The Escherichia coli chaperones, ClpA and ClpX, belong to the AAA+ superfamily of ATPases, which are involved in a variety of cellular functions (1). Many AAA+ proteins mediate non-covalent interactions between macromolecules and participate in such processes as DNA replication, vesicle transport and the secretory pathway, ATP-dependent proteolysis, and many others. Structural domains within the AAA+ family have been identified by primary sequence and predicted secondary structure alignments with the δ′ subunit of DNA polymerase III and the N-ethyl maleimide sensitive factor (NSF) whose structures have been solved at atomic resolution (2–4). Recently, the structure of HslU (ClpY), which is related to ClpX and ClpA, was solved (5, 6). AAA+ proteins have multiple domains. The core region is the ATPase domain itself, which contains an α-β-α-sandwich seen in nucleotide-binding P-loop proteins. An auxiliary component attached on the C-terminal side of the ATPase domain is a mostly helical region variously referred to as the sensor-2 (1) or “sensor and substrate discrimination” domain (7). This C domain contacts its own core ATPase domain and that of the adjacent subunit and is involved in nucleotide binding and hexamer formation in NSF (3, 4) and HslU (5). Proteins such as NSF, p97, ClpA, and ClpB (Hsp104) have two extended (core plus C domain) ATPase domains in tandem, whereas ClpX and ClpY have only one (3, 4, 8–10). A signature motif for Clp ATPases consisting of the triads PE(F/L) and GAR separated by about 59 amino acids is found in the C domain of ClpX and ClpY as well as in the second ATPase domain of ClpA and ClpB (9). A third independently folded domain in AAA+ proteins is formed either by an extension at the N terminus, as in NSF, ClpA, and ClpX, or by insertions in loops within the core ATPase domain (1). In HslU, this third domain is referred to as the intermediate (I) domain. It is located between the equivalent of α3 and β3 in NSF and is attached to the distal ring surface (5, 6, 11).

Many AAA+ proteins interact with functional partners to form complex molecular machines (1). In the case of ClpAP and ClpXP proteases, the ATPases interact with ClpP and catalyze ATP-dependent degradation of specific proteins (12). ClpA and ClpX have autonomous chaperone activity and can catalyze protein unfolding (13–16). The unfolding activity is thought to be important to disrupt the structure of proteins to facilitate their translocation into the proteolytic chamber of ClpP. The proteolytic active sites of ClpP are located in an internal aqueous chamber formed by the conjunction of two concave heptamer rings, and access is obtained by narrow axial channels that can accommodate polypeptides only in an extended conformation (17, 18). ClpA and ClpX associate into hexagonal cylindrical particles in the presence of nucleotide and bind to both external faces of ClpP where they are in a position to interact with protein substrates (18–21). One face of the cylinder makes contact with ClpP, and we refer to it as the protease interface (PI) surface. The outer face of the cylinder is the site of initial substrate binding (21, 22), and we will call it the substrate interface (SI) surface.

In this study, we have used limited proteolysis to investigate

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Functional Domains of the ClpA and ClpX Molecular Chaperones Identified by Limited Proteolysis and Deletion Analysis*

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The abbreviations used are: NSF, N-ethyl maleimide sensitive factor; C domain; C-terminal domain; N domain, N-terminal domain; I domain, intermediate domain; PI, protease interface; SI, substrate interface; PAGe, polyacrylamide gel electrophoresis; Lys-C, lysylendopeptidase; TLCK, 1-chloro-3-tosylamido-7-aminom-2-heptanone.

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changes in the conformation of ClpX and ClpA in response to nucleotide. In addition, we have used these data to deduce the domain organization of ClpA and ClpX and the interaction of different domains with ClpP. ATP binding is needed for assembly of ClpA and ClpX hexamers and for association of ClpA and ClpX with ClpP (19, 20, 23). ATP hydrolysis is essential for both chaperone activity and for protein degradation (13, 16, 19, 24, 25). ClpAP and ClpXP complexes are dynamic in response to nucleotide exchange or hydrolysis, although, in the presence of physiological concentrations of ATP, the complexes are stable and remain associated through multiple rounds of protein binding and degradation (26). Significant conformational changes in ClpA and ClpX are expected to unfold the protein and thread into the proteolytic core.

The domain organization and contacts between components are known for only a few AAA + proteins. This information is essential for understanding the functional interactions between the parts of the complex and the overall mechanism of action. In Clp proteases, the portions of the polypeptide that contribute to ClpP or substrate binding are not known. By analogy with HslUVD (ClpYQ), the N-terminal domains of ClpA and ClpX are expected to be located in a similar position as are the I domains, extending out from the surface distal to the protease (6, 11). Here we show a region in ClpA and ClpX that is exposed to exogenous proteases and can be protected by interaction with ClpP. This region may contain one of the interaction sites for assembly of ClpAP and ClpXP complexes. We also show that the N-terminal domains of ClpX and ClpA are not required for interaction with ClpP and thus are likely to be positioned on the distal, SI surface in ClpAP and ClpXP complexes.

