ClpA and ClpP Remain Associated during Multiple Rounds of ATP-Dependent Protein Degradation by ClpAP Protease

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ABSTRACT: The *Escherichia coli* ClpA and ClpP proteins form a complex, ClpAP, that catalyzes ATP-dependent degradation of proteins. Formation of stable ClpA hexamers and stable ClpAP complexes requires binding of ATP or nonhydrolyzable ATP analogues to ClpA. To understand the order of events during substrate binding, unfolding, and degradation by ClpAP, it is essential to know the oligomeric state of the enzyme during multiple catalytic cycles. Using inactive forms of ClpA or ClpP as traps for dissociated species, we measured the rates of dissociation of ClpA hexamers or ClpAP complexes. When ATP was saturating, the rate constant for dissociation of ClpA hexamers was 0.032 min^{-1} ($t_{1/2}$ of 22 min) at 37 °C, and dissociation of ClpP from the ClpAP complexes occurred with a rate constant of 0.092 min^{-1} ($t_{1/2}$ of 7.5 min). Because the k_{cat} for casein degradation is ∼10 min^{-1}, these results indicate that tens of molecules of casein can be turned over by the ClpAP complex before significant dissociation occurs. Mutations in the N-terminal ATP binding site led to faster rates of ClpA and ClpAP dissociation, whereas mutations in the C-terminal ATP binding site, which cause significant decreases in ATPase activity, led to lower rates of dissociation of ClpA and ClpAP complexes. Dissociation rates for wild-type and first domain mutants of ClpA were faster at low nucleotide concentrations. The $t_{1/2}$ for dissociation of ClpAP complexes in the presence of nonhydrolyzable analogues was ≥30 min. Thus, ATP binding stabilizes the oligomeric state of ClpA, and cycles of ATP hydrolysis affect the dynamics of oligomer interaction. However, since the k_{cat} for ATP hydrolysis is ∼140 min^{-1}, ClpA and the ClpAP complex remain associated during hundreds of rounds of ATP hydrolysis. Our results indicate that the ClpAP complex is the functional form of the protease and as such engages in multiple rounds of interaction with substrate proteins, degradation, and release of peptide products without dissociation.

The *Escherichia coli* ClpAP protease is a 1.3 MDa complex of two oligomeric proteins, ClpA and ClpP (*1*, *2*). ClpA (subunit M_r 84 000) is composed of six identical subunits arranged in a two-tiered hexagonal ring, the tiers probably representing structural domains consisting of the N- and C-terminal ATPase domains (*3*). ClpP (subunit M_r 21 400) is composed of two seven-membered rings, the junction of which forms an internal aqueous chamber that contains the proteolytic active sites (*4*). When sufficient ClpA is present, the major species of ClpAP is a barrel-shaped molecule with a hexameric ring of ClpA bound to each face of ClpP. When ClpA is limiting, complexes with a single ring of ClpA bound to one face of ClpP are formed and are enzymatically active. These architectural features of ClpAP are reminiscent of those of the eukaryotic 26S proteasome (*1*, *5*).

Another similarity between ClpP and the proteasome is the location and arrangement of the proteolytic active sites. X-ray crystal structure analysis has confirmed that each subunit of ClpP has a proteolytic active site and that these sites are distributed along the surface of an interior aqueous chamber (*4*). The only access to the active-site cavity is through a single channel in each ring formed by the conjunction of the subunits at the 7-fold axis. The restricted access to the active sites in ClpP suggested from structural studies is confirmed by experiments showing that, by itself, ClpP can degrade only short peptides, usually of five or fewer amino acids (*6*, *7*). Degradation of larger peptides and proteins requires the presence of ClpA.

The role of ClpA in protein degradation appears to be 2-fold: to promote a conformation in a protein substrate that makes it more susceptible to degradation (probably unfolding) and to actively aid in translocating the protein into the proteolytic chamber in ClpP. Support for this mechanism comes from studies showing that ClpA has ATP-dependent molecular chaperone activity and can modify the conformation of the P1 RepA protein (*8*). ClpA binds protein substrates and, in the presence of ATP, can promote formation of a complex between an enzymatically inactive ClpP and protein substrates (*9*). More recently, studies have shown that protein bound in preformed complexes with ClpAP are partitioned between release/activation and translocation/degradation (*10*).

An important question regarding the mechanism of action of ClpAP is whether the complex remains intact during the entire degradation cycle of a protein and, further, during multiple rounds of substrate binding and degradation. Substrates bound to either ClpA or ClpAP can be degraded without release, confirming the processive nature of protein degradation by ClpAP (*9*). We have now measured the

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kinetics of oligomerization of ClpA and assembly of ClpAP and have determined the rate of dissociation of ClpA and disassembly of ClpAP under assay conditions. Our results indicate that ClpAP remains as an intact complex during multiple rounds of ATP hydrolysis and of polypeptide and protein substrate degradation.

EXPERIMENTAL PROCEDURES

Materials. ATP, ATPγS, α-casein, dithiothreitol (DTT),[1] and diisopropyl fluorophosphate were obtained from Sigma. Acetonitrile was obtained from Burdick & Jackson. Trifluoroacetic acid and Triton X-100 were obtained from Pierce Chemical Co. Mono Q, Mono S, S-Sepharose, and Q-Sepharose columns were from Pharmacia Biotechnology Inc. [^{3}H]Succinimidyl propionate (60 mCi/mmol) was purchased from NEN/Dupont. All other reagents were of the highest quality available. The propeptide, FAPHMALVPV, was synthesized in this laboratory (*6*).

Protein Purification and Assay. Wild-type ClpA and ClpP proteins were purified as described earlier (*11*). The mutant ClpA proteins (ClpA-K220Q, ClpA-K220R, ClpA-K220V, ClpA-K501Q, ClpA-K501R, and the double mutant ClpA-K220Q/K501Q) were purified to homogeneity at 4 °C by ammonium sulfate precipitation, chromatography on S-Sepharose or Mono S, and chromatography on Mono Q, essentially as described in our earlier publication (*12*). His_6-tagged ClpA was prepared at 4 °C by metal chelate affinity chromatography on a Talon matrix (Clontech). After elution with imidazole, the protein was precipitated at 4 °C in 40% saturated ammonium sulfate and dialyzed against buffer B with 0.2 M KCl. His_6-tagged ClpA had about 50% the ATPase activity of wild-type ClpA. Details of the construction and expression of the His-tagged ClpA fusion protein will be published elsewhere (F. Guo and M. R. Maurizi, unpublished results). Previously described methods were used for the assay of protein concentration, ATP-dependent proteolytic activity with [^{3}H]-α-casein as a substrate, and ClpP propeptide cleavage (*11*). ATPase activity was measured by the release of inorganic phosphate under assay conditions described previously. When [γ-^{32}P]ATP was used, phosphate was quantitated by scintillation counting after formation of phosphomolybdate and extraction into toluene/2-butanol (*11*, *13*). A nonradioactive assay of the phosphomolybdate employed a colorimetric method enhanced with malachite green (*14*).

