX substrates. In vivo, specificity is sufficiently stringent that increased stability of target proteins caused by mutations in ClpX confers distinct phenotypes on the cell. Few studies of heterologous complexes of ClpX and ClpP from different organisms have been reported. We show here that eClpX can recognize its specific substrates even in the context of a heterologous complex with hClpP and moreover can efficiently target those substrates to hClpP. Human ClpX, however, does not recognize the same substrates recognized by eClpX, although it can target other specific proteins to hClpP for degradation. Substrate recognition appears not to be dependent on a homologous ClpX-ClpP interaction and not to be specific for the surface or substrate channel of a particular ClpP. If ClpP does influence the rate or specificity of degradation, the effect should be mediated through surface features conserved between *E. coli* and human ClpP.

The specificity of hClpX could in part underlie the low activity observed in vitro; perhaps the substrates used in our studies are simply not optimal ones for hClpXP. In fact, the heterologous complex, eClpX-hClpP, degrades iO protein and GFP-SsrA about as fast as eClpXP and without accumulation of intermediates, indicating that when substrates are recognized by the ATPase component and presented efficiently to hClpP, they can be rapidly degraded. Identifying physiological substrates and particular sequence or structural motifs recognized by hClpX should help in characterization of the enzymatic properties of hClpXP.

hClpP and eClpP also differ somewhat in determinants for positioning the substrate scissile bond for cleavage within the active site. Although there is considerable overlap in the peptide bonds cleaved in model peptide substrates by hClpP and eClpP, the role of the subsites, particularly P-3 and P-2', appear to differ. Human ClpP may require an aliphatic side chain at the P-3 and P-2' positions, because a glycine at P-3 drastically reduced the rate of cleavage of a model peptide, and a glycine at P-2' decreased cleavage at position 6 of insulin B chain. The crystal structure of eClpP suggests that the peptidase binding pocket accommodates an extended chain that interacts with several subsites to place the scissile bond in position to be cleaved. We are currently analyzing the crystal structure of hClpP to compare the substrate-binding cleft with that found in eClpP. It will also be important to determine whether the difference in peptide bond cleavage by hClpP has a physiological role. The difference could simply reflect the composition of the most common substrates cleaved in mammalian mitochondria in contrast to those in *E. coli*. Alternatively, the differences could serve to optimize peptide products for their subsequent metabolism. For example, the peptide products of hClpP might be better suited for presentation of mitochondrial antigens. The length or composition of peptide products could influence whether they are degraded within the mitochondria or transported out of the matrix by the oligopeptide transporter found in the inner membrane (33).

eClpX and eClpA interact equally well with eClpP, but only eClpP can bind to hClpP. eClpA did not activate or show significant affinity for hClpP. The inverse heterologous complex, hClpX-eClpP, cannot be formed under our experimental conditions. These data indicate that the mechanism of binding between Clp ATPases and ClpP may involve more than one region of Clp ATPase. All of the Clp ATPases that interact with ClpP have a conserved motif, IG(L/F), found in a region of otherwise variable length and sequence composition in the D2 ATPase domain near the junction with the small domain (34, 35). In ClpA, this motif lies in a loop located on the surface that interacts with ClpP. 4 The IGL motif in hClpX is in a somewhat longer connecting region than in eClpX, possibly leading to more flexibility and weakened affinity for ClpP. In fact, the

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4 F. Guo, M. R. Maurizi, L. Esser, and D. Xia, unpublished data.
homologous interaction of hClpX with hClpP is also weaker than that between eClpX-eClpP. The inability of eClpA to bind to hClpP suggests either that there is another element involved in these interactions or that some steric interference occurs between eClpA and hClpP that is absent in the eClpX-hClpP interface. Alignments between the two proteins indicate that there are numerous differences in the surface residues in hClpP and eClpP, as well as a long C-terminal extension of 28 amino acids in hClpP. Because the surfaces of both ClpP and ClpA have numerous charged residues, it is possible that they also contribute to binding interaction during complex formation.

We are currently analyzing the contributions of these residues to stability and activity of complexes between Clp ATPases and the ClpP protease.

The high degree of conservation and the retention of chaperone and proteolytic activity of human ClpXP point to a potential role for these proteins in protein quality control and in the regulation of regulatory protein levels within mitochondria. Although no physiological substrates or in vivo functions of hClpXP have been identified, other ATP-dependent proteases play a critical role in mitochondrial functions. In yeast, the matrix Lon protease (36, 37) and the membrane-associated AAA proteases, Yme1 and Yta10/12 (38), are required for assembly of membrane complexes in mitochondria, as well as functioning to remove misfolded proteins. Lon protease also has a regulatory role affecting messenger RNA splicing (39). Protease defects may underlie certain human diseases, such as spastic paraplegia, in which the primary lesion is in the gene for a homolog of the yeast mitochondrial membrane AAA protease (40). In plant chloroplasts, ClpP has been shown to be important for degradation of the partly assembled cytochrome b6f complex (41), and attenuation of ClpP activity resulted in a decreased ability to adapt to elevated levels of CO2. These examples suggest that ClpXP is likely to have important functions in human mitochondria as well.

Considerable attention has also been paid to the role of mitochondria not only as a major metabolic organelle but as a source for critical signaling pathways that lead to apoptosis and cell death. Import and steady state control of regulatory proteins and mitochondrial enzymes thus are profoundly important to the cell. We expect that the purified ClpX and ClpP proteins will be useful for identifying interacting proteins in mitochondrial extracts, providing useful leads to identify physiological roles for human ClpXP.

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