EXPERIMENTAL PROCEDURES

Reagents—Adenosine 5’-(thiotriphosphate) was obtained from Roche Molecular Biochemicals. ATP, diisopropyl fluorophosphate, tosyl lysine chloromethyl ketone, Staphylococcus aureus V8 protease, trypsin, and chymotrypsin were obtained from Sigma. Lysylendopeptidase C was obtained from Wako Scientific. Reagents for SDS-PAGE were obtained from Bio-Rad.

Protein Purification and Assay—ClpA (27), ClpX (19), and ClpP (27) were purified as described. Assays for ATPase of ClpA (27) and ClpX (19) as well as for ClpAP (27) and ClpXP (19) protease activity were described previously. ClpAΔI53 was purified from overexpressing by the same procedure used for wild-type ClpA (27). The coding region for ClpAΔI53 was amplified by the polymerase chain reaction using oligonucleotide primers, CTAGC GCTAGC AGG AGA TAT A CAT ATG GCC AGC CAG CCA AAC AGC GAA GAA CAA GC and ACCTAG GCTAGC GA TTA ATG CGC TGC TTC CGC CTT GTG C. The polymerase chain reaction product included an NdeI site, codons 154–786 of ClpA in frame with the initiator methionine, and an in-frame stop codon followed by a SpHI site. The amplified DNA was cloned into the expression plasmid, pBAD33, modified by insertion of a ribosomal binding site and restriction sites for NdeI and SpHI.

Conditions for Limited Digestion—ClpA (10 µg) was incubated in 50 mM Tris/HCl, pH 7.5, 0.2 mM KCl, 25 mM MgCl2, and 10% glycerol (digestion buffer). ATP (4–8 mM) or ATPγS (1–2 mM) was included as desired. When ClpA was added, it was present to give a 1:4 molar excess of ClpP tetradecamers over ClpA hexamers, e.g. 5 µg of ClpA with 10 µg of ClpP. The final reaction volume was 50 µl. Aliquots of 10 µl were withdrawn and immediately added to 10 µl of 2X SDS sample buffer sitting in a boiling water bath. The entire sample was loaded in a lane, and proteins were separated on a 12% polyacrylamide gel. Protein bands were stained with Coomassie Blue. ClpX digestion was performed under similar conditions. To isolate digestion products under non-denaturing conditions after trypsin or lysylendopeptidase treatment, reaction mixtures were stopped with 1 mM tosyl-lysine chloromethyl ketone and 100 mM EDTA. Particles were extracted using X3D (28), aligned, and averaged as described (29). The resulting averages were subjected to further 6-fold and 4-fold (top-bottom and left-right) averaging for top views and side views, respectively.

Sequence Analysis—Secondary structure predictions in ClpA and ClpX were obtained through a web site employing the program Predict Protein (dodo.epmc.columbia.edu/predictprotein/predictprotein.html) (30).

RESULTS

Predicted Secondary Structure and Domain Organization of ClpA and ClpX—Multiple sequence alignments of >25 ClpAs and ClpBs and >20 ClpXs allowed prediction of the distribution of secondary structural elements (data not shown; cf. Fig. 8). The predictions agree quite well with the known structures of the ATPase domains of other AAA proteins, such as HslU, and with preliminary structural data for both the N terminus and ATPase domains of ClpA obtained by x-ray crystallography.2 In addition to the nucleotide fold, notable features include a helix-rich N-terminal domain connected to the ATPase domain by a flexible loop in ClpA; the N domain of ClpX has a putative zinc binding motif but is otherwise relatively featureless. A sensor-2 or sensor and substrate discrimination domain with 3–4 helices followed by 2 β strands is associated with the ATPase core in ClpX and in both domains of ClpA. Finally, a variable length region interrupting a motif referred to as the second region of homology in AAA proteins (1) is located C-terminal to the Walker B motif in ClpX and ClpA domain II. This region contains a “ClpP-loop” implicated in ClpP-mediated reactions (31) and, as shown below, may directly interact with ClpP.

The N-terminal Region of ClpA Is More Stable to Proteolysis—ClpA without protective ligands is rapidly cleaved by trypsin, chymotrypsin, V8 protease, and Lys-C (data for Lys-C are shown in Fig. 1A). The products obtained were separated on SDS gels, blotted to membranes, and N-terminally sequenced (Table I). Transient intermediates that appeared resulted from internal cleavage at many sites: Tyr-388 (35 kDa), Asp-400 (40 kDa), Glu-438 (21 kDa), Leu-449 (28 kDa), Lys-450 (50 kDa), Glu-631 (14 kDa), Glu-639 (13 kDa), and Met-704 (6 kDa). The most prominent cleavage products were a ladder of N-terminal fragments, and the most stable product invariably retained the original N terminus (cf. Fig. 1A). The size of the final product varied with the protease used: 41 kDa with V8 protease, 25 kDa with Lys-C, and approximately equal amounts of 20 and 13 kDa N-terminal products with trypsin or chymotrypsin, respectively (data not shown). The N-terminal 150 amino acids of ClpA are relatively rich in Glu (18), Arg (12), and aromatic (eight) residues, and therefore its resistance to digestion indicates that the N terminus is a stably folded structure. Secondary structure predictions indicate that the ClpA N terminus contains a tandem duplication of a domain containing four α-helices (cf. Fig. 8), a feature also noted in another recent study (32). In separate experiments, the ClpA-N terminal 153 amino acids was expressed and found to be a soluble, well folded protein, which behaved as a monomer during gel filtration. 3

2 D. Xia, F. Guo, and M. R. Maurizi, manuscript in preparation.

3 S. K. Singh, P. Guo, and M. R. Maurizi, unpublished data.
other ClpA site cleaved in the presence of ATPγS is also unique in different Clp ATPases. This site lies in a loop of variable size found in the C-terminal part of the ATPase core between the Walker B motif and the Clp signature motif, PEF. By alignment with the known structure of ClpY (HalU), this loop is expected to be on the outside surface of the ClpA domain II ring, away from the junction between the two ATPase domains. Leu-619 is part of a conserved triad, IGF(L/F), found in other Clp ATPases that interact with ClpP (Ref. 31, and below).