Inactivation of ClpP. Inactive ClpP (DIP-ClpP) was prepared by chemical modification with DFP (*15*). Typically, 3−5 mg of ClpP was incubated in 1 mL of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 10% glycerol (buffer B) with 10−20 mM (final concentration) DFP at room temperature for 3 h. The DFP (1 M dissolved in dry 2-propanol) was added in increments of 5 mM at 45 min intervals. The reaction was stopped, and excess DFP was removed by precipitating the ClpP with 2 volumes of chilled acetone. ClpP was dissolved in buffer B and passed through a Bio-Rad P-10 column equilibrated with buffer B (containing 0.2 M KCl and 1 mM DTT) to remove acetone and residual DFP. The remaining activity of DIP-ClpP with α-casein as a substrate was less than 1−2%.

Preparation of [^{3}H]-Labeled ClpA and ClpP. ClpA, ClpP, or DIP-ClpP (1−2 mg) in 0.5 mL of 25 mM Hepes/KOH, pH 7.5, 0.3 M KCl, 1 mM EDTA, and 10% (v/v) glycerol was incubated with 100 μCi of [^{3}H]succinimidyl propionate (specific radioactivity 60 mCi/mmol) on ice for 1 h. ClpA was recovered by precipitation on ice with 40% saturated ammonium sulfate, dissolution in buffer B with 0.3 M KCl, and removal of excess ammonium sulfate by gel filtration on a small Sephadex G-50 column in the same buffer. ClpP was recovered by acetone precipitation as described above. The specific activity for ClpA was ∼40 000 cpm/μg and for ClpP was ∼20 000 cpm/μg.

Inhibition of Active ClpA Species by Premixing with ClpA-K220Q. Wild-type ClpA or mutant ClpA proteins were mixed on ice with increasing amounts of ClpA-K220Q in buffer B containing 1 mM DTT and 0.05% (v/v) Triton X-100. Equal amounts of active ClpA (0.2 μg of wild-type or domain I mutants or 1 μg of domain II mutants) were added to assay mixtures (50 μL) and ATP hydrolysis was measured after a fixed time. When mutant ClpA was present in ≥10:1 molar ratio over active ClpA, the resulting mixed hexamers had no remaining ATPase activity.

ClpA Subunit Exchange Kinetics. To study the rate of subunit dissociation from ClpA hexamers under assay conditions, a trapping assay in which dissociated subunits were incorporated into inactive mixed hexamers with ClpA-K220Q (*12*) was devised. Assays were conducted at 37 °C in solutions containing 50 mM Tris-HCl, pH 8.0, 0.1 M KCl, 0.05% Triton X-100, 25 mM $MgCl_2$, 1 mM DTT, and 1 or 5 mM [γ-^{32}P]ATP (1−2 mCi/mmol) in a total volume of 50 μL. Parallel assays were started by the addition of 0.2−1 μg of active ClpA (wild-type or partially active mutants). After 2−5 min, a 10−20-fold excess of ClpA-K220Q was added to one of the reactions, and ATP hydrolysis was measured during the subsequent incubation. The other reaction served as the control to determine the ATP hydrolyzed as a function of time. The rate of decrease in activity in the reaction was used to calculate the rate of dissociation ClpA hexamers (see below).

Kinetics of ClpP Exchange from Active ClpAP Complexes. To study ClpP exchange from the active ClpAP proteolytic complex, [*methyl*-^{3}H]casein degradation was assayed with limiting ClpA at 37 °C in 250 μL (final volume) of 50 mM Tris-HCl, pH 8.0, 25 mM $MgCl_2$, 1 mM DTT, 4 mM ATP, 10−15 μg of [*methyl*-^{3}H]casein (∼80 000−120 000 cpm). Duplicate reactions were started with the addition of ClpP (2−5 μg) and ClpA (0.2−2 μg), and after a fixed time DIP-ClpP (1−4-fold excess) or buffer was added. Casein degradation was measured at different times as described above. Inhibition kinetics were also measured with an assay that requires ClpA but not ATP hydrolysis. Degradation of the peptide, FAPHMALVPV, was assayed in the above buffer with either 1 mM ATPγS or 4 mM ATP and limiting ClpA (0.5 μg). Reactions were started with ClpA and ClpP (2−5 μg) and excess DIP-ClpP were added as described above. Products were quantitated after C18 reverse-phase chromatography as described earlier (*6*).

Calculation of the Half-Time for ClpA Hexamer Dissociation. The time course for appearance of product [P] in a reaction in which the enzyme is inactivated by a first-order process (in this case dissociation of components) with a rate constant, k, is given by the equation $[P] = (1/k)(1 - e^{-kt})$.

[1] Abbreviations: DTT, dithiothreitol; DFP, diisopropyl fluorophosphate; DIP-ClpP, diisopropyl phosphoryl-ClpP.

For each experiment, the data were normalized to unit activity and the curve drawn is the best fit of the experimental data points to this function. To facilitate analysis of the dissociation of ClpA hexamers we made the simplifying assumption that ClpA hexamers dissociate in a first order process with no partially active species generated. This model would hold if dissociation of hexamers were a highly cooperative, all-or-none process or if only the smallest dissociating species (monomer or dimer) were inactive and the subunits remaining in associated species (with three, four, or five subunits in a mixed hexamer with mutant ClpA) were fully active. We have not been able verify or distinguish between these models experimentally, but the kinetic data are consistent with either, and this treatment allowed us to quantitatively compare the rates of dissociation for different ClpA species under different conditions. For ClpAP dissociation, the model assumes that loss of activity follows separation of ClpP and ClpA oligomeric species.

Separation of ClpP and ClpAP by Ultrafiltration. Complexes of ClpA (24 μg/mL) and [^{3}H]DIP-ClpP (6 μg/mL) were assembled in 50 mM Tris-HCl, pH 8.0, 0.2 M KCl, 0.025% Triton X-100, 25 mM $MgCl_2$, 1 mM DTT, and 4 mM ATP at 37 °C. After 2 min, excess nonradioactive ClpP (60 μg/mL) was added and aliquots of 100 μL were centrifuged through UF300 ultrafiltration membranes (Millipore) at 1000*g* for 2 min at 4 °C. Radioactivity in the filtrate was quantitated by scintillation counting. A maximum of 80% of ClpP in the ClpAP complex was retained on the membrane, and 75% of the free ClpP was recovered in the filtrate.

Light Scattering. Oligomerization and disassembly of ClpA and ClpAP was monitored by changes in 90° light scattering of protein solutions. Measurements were made in 1 cm fluorescence cuvettes in an SLM/Aminco Bowman spectrofluometer with excitation and emission monochromators set at 360 nm with 4 nm slits. Usually 25−90 μg of ClpA or 10−50 μg of ClpP was added to 1 mL of buffer B containing 0.2 M KCl, 25 mM $MgCl_2$, and 1 mM DTT. ATP or ATPγS was added before or after the proteins as needed. Solutions were maintained at room temperature (22−24 °C) or at 37 °C and recordings were made for 30−300 s until stable readings were obtained.

RESULTS

Hexameric State of ClpA under Assay Conditions. ClpA exists as an equilibrium mixture of monomers and dimers in the absence of nucleotide and forms hexamers in the presence of ATP or an analogue of ATP. Previous analyses showing hexamer formation of wild-type ClpA were performed at concentrations of 15 μg/mL or higher (*1*, *2*). To confirm that ClpA was hexameric at the lower concentrations used in assays, we ran radioactively labeled ClpA over a Superdex 200 gel-filtration column in the presence of MgATP. About 40% of the ClpA ran as a hexamer at 1 μg/mL and less than 10% was hexameric at 0.2 μg/mL (data not shown). As the specific activity of ClpA begins to decrease below 1 μg/mL (12 nM subunit) in assays, the gel-filtration results support our model that the active form of ClpA is hexameric.

Assembly of Wild-Type ClpA in the Presence of the Mutant, ClpA-K220Q, Inhibits ATPase Activity. In an earlier study (*12*) we reported that the ATP binding-site mutant, ClpA-K220Q, could not self-associate to form hexamers and showed no ATPase activity. Surprisingly, when ClpA-K220Q was added to reaction assay solutions, it interfered with the basal ATPase activity of wild-type ClpA (*12*). Maximum inhibition required mixing ClpA-K220Q with wild-type ClpA prior to addition of nucleotide (Figure 1A). Inhibition was dependent on the ratio of mutant ClpA to wild-type ClpA and was complete when the mutant was added in a 10−20-fold molar excess over wild type. With a 2−5-fold excess of ClpA-K220Q, inhibition was less complete at 5 mM ATP than at 1 mM ATP (Figure 1A), suggesting that wild-type ClpA either had slightly higher affinity for itself or preferentially assembled with itself and was resistant to inhibition. When mutant was added in amounts that gave partial inhibition, the remaining activity was linear for at least 1 h (Figure 1B), indicating that loss of activity was dependent only on the amount of ClpA trapped in a complex with the mutant ClpA-K220Q and there was no further time-dependent change in activity.

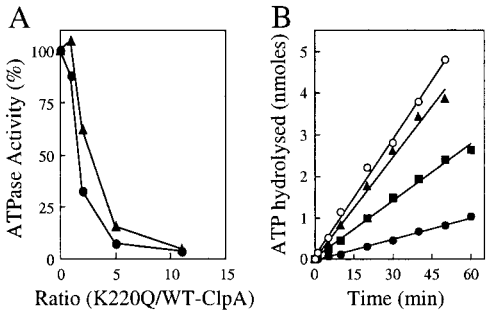

FIGURE 1: Inhibition of basal ATPase activity of ClpA by ClpA-K220Q. (A) Premixing of WT-ClpA and ClpA-K220Q. ClpA (40 μg/mL) was mixed with increasing amounts of ClpA-K220Q in buffer B plus 1 mM DTT and 0.05% Triton X-100 prior to adding to assay solutions containing either 1 mM (●) or 5 mM (▲) ATP. ATP hydrolysis was measured after 10 min. Results are shown for a single experiment, but similar results were obtained in four other assays. (B) Time course of ATPase reaction with partially inhibited ClpA. ClpA and ClpA-K220Q were mixed as described above to give different degrees of inhibition and added to ATPase assay mixtures. Aliquots were removed at the times indicated and the amount of ATP hydrolyzed was measured. The ratios of ClpA-K220Q to wild-type ClpA were 1:1 (▲), 2:1 (■), 5:1 (●), and no ClpA-K220Q (○).

Because inhibition was most readily seen when ClpA-K220Q was added to unassembled ClpA, it was possible inhibition occurred because ClpA-K220Q trapped the wild-type protein in inactive mixed dimers or that ClpA-K220Q and wild-type ClpA formed mixed hexamers in which ClpA was in an inactive conformation. We confirmed that wild-type ClpA and ClpA-K220Q formed a complex that had the same mobility as wild-type ClpA hexamers when run on a gel-filtration column in the cold (data not shown). The ratio of ClpA-K220Q to wild-type subunits in the mixed complex is about 2:1. Exact characterization of the complex has been difficult because of its tendency to dissociate upon dilution and eventually to aggregate over time. The complex between wild-type ClpA and ClpA-K220Q could not be observed after gel filtration at this low concentration (data not shown).

Dissociation Rate of ClpA Subunits from ClpA Hexamers. To measure the stability of ClpA hexamers under reaction conditions, we took advantage of the ability of ClpA-K220Q to trap the active ClpA subunits in an inactive form. We