ClpP Protects the C-terminal Portion of ClpA—To determine whether either cut site lies in regions that interact with ClpP, digestion of ClpA-ATPγS was carried out with ClpP present. ClpA was cleaved by Lys-C at Lys-416 but not at Lys-615 (Fig. 1D). ClpP itself was resistant to degradation during extended incubations. ClpP also prevented cleavage by trypsin, chymotrypsin, and V8 protease in the vicinity of Lys-615 (Fig. 2). Thus, ClpP either interacts with this portion of ClpA, blocks access to it, or induces a conformational change that stabilizes this region. ATPγS-stabilized complexes were resistant to further digestion for at least 1 h after cleavage at Lys-416 had occurred (data not shown). ClpP also reduced the rate of Lys-C digestion of ClpA in the presence of ATP. Although eventually all of the ClpA was degraded with ATP present, the products of limited cleavage at Lys-416 were observed early on (data not shown). ClpP did not prevent the slow cleavage at Tyr-122 and Phe-137 by chymotrypsin (Fig. 2), suggesting that ClpP does interact with the N-terminal domain of ClpP.

Nucleotide-induced Protection of ClpA Reflects Both Local Conformational Changes and Assembly—Stable assembly of ClpA requires the continuous presence of ATP. We wanted to know if the protection from proteases afforded by ATP or ATPγS binding was due to local conformational changes or to subunit interactions within the hexamer, and we wanted to compare the conformational effects produced by ATP and ATPγS binding. To address these issues, we digested wild-type and mutant ClpA proteins in the presence or absence of ATP, ATPγS, or ADP.

First, we compared two mutants that cannot form hexamers. ClpA-K220Q, with a single mutation in the ATP-binding consensus sequence in domain I, cannot assemble and expresses no activity (25) although its domain II site is wild type and can bind nucleotide. ClpA-K220Q/K501Q, with mutations in the ATP-binding consensus in both domains, has no activity and does not bind nucleotide (25). ATPγS partially protected ClpA-K220Q from Lys-C (Fig. 3A), whereas it did not protect ClpA-K220Q/K501Q (Fig. 3B). Thus, nucleotide binding to domain II, without hexamer formation, suffices to promote conformational stability in ClpA. However, with ATPγS, ClpA-K220Q was degraded more rapidly and extensively than wild-type ClpA (cf. Figs. 1C and 3A), suggesting that nucleotide binding to domain I provides additional protection. ClpP had no protective effect with either mutant (Fig. 3, A and B). In contrast to wild-type ClpA, ClpA-K220Q was protected to the same extent by ATP and ATPγS, indicating that, in the absence of ATP hydrolysis, both nucleotides induce similar conformations. In fact, ATP afforded better protection of ClpA-K220Q than of wild-type ClpA, emphasizing the dynamic state of the wild-type protein produced by ATP hydrolysis.

Next, we tested domain I mutants that are able to form hexamers. These mutants express ATPase and proteolytic activity, but the hexamers are less stable (25). ClpA-K220V (Fig. 3C) and ClpA-K220R (data not shown) were both protected by ATPγS, and ClpP provided additional protection as seen with wild type. With ATP, cleavage was faster for the mutants than

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4 S. K. Singh and M. R. Maurizi, unpublished data.
TABLE I

Cleavage sites in ClpA and ClpX

ClpA or ClpX was incubated at 37 °C with 5% by weight of the protease in buffer B containing 25 mM MgCl₂ and 2 mM ATPγS and a molar excess of ClpP where indicated. The products generated within 5 min without nucleotide or after 1 h with nucleotide were separated by SDS-PAGE, blotted, and sequenced to identify the cleavage sites (see "Experimental Procedures.") ND, not determined.

| Clp + addition      | Lys-C  | Trypsin | Chymotrypsin | V8 protease |
|---------------------|--------|---------|--------------|-------------|
| no nucleotide       | Lys-220*| Lys-220*| Tyr-388*     | Asp-400*    |
| + ATPγS             | Lys-416*| Lys-615*| Leu-619*     | Glu-613*    |
| + ATPγS + ClpP      | Lys-416*| trace   | trace        | trace       |
| no nucleotide       | Lys-6*  | ND      | ND           | ND          |
| + ATPγS             | Lys-6*  | Arg-61* | His-58*      | Glu-53*     |
| + ATPγS + ClpP      | Lys-275*| Lys-275*| Phe-270      | Glu-53*     |

*a Cleavage sites were determined by N-terminal sequencing of the products. Not listed in the Table are numerous N-terminal fragments of ClpA obtained in the absence of nucleotide; the sizes were: for chymotrypsin, 78, 50, 43 (major), 21 (major), and 12 kDa; for V8, 68, 48, 43, and 41 (major) kDa; and for KC and trypsin, 68, 50, 42 (major), and 25 (major) kDa.