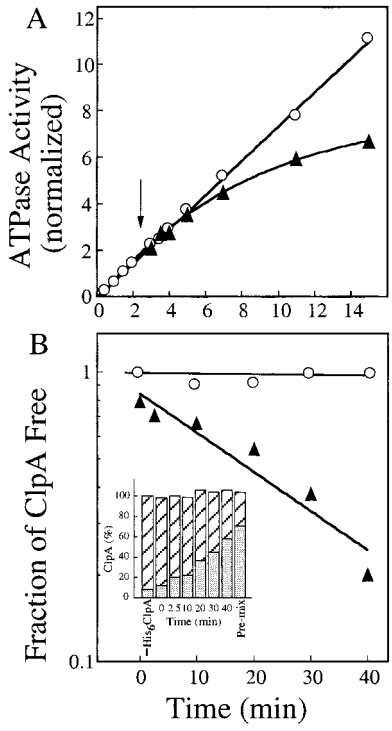

FIGURE 2: Rate of ClpA hexamer dissociation. (A) Rate of loss of ATPase activity by subunit exchange with inactive mutant. ATPase assays were started in parallel by addition of wild-type ClpA (0.2 μg). After 2.5 min (arrow), a 10-fold excess of ClpA-K220Q was added to one solution (▲) and buffer was added to the other as a control (○). Reactions were continued and aliquots were removed to measure the extent of ATP hydrolysis. Similar results were obtained in three separate experiments. Assay data were normalized to unit activity for ease of display and curve fitting. The curve drawn represents a fit to the integrated equation [activity] = $(1/k)(1 - e^{-kt})$, where k is the first-order rate constant for loss of activity (dissociation). (B) Subunit exchange between ClpA and His_6-tagged ClpA hexamers. [^{3}H]Wild-type ClpA (10 μg) was incubated in 0.5 mL of 50 mM Tris/HCl, pH 7.5, 0.2 M KCl, 1 mM DTT, 0.05% Triton X-100, 4 mM ATP, and 10 mM $MgCl_2$ for 1 min. His_6-tagged ClpA (50 μg) preassembled in 0.5 mL of the same solution was then added and 0.1 mL aliquots were removed at the times indicated. The samples were passed over a 0.1 mL bed of metal-chelate gel (Talon) and washed with 0.5 mL of the same buffer containing 10 mM imidazole. Bound protein was then eluted with 0.6 mL of the buffer containing 0.5 M imidazole. Radioactivity in aliquots of the unbound effluent and in the imidazole eluate was determined by scintillation counting. The plot shows the first-order loss of [^{3}H]wild-type ClpA from the unbound fraction as it was incorporated into mixed hexamers with His_6-tagged ClpA and trapped on the column (▲). Control incubations with ATPγS (○) show lack of exchange in the presence of nonhydrolyzable analogues of ATP. The inset shows the distribution of counts between the unbound fraction (hatched) and the column-bound fraction (gray) at each time. The control ($-His_6$ClpA) shows background binding of [^{3}H]wild-type ClpA in the absence of His_6-tagged ClpA. For the premixed sample, [^{3}H]ClpA and His_6-tagged ClpA were mixed in the same proportions before the addition of ATP.

found that if ClpA-K220Q was added after wild-type ClpA was allowed to assemble, inhibition of wild-type ClpA was delayed. Figure 2A shows the progress of ATP hydrolysis when excess ClpA-K220Q was added to an ATPase assay solution 2.5 min after the reaction was started with wild-type ClpA. The rate of ATP hydrolysis did not decrease immediately, and several minutes were required before ATP hydrolysis was completely inhibited. Thus, ClpA-K220Q did not interfere with the activity of assembled ClpA hexamers. Because ClpA-K220Q completely inhibited unassembled ClpA without a lag, the time dependence of inhibition was taken as the time required for wild-type ClpA hexamers to dissociate and be trapped by ClpA-K220Q.

Table 1: Half-Times for Dissociation of ClpA Mutants[a]

ClpA protein	half-time for dissociation (min)	
	1 mM ATP	5 mM ATP
wild type	6 ± 0.5	22 ± 2
K220V	1 ± 0.5	15 ± 1 (25 ± 1)[b]
K220R	1 ± 0.5	5 ± 0.5
K501R	>60[c]	>60[c]
K501Q	>60[c]	>60[c]
His_6-tagged	8 ± 1	15 ± 1

[a] Half-times were calculated from the dissociation rates determined from the kinetics of inhibition of ATPase activity after addition of excess ClpA-K220Q as described under Experimental Procedures. [b] Rate in parentheses was determined with a ClpA-K220V mutant carrying a second mutation, F172S. [c] No significant inhibition was observed when the assay was continued for 1 h after addition of ClpA-K220Q.

To measure hexamer dissociation directly, we determined subunit exchange between wild-type ClpA hexamers and His_6-tagged ClpA hexamers. We first confirmed that preassembled His_6-tagged ClpA was inhibited with a similar half-life as the wild-type enzyme upon addition of ClpA-K220Q (Table 1). Thus, His_6-tagged ClpA hexamers appear to be as stable as wild-type ClpA hexamers. For the direct exchange measurements, we used [^{3}H]wild-type ClpA instead of ClpA-K220Q, because the latter tended to aggregate when complexed with His_6-tagged ClpA. To show mixed hexamer formation, His_6-tagged ClpA and [^{3}H]wild-type ClpA were mixed prior to addition of ATP and then passed over a small metal chelate column. [^{3}H]Wild-type ClpA was retained when His_6-tagged ClpA was present, whereas alone it did not bind to the column (Figure 2B, inset). The His_6-tagged ClpA and [^{3}H]wild-type ClpA were then preassembled in separate solutions by addition of ATP, and the two solutions were mixed. At different times, aliquots were removed and passed over a small metal chelate column, and the amount of radioactive wild-type ClpA retained was measured. As shown in Figure 2B, incorporation of [^{3}H]ClpA into mixed hexamers with His_6-tagged ClpA was time-dependent and proceeded smoothly to reach a level equal to that obtained when the samples were mixed prior to addition of the nucleotide (Figure 2B, inset). The rate of exchange of the ClpA into mixed hexamers ($t_{1/2}$ = 22 min) was similar to that measured for inhibition by ClpA-K220Q, suggesting that both processes were dependent on dissociation of preformed ClpA hexamers.

Dissociation of N- and C-Terminal ATPase Domain Mutants. The kinetics of inhibition by ClpA-K220Q was used to test the stability of four different ClpA mutants under reaction conditions. All mutants were completely inhibited when they were assembled in the presence of >10-fold excess ClpA-K220Q (Figure 3A). However, they showed very different rates of inhibition when ClpA-K220Q was added to reaction mixes after assembly of the mutant ClpA hexamers (Figure 3B, C). In 1 mM ATP, the N-terminal domain mutants (ClpA-K220V and ClpA-K220R) dissociated faster than wild-type ClpA (Figure 3B and Table 1). In contrast, C-terminal domain mutants (ClpA-K501R and

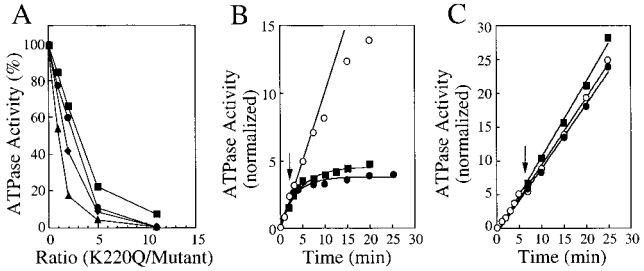

FIGURE 3: Dissociation of mutant ClpA hexamers. ATP hydrolysis was measured under standard conditions with 0.2 μg of wild-type ClpA or domain I mutants or 1.0 μg of domain II mutants. Assay data were normalized to 100%; the specific activities were 130, 78, 85, 10, and 9 μmol h^{-1} (mg of protein)$^{-1}$ for the K220R, K220V, K501R, and K501Q mutants, respectively. In panels B and C, the curves are theoretical fits to the equation given in the caption to Figure 2A. (A) Inhibition of mutant ClpAs by premixing with ClpA-K220Q. Mutant ClpA proteins were premixed with increasing amounts of ClpA-K220Q in buffer B plus 1 mM DTT and 0.05% Triton X-100 for 5 min on ice prior to addition to assay solutions containing 5 mM ATP. ClpA-K220V (■); ClpA-K220R (●); ClpA-K501R (◆); ClpA-K501Q (▲). (B) Inhibition of preassembled domain I mutants. ClpA-K220R (●) or ClpA-K220V (■) was added to assay solutions containing 1 mM ATP to initiate ATP hydrolysis. After 2.5 min (arrow), 20-fold excess ClpA-K220Q was added to one and buffer was added to another as a control. Aliquots were removed at the indicated times to measure the extent of ATP hydrolysis. Control activity for ClpA-K220V (○) and ClpA-K220R (not shown) were the same. (C) Inhibition of preassembled domain II mutants. The experiment was conducted as in panel B except that ClpA-K220Q was added at 7.5 min (arrow) to assay solutions with ClpA-K501R (●) and ClpA-K501Q (■). Control activity (○), obtained by adding buffer instead of ClpA-K220Q, is shown for ClpA-K501R only.

ClpA-K501Q) were nearly completely resistant to inhibition once they were assembled (Figure 3C and Table 1), indicating that they dissociated very slowly under these conditions.

Earlier studies showed that mutations in the domain I site weakened ATP or ATPγS binding 4−5-fold at the mutated site while not affecting binding at the nonmutated domain II site (*12*). Conversely, mutations in the domain II site weakened ATP binding 2−3-fold and ATPγS binding >10-fold at the mutated site while not affecting binding at the nonmutated domain I site. The dissociation rates measured above suggest that nucleotide binding to the N-terminal domain affects hexamer stability. Mutants with weaker nucleotide binding at domain I dissociated more rapidly. In contrast, mutations at the C-terminal site led to slower rather than faster ClpA dissociation. Because the C-terminal domain mutants bind nucleotides less tightly and have much lower ATPase activity than wild-type ClpA (*12*), the slower dissociation of domain II mutants suggests that, at the C-terminal domain, hydrolysis of ATP favors hexamer dissociation. One explanation for this effect could be that ClpA dissociation is favored from a conformational state that appears transiently during cycles of ATP hydrolysis. This loose conformation may represent a state of ClpA required for efficient unfolding of substrates or transfer of substrates to ClpP. Hexamer dissociation must be infrequent from the transient conformational state, because the k_{cat} for ATP hydrolysis (~140 min^{-1} per subunit of ClpA) is >1000-fold higher than the rate constant for subunit dissociation.

Nucleotide Concentration Dependence of Dissociation. The rate of ClpA dissociation depended on the concentration of ATP used in the assay. Increasing the concentration of

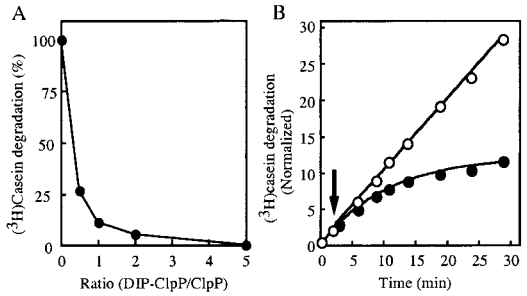

FIGURE 4: Exchange of ClpP from the ClpAP complex. (A) Competition between ClpP and DIP-ClpP. Varying amounts of proteolytically inactive DIP-ClpP were mixed with active ClpP in buffer B containing 1 mM DTT. Aliquots containing 2 μg of active ClpP were then added to standard casein degradation assay solutions, and reactions were started by the addition of 0.2 μg of ClpA. (B) Time-dependent displacement of ClpP by DIP-ClpP. Standard casein degradation assays were started with 0.2 μg of ClpA and 2 μg of ClpP. After 2 min, 8 μg of DIP-ClpP was added (●); buffer was added to control assays (○). Samples were removed at different times, and the amount of casein degraded was measured. Activity was normalized to unity [1 unit is 0.42 μg of casein min^{-1} (μg of ClpA)$^{-1}$] and fit to the integrated equation for a first-order dissociation (rate constant, k) of the active ClpAP species, [activity] $= (1/k)(1 - e^{-kt})$.

ATP from 1 to 4 mM in the assay did not have a large effect on the ability of ClpA-K220Q to inhibit active ClpA (Figure 1A), but it decreased the dissociation rate of preassembled ClpA by more than a factor of 2 (Table 1). This effect was very pronounced with the N-terminal domain mutants, especially ClpA-K220V, which dissociated very rapidly ($t_{1/2}$ ~ 1 min) in the presence of 1 mM ATP but behaved more like wild-type ClpA in 5 mM ATP (Table 1). The C-terminal domain mutants did not dissociate in the time of the assay with either concentration of ATP (Figures 3B and Table 1). These results confirm that nucleotide binding at the N-terminal ATP-binding site is essential for stability of ClpA hexamers and is consistent with our earlier conclusion, based on the behavior of ClpA-K220Q itself, that ATP binding to the N-terminal domain is needed for ClpA assembly (*12*).