*b Minor cleavage (< 15%) occurred at these sites after 40 min.

*c Several N-terminal fragments (starting at Asp-7) were obtained with sizes 31, 27, and 24 kDa.

*d Indicates significant accumulation (60–90%) of fragment following cleavage at this site.

Fig. 2. Schematic diagram of cleavage sites in ClpA and ClpX. ClpA and ClpX linear sequences are shown as elongated blocks and separated into “domains” defined from sequence alignments and secondary structure predictions. For ClpA the domains are: N domain (1–169); AAA-1 (170–350); C domain (C-I); a helix-rich region related to sensor-2 domains of AAA proteins (351–433); AAA-2 (434–637); and C domain (302–424). For ClpX, the domains are: N domain (1–61); AAA (62–301); and C domain (302–424). The major sites of cleavage by trypsin (red), chymotrypsin (orange), V8 protease (lavender), and protease Lys-C (brown) are shown by the arrowheads. The sequences in the loops in ClpA and ClpX protected by ClpP are shown. A three-amino acid motif found in Clp ATPases that interacts with ClpP is highlighted. (Alignments are shown in reference 31.) Also shown is the sequence in the basic loop in the middle of ClpX cleaved by Lys-C. This latter site corresponds to the point of insertion of a large 120-amino acid domain in ClpB/Hsp104 orthologs and smaller (50–70 amino acid) insertions in Clp ATPases from plants.

for wild-type ClpA, especially for K220R (data not shown).

We then tested the protective effect of ATP on domain II mutants of ClpA, which form stable hexamers and hydrolyze ATP at 5–10% of the rate seen with wild-type ClpA (25). Cleavage of ClpA-K501R and ClpA-K501Q in the presence of ATP resembled cleavage in the absence of ATPγS (Fig. 3D). Electron microscopy of ClpA cleaved with trypsin showed
most of the ClpA in normal ring-like particles (data not shown). Of particular note, the C-terminal region following Lys-615 remained with the ClpA hexamer. These results are consistent with crystal structure data for other AAA-\(^+\) proteins, such as HslU (ClpY), which show that C-terminal sensor-2 domains are directly involved in inter-subunit contacts that stabilize the hexameric state. When ClpP was added to trypsin-cleaved ClpA, no complexes were seen by electron microscopy (data not shown), and there was no change in the elution position of ClpP or ClpA during gel filtration (Fig. 4B). Thus, cleavage at that Lys-615 alone is sufficient to prevent or weaken binding to ClpP.

About 35\% of the ATPase activity was retained after cleavage at Lys-615 in the presence of ATP\(\gamma\)S, whereas activity was almost completely lost when cleavage occurred in the presence of ATP\(\gamma\)S (Table 1). These results suggest that loss of activity may be due partly to dissociation of the subunits during digestion in ATP. ClpA cleaved at Lys-615 could no longer activate ClpP for casein degradation; however, about 15–20\% of propeptide cleavage activity remained (Table 1). Thus, after cleavage at Lys-615, ClpA can interact weakly with ClpP and allosterically activate peptidase activity, but that interaction is not sufficient to allow translocation of protein substrates or to allow isolation of complexes by gel filtration. Cutting of ClpA at Leu-619 completely inactivated ClpA for activation of propeptide degradation, indicating that the site near Leu-619 is important for interacting with and activating ClpP.

**Structure and Interactions of ClpA without Its N-terminal Domain**—Earlier studies had shown that a ClpA lacking the N-terminal 168 amino acids formed an oligomer in the presence of nucleotide, but had lost \(>90\%\) of its ATPase activity and was not able to promote casein degradation by ClpP (33). To determine the influence of the N-terminal domain on assembly of ClpA hexamers and on interaction between ClpA and ClpP, we cloned and purified ClpA\(\Delta\)N153, and the protein alone or mixed with ClpP was run over a gel filtration column in the presence of ATP\(\gamma\)S. ClpA\(\Delta\)N153 ran in the position expected for a hexamer (data not shown). With ClpP, both proteins eluted near the position expected for a 2:1 ClpA:ClpP complex (data not shown). ClpA\(\Delta\)N153 and the ClpA\(\Delta\)N153:ClpP were isolated by gel filtration and were examined by electron microscopy. ClpA hexamers formed disc-shaped particles with the same diameter as wild-type ClpA and an apparent 6-fold symmetry (Fig. 5A). After addition of ClpP, complexes were formed that had the same appearance as wild-type ClpA and an apparent 6-fold symmetry (Fig. 5B). Similar results were obtained with ClpA\(\Delta\)N162 (data not shown), another N-terminally deleted ClpA protein (32). Thus, the N-terminus of ClpA is not required for tight interaction between ClpA and ClpP.