Stability of the ClpAP Complexes. Protein degradation requires a complex of ClpA and ClpP, which earlier measurements indicated has a K_d of 4−6 nM in the presence of MgATP at 37 °C (*2*). To address the question of how rapidly the ClpAP complex dissociates under reaction conditions, we employed a proteolytically inactive form of ClpP, DIP-ClpP, as a trap to bind ClpA released from the ClpAP complex. DIP-ClpP binds ClpA with high affinity. When equal amounts or slight excesses of DIP-ClpP were added simultaneously with active ClpP, the inactive ClpP preferentially bound to ClpA, preventing it from promoting proteolysis by active ClpP (Figure 4A). However, competition by excess DIP-ClpP added to pre-assembled ClpAP was time-dependent, and complete inhibition required more than 30 min (Figure 4B). The time-dependent decrease in casein degradation is consistent with a requirement for dissociation of the ClpAP complex before DIP-ClpP can bind to ClpA, blocking further activity. The kinetics of inhibition was analyzed as described above for the time-dependent inhibition of ClpA ATPase activity, and a half-time for dissociation of ClpAP of 7−8 min was calculated (Table 2).

To show that loss of activity did not result from nonspecific inhibition of ClpAP by DIP-ClpP, the exchange assay was done in reverse, starting with complexes made with ClpA

Table 2: Summary of Half-Times for ClpAP Complex Dissociation

ClpA variant in complex	half-time for dissociation (min)
(A) With Active ClpP	
wild-type[a]	7.5 ± 0.5
K220R[a]	6 ± 0.5
K220V[a]	6 ± 0.5
K501R[a]	25 ± 1
K501Q[a]	23 ± 1
wild type[b]	6 ± 1
wild type + ATPγS[b]	29 ± 2
(B) With DIP-ClpP	
wild type[c]	6 ± 1
wild type + ATPγS[c]	>30

[a] Half-lives were measured at 37 °C in standard casein degradation assay solutions with 4 mM ATP. [b] Dissociation was measured at 37 °C in the propeptide degradation assay solution with either 4 mM ATP or 1 mM ATPγS. [c] Dissociation was measured by exchange of [^{3}H]DIP-ClpP out of ClpAP complexes in the presence of 4 mM ATP or 2 mM ATPγS and excess nonradioactive ClpP.

and DIP-ClpP. When active ClpP was added to assay solutions containing ClpA/DIP-ClpP complexes, there was a long lag period during which activity slowly increased (data not shown). After about 20 min, activity was about 50% of that expected; however, the reaction was not carried out long enough to observe a linear slope indicative of full exchange. The time course of appearance of activity correlated well with the time required for dissociation of DIP-ClpP and exchange of active ClpP into the complex (see below) and was similar to that observed for the reverse exchange reaction.

ClpAP Complex Dissociation Is Slow without ATP Hydrolysis. The association of ClpA and ClpP is promoted by the ATP analogue, ATPγS, which is not hydrolyzed rapidly by ClpA (data not shown). To determine ClpAP dissociation rates in the presence of this analogue, we employed an assay that requires the ClpAP complex but not ATP hydrolysis. Cleavage of the peptide FAPHMALVPV is very rapidly catalyzed by ClpAP in the presence of ATPγS (*6*). DIP-ClpP was a very effective inhibitor of peptide degradation when added together with active ClpP (data not shown). In contrast, when DIP-ClpP was added to peptide degradation assay solutions after preassembly of active ClpAP complexes, inhibition was not complete even after 1 h of incubation (Figure 5). The half-time for dissociation of ClpAP in ATPγS was calculated by curve fitting to be about 29 min. When ATP was used instead of ATPγS in the same peptide degradation assay, the time course of inhibition after addition of DIP-ClpP was similar to that observed in the casein degradation assay (Figure 5) and gave a calculated $t_{1/2}$ of 6 min.

Direct Demonstration of DIP-ClpP Exchange out of ClpAP Complexes. To confirm that the kinetics of inhibition corresponded to the kinetics of dissociation of ClpAP, direct exchange between free ClpP and ClpP in complexes with ClpA was measured. ClpAP complexes were assembled in the presence of ATP and [^{3}H]DIP-ClpP and then mixed with excess nonradioactive ClpP. Samples removed at different times were centrifuged through Millipore membranes (300K molecular weight cutoff), which retain ClpAP complexes but allow passage of free ClpP oligomers. Table 2 shows that [^{3}H]DIP-ClpP was exchanged out of the complex and recovered in the filtrate with a half-time of about 6 min.

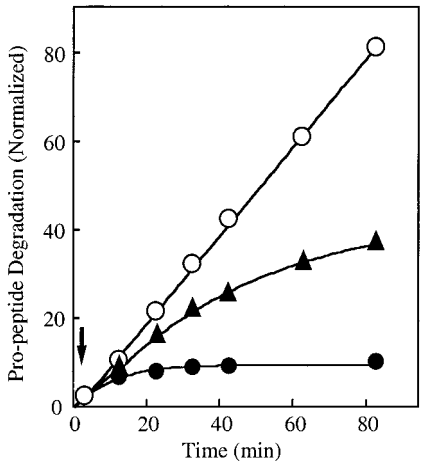

FIGURE 5: Exchange of DIP-ClpP for ClpP is slower in the absence of ATP hydrolysis. Propeptide degradation reactions were initiated with 0.2 μg of ClpA and 1 μg of active ClpP in the presence of either 4 mM ATP (●) or 1 mM ATPγS (▲). After 2.5 min, 2 μg of DIP-ClpP was added and samples were taken at different times to measure cleavage of the peptide. Buffer was added to control assays (○). Controls with ATP and ATPγS were nearly identical, and only data with ATP are shown. Data were normalized to unit activity and analyzed as described in the legend to Figure 4.

When ATPγS was used to assemble the complex of ClpA with [^{3}H]ClpP complex, no exchange was detectable 30 min after addition of excess ClpP. Thus, the ClpAP complexes dissociate slowly in the presence of ATP and are considerably more stable in the presence of ATPγS. The slower dissociation of DIP-ClpP from ClpAP complexes compared to dissociation of ClpP measured by kinetics of inhibition reflects the higher affinity with which inactivated ClpP binds to ClpA.

ClpAP Complexes Made with C-Terminal ATPase Mutants Are More Stable Than Those Made with N-Terminal Mutants. On the basis of previous results, we expected that ClpAP complexes formed with mutants with reduced ATPase activity would be more stable, and that is exactly what we observed. The half-times for ClpAP dissociation with the various ClpA mutants are shown in Table 2. The N-terminal ClpA mutant complexes had half-times of dissociation similar to that of wild-type ClpA, and the C-terminal mutant complexes were very stable. These data are consistent with those obtained by measuring ClpA hexamer dissociation and suggest that dissociation of ClpAP complexes is favored by a conformation of ClpA that may occur during cycles of ATP hydrolysis.

Dissociation of ClpA Subunits from the ClpAP Complex. We wanted to measure ClpA exchange out of complexes with ClpP, but we were unable to use ClpA-K220Q for this purpose because mixed hexamers of K220Q and wild-type ClpA have activity in the presence of ClpP (*12*). We therefore used another mutant, K220Q/K501Q, which interferes with wild-type ClpA activity even in the presence of ClpP. Dissociation of ClpAP complexes could proceed by one of two pathways: the ClpA hexamers could dissociate from ClpP tetradecamers, or ClpA subunits could dissociate from hexamers within the ClpAP complex. To distinguish between these pathways, we tried to measure dissociation of ClpA subunits from ClpAP by adding an inactive mutant of ClpA to the preassembled ClpAP complexes. Because activities of ClpA-K220Q in hybrid complexes with active ClpA are

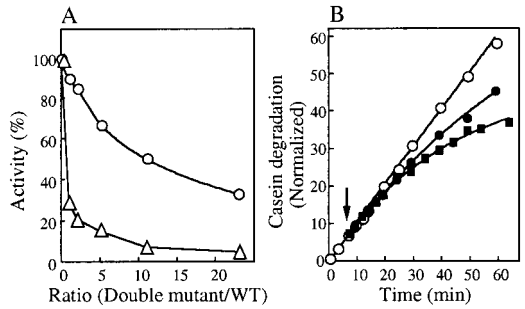

FIGURE 6: Exchange of ClpA subunits from the ClpAP complex. (A) Presence of the double mutant, ClpA-K220Q/K501Q, during assembly of wild-type ClpA inhibits activity. Wild-type ClpA was mixed with increasing amounts of ClpA-K220Q/K501Q in buffer B containing 1 mM DTT, and 0.05% Triton X-100, and aliquots (0.2 μg of wild-type ClpA in each) were added to ATPase assay solutions (○). For casein degradation assays (△), the mixtures of wild-type and mutant ClpA were added to assay solutions containing 5 μg of ClpP. (B) ClpA-K220Q/K501Q does not displace active ClpA from the ClpAP complex. Casein degradation assays with 1 mM (■) or 4 mM (●) ATP were initiated with 0.2 μg of wild-type ClpA and 1 μg of ClpP. After 2 min, excess ClpA-K220Q/K501Q was added to one set of assays (■, ●) and buffer was added to control assays (○). Aliquots were removed and the amount of casein degraded was measured. Data were normalized as described in Figure 4.

partly restored by interaction with ClpP (*12*), we could not use this mutant in the presence of ClpP. We were able to use the double mutant ClpA-K220Q/K501Q, which has virtually no activity itself but interferes with wild-type ClpA activity even in the presence of ClpP. Figure 6A shows that adding excess ClpA-K220Q/K501Q simultaneously with active ClpA resulted in partial inhibition of ATPase activity and complete inhibition of ClpP-dependent casein degradation. It is important to note that ClpA-K220Q/K501Q does not appear to interact with ClpP as judged by electron microscopy and gel filtration (data not shown). Thus, interference with activity requires interaction between subunits of the double mutant and wild-type ClpA. To measure the kinetics of inhibition, reactions were initiated with preformed ClpAP complexes, and ClpA-K220Q/K501Q was added after several minutes. Figure 6B shows that inhibition of casein degradation after addition of excess ClpA-K220Q/K501Q was time-dependent. The rate of inhibition was slow, implying that ClpA subunit dissociation from ClpAP complexes is also slow. The rate was much slower than seen for ClpA dissociation from hexamers, indicating that ClpP stabilizes the hexamer state of ClpA. Dissociation was slower at 4 mM ATP ($t_{1/2} \sim 56$ min) than at 1 mM ATP ($t_{1/2} \sim 29$ min), paralleling the effect of ATP on ClpA dissociation. The half-time for inhibition at 4 mM ATP can be converted to a k_{off} of 0.012 min^{-1} for ClpA subunit dissociation from the complex compared to a k_{off} of 0.092 for ClpP (calculated from Table 2). Thus, we can conclude that dissociation of ClpAP complexes proceeds by separation of ClpP from intact ClpA hexamers followed by dissociation of ClpA hexamers.

Assembly and Disassembly of ClpA and ClpAP Measured by Light Scattering. Formation of ClpA hexamers and association between ClpA and ClpP was monitored by changes in light scattering, which allowed us to estimate the rates of assembly and disassembly. Addition of saturating ATP or ATPγS to ClpA led to a 3−4-fold increase in light scattering, in good agreement with the increase expected for

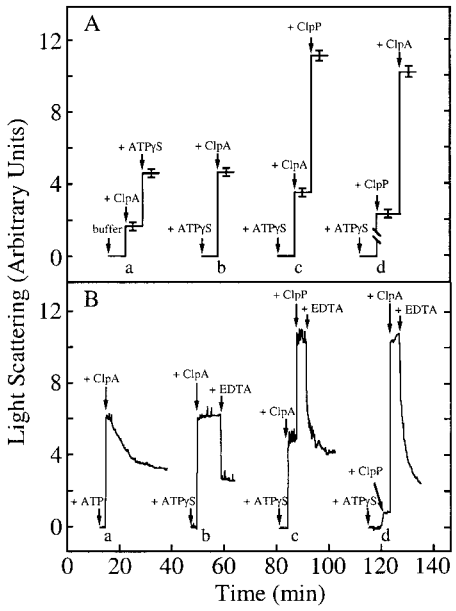

FIGURE 7: Light scattering changes upon assembly and disassembly of ClpA and ClpAP. Light scattering was measured at room temperature (except where noted) in 50 mM Tris, pH 7.5, 0.2 M KCl, 1 mM DTT, 10% (v/v) glycerol, and 25 mM $MgCl_2$. ATPγS (2 mM) or ATP (4 mM) was added as indicated. (A) Assembly of ClpA hexamers and ClpAP complexes. Light scattering signals were normalized to correct for variations in signal intensities obtained at different times. The values shown represent averages of 2−4 measurements made on separate samples; error bars reflect variations (usually 5−10%) between experiments. (a) ClpA (100 μg/mL) was added to buffer followed by ATPγS. (b) ATPγS was added before ClpA. (c) ClpA (90 μg/mL) was added to buffer with ATPγS followed by ClpP (27 μg/mL) to generate 2:1 ClpAP complexes. (d) ClpP (220 μg/mL) was added to buffer with ATPγS, followed by addition of ClpA (90 μg/mL) to generate 1:1 ClpAP complexes. (B) Time traces of assembly and disassembly of ClpA hexamers and ClpAP complexes. Buffer was equilibrated in a cuvette, proteins were added, and light scattering measurements were initiated within 15 s after each addition. (a) ClpA (100 μg/mL) was added to buffer containing 4 mM ATP equilibrated at 37 °C. (b) ClpA (100 μg/mL) was added to buffer with ATPγS, and after 20 min, EDTA was added in 2 mM excess over Mg. (c) ClpA (90 μg/mL) was added to buffer with ATPγS; after 5 min, ClpP (55 μg/mL) was added; after another 5 min, EDTA was added in excess over Mg. (d) ClpP (27 μg/mL) was added to buffer with ATPγS, and after 3 min, ClpA (90 μg/mL) was added, followed by excess EDTA after another 5 min.