**ClpA\(\Delta\)N153 Has ATPase Activity and Activates ClpP Peptidase Activity**—In contrast to properties reported for ClpA\(\Delta\)168 (33), ClpA\(\Delta\)N153 had ATPase activity similar to that of wild-type ClpA (Table 1). The ability to unfold proteins and activate ClpP proteolytic activity depended on the substrate used, but was between 50 and 100\% of wild type (32). ClpA\(\Delta\)N153:ClpP also degraded the 10-amino acid propeptide, FAPHMALVPV, which is the most rapidly degraded substrate of ClpAP (\(k_{\text{cat}} \approx 10,000\) min\(^{-1}\)) at wild-type levels. Thus, the N-terminal region is not essential for enzymatic activity of ClpA.

**Limited Proteolysis of ClpX Divides It into Three Identifiable Domains**—ClpX was cleaved with trypsin, V8 protease, chymo-
The presence of ATP...the N domain fragment by gel filtration (data not shown).

were obtained by digesting assembled ClpXP and separating the complex from the N domain fragment by gel filtration (fraction 15 from the column shown in Fig. 7, A and B), and imaged. Inset, averaged image (n = 1000, r = 24 Å). D, ClpXΔN-ClpP. ClpP was added to isolated ClpXΔN in the presence of ATPγS. Inset, averaged image (n = 1888, r = 26 Å). Identical images were obtained by digesting assembled ClpXP and separating the complex from the N domain fragment by gel filtration (data not shown). Scale bar in micrograph is 250 Å and in the inset, 100 Å. n is the number of averaging operations, i.e. the number of particles times the order of symmetry; r is the resolution.

Fig. 5. Electron microscopy of complexes made with ClpA and ClpX lacking their N-terminal domains. Proteins were applied to grids, and micrographs of the negatively stained particles were recorded as described under "Experimental Procedures." The fields show representative images of particles indicating that ClpAΔ153 and ClpXΔN formed intact oligomers (A, C) and were able to complex with ClpP (B, D). A, ClpAΔ153 was purified over a gel filtration column in the presence of ATPγS (see Fig. 6A). The protein ran as a hexamer, and an aliquot from the peak fraction was used for microscopy. Inset, averaged image (n = 1200, r = 35 Å). B, ClpAΔ153-ClpP. ClpAΔ153 was mixed in 2:1 molar ratio with ClpP in the presence of ATPγS prior to electron microscopy. Inset, averaged image (n = 400, r = 29 Å). C, proteolytically generated ClpXΔN. An aliquot of the ClpX was cleaved with V8 in the presence of ATPγS, separated from its N-terminal domain by gel filtration (fraction 15 from the column shown in Fig. 7, A and B), and imaged. Inset, averaged image (n = 1000, r = 24 Å). D, ClpXΔN-ClpP. ClpP was added to isolated ClpXΔN in the presence of ATPγS. Inset, averaged image (n = 1888, r = 26 Å). Identical images were obtained by digesting assembled ClpXP and separating the complex from the N domain fragment by gel filtration (data not shown). Scale bar in micrograph is 250 Å and in the inset, 100 Å. n is the number of averaging operations, i.e. the number of particles times the order of symmetry; r is the resolution.

Fig. 6. Cleavage of ClpX by V8 protease. ClpX (10 µg) was incubated in digestion buffer with 0.5 μg V8 protease in buffer B, 0.2 M KCl, 10 mM MgCl₂, and 10% glycerol with the additions noted. Digestion products were detected following SDS-PAGE as described in Fig. 1: A, + 4 mM ATP; B, + 2 mM ATPγS; C, + 2 mM ATPγS and 10 µM ClpP. The times of incubation were 0, 5, 10, and 20 min in each case.

trypsin, and Lys-C in the presence and absence of ATP, ATPγS, and ClpP. Both ATP and ATPγS limited cleavage of ClpX by trypsin to two sites: the first after Arg-61 and the second after Lys-275 (Fig. 6 and Fig. 2). Others proteases cut near one or both of these sites; Lys-C cut only after Lys-275 and chymotrypsin cleaved at the nearby residue, Phe-270 (Figs. 5 and 2). V8 protease cut only after Glu-54 and chymotrypsin cut very slowly after His-56 (Fig. 2). There was little difference between the protective effects of ATP and ATPγS, perhaps reflecting a lesser tendency for ClpX hexamers to dissociate in the presence of ATP. Glu-54, His-58, and Arg-61 fall in a region separating an N-terminal cysteine-rich domain from the conserved core of the ATPase domain (Fig. 2). Phe-270 and Lys-275, on the other hand, are predicted to be in a loop preceding the C-terminal helix-rich domain, comparable with the loop containing Lys-615 in ClpA. ClpP blocked cleavage at Phe-270 and Lys-275, whereas the N-terminal sites, Glu-54, His-58, and Arg-61 were not protected (Fig. 2). Thus, as seen with ClpA, ClpP binding affects the conformation of ClpX in the C-terminal loop preceding the helix-rich domain but does not interact with the N-terminal sites.

ClpX Cleaved at Lys-275 or Phe-270 Does Not Activate ClpP—ClpP was cut by Lys-C and run over a gel filtration column. As seen with ClpA, the C-terminal domain (276–424) remained associated with the ATPase domain, and the hexamer remained associated (data not shown). ClpP did not co-migrate with Lys-275-cleaved ClpX upon gel filtration in the presence of ATPγS, and no ClpX complexes were detected by electron microscopy although individual rings of ClpP and cleaved ClpX were seen in abundance (data not shown). ClpX cut at Lys-275 or Phe-270 retained about 25% ATPase activity but was no longer able to activate degradation of λO protein or other protein substrates (Table II). λO protein binding appeared to be nearly normal (data not shown). ClpX cut at Lys-275 or Phe-270 lost virtually all activity in the propeptide cleavage assay, indicating that if transient interaction with ClpP occur they are not sufficient to activate ClpP. Thus, the loop containing Phe-270 and Lys-275 of ClpX is important for stable interactions with ClpP and activation of its proteolytic and peptidase activities.

Structure of ClpX with Its N-terminal Domain Removed—Quite different results were obtained when the effects of cleavage at the N-terminal site of ClpX were examined. After treating ClpX with V8 protease, the N-terminal 54-amino acid domain separated completely from the main peak of ClpX and ran with an apparent molecular weight expected for a monomer

Table II

| Clp ATPase | Activity* | Protein degradation | Propeptide cleavage |
|------------|-----------|---------------------|---------------------|
| ClpA       |           |                     |                     |
| control    | 100       | 100                 | 100                 |
| cut at Lys-615 + ATPγS | 35 ± 5 | <1          | 15 ± 5             |
| cut at K615 + ATP  | <5   | <1          | ND                 |
| cut at Leu-619 | ND | ND         | <1                 |
| ClpAΔ153   | >100      | 50 ± 5             | <100                |
| ClpX       |           |                     |                     |
| control    | 100       | 100                 | 100                 |
| cut at Lys-275 | 25 ± 1 | <1          | <5                 |
| cut at Phe-270 | 21 ± 1 | <1       | <2                 |
| ClpXΔN     | 50 ± 2   | 30–80              | >95                 |

* Activities were normalized to control Clp protein not incubated with protease. Protein degradation was measured with casein for ClpAP and with λO protein for ClpXP.

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FIG. 7. Gel filtration of digestion products of ClpX and ClpXP. ClpX alone (10 μg) (panel A) or a mixture of 10 μg ClpX and 12 μg ClpP (panel B) was incubated with 0.5 μg V8 protease for 1 h at room temperature. The reaction was stopped by addition of 1 mM diisopropyl fluorophosphate, and the mixture was loaded onto a Superdex200 column as described in the legend to Fig. 6. Aliquots of the samples were separated by SDS-PAGE and stained.

FIG. 8. AO protein binds to ClpxAN. Samples of ClpX, ClpxAN, ClpX1–53, and a mixture of ClpxAN and ClpX1–53 were applied to poly(vinylidene difluoride) membranes, incubated with AO protein, and probed with anti-AO antibody, which were detected by a chemiluminescence procedure. For undiluted samples, 1 pmol of protein was spotted along with one to three and one to nine dilutions.

**DISCUSSION**

*N-terminal Domains Are Away from ClpP—The Clp ATPases, ClpA, ClpB, ClpX, and ClpY (HslU), share several properties with proteins in the AAA+ superfamily as well as features unique to themselves. The core of the ATP-binding domain and a C-terminal helix-rich sensor-2 subdomain are structurally well conserved, although there is very little homology at the amino acid level. In Clp ATPases, the C-terminal has been called a sensor and substrate discrimination domain because isolated forms are able to interact with specific substrate proteins in vitro. Non-homologous domains are located at the N terminus of ClpA, ClpB, and ClpX, whereas the equivalent domain in ClpY is a separately folding domain that protrudes out of the ATPase domain. Because all the Clp ATPases have unique substrate specificity, this N-terminal domain (or the I domain of ClpY) is another attractive candidate for a specific substrate recognition region or one that affects access to specific binding sites. In support of this model, we have presented data that localize the N-terminal domain of ClpX and ClpA on the distal surface of holoenzyme complexes away from ClpP Fig. 9. This SI surface has been shown to be the site of initial binding of protein substrates to ClpXP and ClpAP (21, 22), although a direct role for the N-terminal region in substrate binding has not been shown.

We have shown that both ClpX and ClpA lacking their N-terminal domains can assemble into hexamers and form stable complexes with ClpP. ClpX remains bound to ClpP when the N-terminal domain is missing.
of the PI surface in NSF, p97, and HslU. Thus, the protected proteins possessing an “arginine finger” and lie on the equivalent influence the oligomerization-dependent ATPase in AAA proteins (1). Residues within the second region of homology referred to as the second region of homology found in AAA the loop/insertion in Clp ATPases align with a signature motif protease, ClpQ (HslV) (5, 6, 34). Second, the residues flanking residues and secondary structure aligns with ClpY residues ClpA and residues 263 and 283 of ClpX. The surrounding reasons. First, the loops lie between residues 605 and 625 of ClpP lie on the surface opposite the N domains for the following-between ClpA, ClpX, and ClpY suggest that the loops protected by ClpP.

**FIG. 9. Comparison of the published structure of ClpY (HslU) with the predicted structure of ClpX and ClpA.** Alignment of the predicted secondary structures of ClpA and ClpX with the structure of ClpY (HslU) obtained from x-ray crystallography are shown. Clp domains were defined in the legend to Fig. 2; WA and WB mark the positions of the nucleotide-binding consensus sequences referred to as Walker A and B. Sen-I is the sensor I motif of AAA proteins (1). The secondary structure predictions were obtained from an alignment of 29 ClpA and 24 ClpX sequences using the Predict Protein program (30). Dashed lines indicate regions were a protein lacks a region of at least 10 amino acids found in one of the other proteins, such as the AAA domain I in ClpA (not in ClpX) or the I domain of ClpY (not in ClpA or ClpX). Two triads (PE(F/L) and GAR) that constitute a Clp family signature in the C domain (41) are indicated. The region protected by ClpP is labeled “ClpP-loop.” B, cartoons of ClpX and ClpA derived by alignment with the published structure of ClpY (HslU) (35). The subunits in ClpY are oriented in the same direction so that structural and functional sites are distributed in different layers along the 6-fold axis. The I domains extend out from the distal surface of the ATPase domain (6, 11), and the N terminus is also located on this distal surface. In ClpX and ClpA, the N-terminal domains are not involved in ClpP contacts, which should place them on the distal face of the complex. This surface is the site at which substrate is seen bound to the holoenzyme, and we refer to it as the substrate interface (SI) surface. In ClpY, the C terminus is on the opposite surface and makes contact with the protease in the ClpYQ holoenzyme complex. We refer to this surface as the PI (for protease interface) surface. Residues 310–320 of HslU lie near the PI surface. In ClpX and ClpA, there are insertions of 20–40 amino acids in the region equivalent to residues 310–320 of ClpY, and this insertion would be predicted to be located on or extend from the PI surface. These insertions include a highly accessible loop that is protected by ClpP.

The N terminus is proteolytically removed. Thus, the N-terminal regions of ClpX and ClpA are not involved in the ring-ring contacts that hold the ClpXP or ClpAP complexes together. In Fig. 9, we show the secondary structure of ClpY (HslU). By aligning homologous regions of ClpA, ClpX, and ClpY and taking into account predicted secondary structures based on multiple sequence alignments (30), we have derived models of ClpA and ClpX and marked the possible positions of structural regions referred to in this study. The N-terminal region of ClpY aligns with the junction between the N terminus and the ATPase domain in ClpA and ClpX, and thus the N domains can be expected to extend from the same surface as the I domain of ClpY (21).

A ClpP-binding loop in ClpA and ClpX—Alignments between ClpA, ClpX, and ClpY suggest that the loops protected by ClpP lie on the surface opposite the N domains for the following reasons. First, the loops lie between residues 605 and 625 of ClpA and residues 263 and 283 of ClpX. The surrounding residues and secondary structure aligns with ClpY residues that are located on the ring surface that makes contact with the protease, ClpQ (HslV) (5, 6, 34). Second, the residues flanking the loop/insertion in Clp ATPases align with a signature motif referred to as the second region of homology found in AAA proteins (1). Residues within the second region of homology influence the oligomerization-dependent ATPase in AAA proteins possessing an “arginine finger” and lie on the equivalent of the PI surface in NSF, p97, and HslU. Thus, the protected loops should be on the ClpP proximal surface of both ClpX and ClpA. We cannot know from our data whether the protected region makes direct contact with ClpP or is simply in a region covered or sterically hindered when ClpP is bound. One argument in favor of direct contact is the position of the loop at the surface of both ClpX and ClpA. Most of the residues present in the loop are ones predicted to be surface-exposed. In addition, three residues at the point where the loops are connected to the remainder of the ATPase are universally conserved in all Clp ATPases and constitute part of a Clp family signature motif (PE(F/L)-GAR) (9). In the crystal structure, the Glu residue in the ClpY motif is directly involved in subunit-subunit interactions within the ATPase ring (6, 35). Thus, the protected loop would be conformationally sensitive to assembly of the hexamer and is thus in a position to mediate interaction with ClpP, which only occurs with the hexameric state of the ATPase. Finally, only three residues within the loop (IG(F/L)) are conserved in ClpX and ClpA but not in ClpB or in ClpX species that do not interact with ClpP, such as yeast (31). This triad is the most protease-accessible region of ClpA and ClpX, cuts coming after the Leu in ClpA and the Phe in ClpX, which presumably are located at a point in the loop that projects from the surface. Recent data support a role for this IG(F/L) motif in interacting with ClpP (31).

**N Domains Are Not Needed for Most Activities—**Both ClpX and ClpA without their N-terminal domains retain ATPase activity and the ability to activate ATP-dependent protein deg-
Domain Organization in ClpA and ClpX

radiation by ClpP. Although there are some changes in activity, most notably the loss of λO protein degrading activity by ClpXΔN-ClpP, most interactions and activities are at least partially retained. Although no fundamental activity requires the N-terminal region, we can ascribe an auxiliary role or role more specific for certain classes of substrates to it. Binding of λO is weaker to ClpXΔN suggesting that the N terminus of ClpX may affect the site of interaction on ClpX or the orientation of the bound protein necessary for its translocation. These issues are under further study.

The N domains are linked to their respective ATPase domains through the polypeptide chain, but do not associate strongly with the ATPase domain. The N domain of ClpX is cleaved and easily dissociates from the remainder of the protein, suggesting that it is an independently folded domain that does not interact strongly with the core ATPase domain. The N terminus of ClpA is also a stable, protease-resistant domain, but apparently the connector between it and the ATPase core is sterically inaccessible to proteases in the presence of nucleotides. In separate studies, we have expressed the N-terminal domain of ClpA and shown that it forms a stable, well folded monomeric domain that does not associate with intact ClpA or with ClpΔA153. We propose that the N-terminal domains of ClpA and ClpX may be functionally equivalent to the I domain of ClpY (HslU). In HslU, two α-helices extend from the I domain into the core ATPase serving as the connection between them, but there are relatively few surface contacts between the regions (6, 35). Nucleotide binding and hydrolysis in the core ATPase domain produce conformational effects that can mediate changes in the position of the I domain (5, 34) or by analogy the N domains, thereby affecting substrate binding or access.

In AAA proteins such as NSF and p97, the N domains bind adapter proteins that mediate or modulate interactions with different substrates (36, 37). The contribution of the N domain can be expected to vary depending on the substrate examined and the type of reaction being measured. Such possibilities for the N domains of ClpA and ClpX are currently being investigated. Another possible, more direct, role for the N domains is that they act as a lid or similar barrier controlling dissociation of substrates. In this model, the N domain (or I domain) of Clp ATPases would act similarly to the GroES-like N-terminal domain of the thermosome (38) or CCT (39, 40). In those chaperonins, the attached N domain may move into and out of position in response to the nucleotide state and in this way help regulate substrate association and dissociation.

Conformational Dynamics in Response to Nucleotide—Previous studies suggested that assembly of ClpA required binding of ATP or an analog of ATP to the domain I ATP binding site, but it was not known if nucleotide could bind to domain I or domain II prior to hexamer formation. The studies presented here show that nucleotide binding can partially protect ClpA from proteolysis under conditions in which the nucleotide binds poorly to the domain I site and hexamer formation does not occur. Thus, ATP can bind to the domain II site before assembly occurs. Earlier studies indicated that hydrolysis of the bound nucleotide either does not occur or is very slow until nucleotide binds to the domain I site and hexamer assembly occurs. Our results also show that assembly of hexamers protects ClpA even with mutants that do not bind nucleotide tightly at the domain II site. The protection pattern of the mutants parallels the effects of the mutations on hexamer dissociation rates. Thus, interactions in the ClpA hexamers are also major factors stabilizing the conformation of both domains. These results are consistent with a dynamic model of ClpA in which cycles of ATP hydrolysis are accompanied by transient destabilization of subunit contacts within the ClpA rings. This loosening of subunit interactions does not lead to complete dissociation because subunit exchange rate constants have been shown to be <5% of ATP hydrolysis turnover numbers (26). Nevertheless, cyclical loosening and tightening of the hexamer could play a role in binding and release of protein substrates during substrate unfolding and translocation. Such breathing of the ATPase could also allow periodic changes in interaction with ClpP, such as altering the rotational orientations of the six- and seven-membered rings.

ClpX hexamers are relatively stable in the presence of either ATP or ATPγS. ClpX differs from ClpA in being partially assembled into hexamers even in the absence of nucleotide. The explanation behind these differences is not yet clear. Perhaps, ClpX undergoes less structural perturbation during catalytic cycles. Substrates bound to ClpX are passed directly to ClpP, whereas, with ClpA, substrates must be passed from the domain I to domain II before they go to ClpP. This additional step in ClpA may allow an additional proofreading process whereby substrates are subject to further screening before being translocated to the protease. The additional domain and interaction step could also reflect the types of substrates recognized by ClpA and ClpX. ClpA apparently binds a wider range of unfolded proteins than does ClpX (14) and thus may need to undergo conformational changes of a greater magnitude to bind and release hydrophobic portions of bound substrates.

In summary, our results establish the orientation of ClpA and ClpX with respect to ClpP in the assembled proteolytically active complexes and point to a region in ClpX and ClpA that is involved in interactions with ClpP. Together with earlier data showing the initial site for binding of substrates, our results provide a structural framework for considering the mechanism by which Clp ATPases activate proteolysis. Substrates bind to the distal surface of the complex from which also extends a modular domain composed from either an N-terminal extension or an I domain. These modular domains vary widely in different Clp ATPases and may contribute to substrate binding or may affect access to other binding sites. The opposite surface of the ATPase rings binds to the protease. At least one variable loop is found on this surface and is protected from proteases by assembly in the holoenzyme complex. These loops or other extensions from this surface may act as grappling hooks to attach the ATPase to the protease. For example, the crystal structure of ClpYQ shows that a major contact with ClpQ is made through the C-terminal 10 amino acids of ClpY (6). ATP hydrolysis promotes unfolding of the substrate, which then boards the associated protease through axial access channels. Because ClpAP and ClpXP interactions are more dynamic when ATP hydrolysis occurs, ATP hydrolysis may lead to localized breaking and making of new contacts between the grappling lines and the protease, which could be necessary for efficient translocation of the substrate. Further studies, including mutagenesis of the residues within the surface loops and surrounding regions, is underway to identify the specific sites involved in contacts between the ATPases and the proteases and to define the mechanism by which these contacts are altered during catalysis.

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Functional Domains of the ClpA and ClpX Molecular Chaperones Identified by Limited Proteolysis and Deletion Analysis
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