formation of hexamers from mixtures of monomers and dimers (Figure 7A). Oligomerization occurred within the time of mixing (~10 s) at 37 and at 5 °C regardless of the order of addition of ClpA and ATPγS (Figure 7B). Titrations with nucleotides indicated that half-maximal light-scattering was obtained with ~100 μM ATP or ATPγS (data not shown). These values are close to the $S_{0.5}$ for these nucleotides in enzymatic assays and suggest that association of ClpA can be rate-limiting at low nucleotide concentrations. Mg^{2+} was required for complete hexamer formation, and addition of EDTA to chelate the Mg^{2+} led to a rapid dissociation of preassembled ClpA (Figure 7B).

ClpA assembled in ATP slowly dissociated as the ATP was hydrolyzed (Figure 7B), whereas in ATPγS hexamers were stable for up to 1 h. Lowering the ATPγS concentration to 25 μM after formation of ClpA hexamers led to partial dissociation within 2−5 min (data not shown). At this concentration, nucleotide may still be bound to the domain

II site but should not be saturating at the domain I site (*12*). When ClpA assembled in the presence of ATPγS was run over a gel-filtration column in the absence of nucleotide, about 70% of the ClpA dissociated to a species with the mobility expected for dimers (data not shown). Thus, nucleotide binding converts ClpA to a stable conformation in which hexamer associations are favored, and this conformation requires the continued presence of nucleotide at the domain I ATP site.

Mixing ClpA and ClpP produced a further increase in light scattering. Association was complete within a few seconds even at reduced temperatures. When ClpP was titrated into a solution of ClpA, the light scattering reached a plateau at ~3.5 times that of the ClpA hexamer alone (Figure 7A), a value consistent with a complex of two ClpA hexamers with one ClpP tetradecamer. When ClpA was added to an excess of ClpP, the light scattering increased to only 2.5 times that of the ClpA hexamer (Figure 7A), as expected for a 1:1 complex of ClpA hexamers and ClpP tetradecamers. These light scattering results are consistent with electron microscopic data and sedimentation velocity experiments, which show that both 1:1 and 2:1 complexes of ClpA hexamers and ClpP tetradecamers can be formed depending on the ratio of the proteins. Addition of EDTA to ClpAP complexes led to a slower dissociation than seen with ClpA alone (Figure 7B), suggesting that ClpP binding stabilizes the nucleotide-bound state of ClpA and may alter the accessibility of the active site to the chelating agent.

DISCUSSION

ATP-dependent protease complexes, such as ClpAP and the 26S proteasome, have been likened to molecular organelles, because the proteolytic active sites are sequestered within an internal aqueous chamber accessible by narrow axial channels (*5*). Entry of protein substrates requires an energy-intensive mechanism for selection, preparation, and presentation of appropriate substrates for degradation. Conformational changes within ClpA and ClpP and changes in interaction upon nucleotide binding and hydrolysis can be expected to alter the number and possibly the nature of substrate binding sites, to facilitate substrate unfolding (*9*), and to open channels and internal chambers within the complex, allowing translocation of substrates to the proteolytic component. The more complex 26S proteasome has additional enzymatic and regulatory components that make that enzyme more responsive to cell-specific signals and other controls over proteolysis (*16*). The oligomeric structures of ClpA and the ClpAP complex are stabilized by nucleotide binding (*2*), and a similar dependence on nucleotide binding is observed for the 26S proteasome (*17*). A central issue is the relative stability of these multiprotein complexes during catalytic turnover of protein substrates.

We designed experiments to test this aspect of the model for ATP-dependent protease action, specifically, whether the cycle of protein binding, unfolding, translocation, and degradation occurs within the lifetime of the intact ClpAP complex. Recent data (*9*) showed that a significant fraction of a protein substrate bound in a stable complex to ClpA or ClpAP could be degraded without release and rebinding. In this paper we have presented data showing that the ClpAP complex, once assembled, remains as an intact complex through multiple rounds of protein substrate turnover. Thus, the intact complex is the enzymatically active form of ClpAP, and no steps in the degradation pathway require separation of the components to a degree that allows exchange of subunits or components.

The data suggest that the conformation of ClpA and the interaction between ClpA and ClpP are altered by ATP hydrolysis and ADP/ATP exchange, even if ClpA and ClpP do not dissociate during the catalytic cycle. Dissociation of ClpA and ClpAP is faster in the presence of ATP and ATPγS (Figure 5 and Table 2), which suggests that ATP hydrolysis does produce a transient conformation from which dissociation might occur. However, if this state is generated during each cycle of ATP hydrolysis (k_{cat} = 140 min^{-1}), dissociation from this state is still very unfavorable and occurs in fewer than 1 in 100 cycles. The symmetry mismatch at the bonding interface between rings of hexameric ClpA and heptameric ClpP implies that some degree of movement of one ring about the other may occur during catalysis. Partial or complete rotation of the two rings (either by an oscillatory or a ratcheting motion) would require contacts that are sufficiently stable to keep the two rings associated throughout a degradation cycle but fluid enough to allow the rings to slip from one position to the next. The results in this paper show that the association has a long enough lifetime to allow such rotational motions to be repeated many times. It may be that the partial separation of ClpA and ClpP during cycles of ATP hydrolysis is required for such movement of the rings about each other, and that this in turn facilitates translocation of substrates.

The high affinity of ClpA and ClpP for each other and the slow rate of exchange suggests that ClpA hexamers and ClpAP complexes should be stably maintained under physiological conditions in which ATP concentrations are high (2−4 mM). While ClpA can be shown to have chaperone activity on its own in vitro, it has not been shown that any activities in vivo require ClpA free of ClpP. Recent data have shown that in vitro the intact ClpAP complex can bind inactive RepA and release a significant fraction of it in an active conformation before it is degraded (*10*). Thus, ClpAP complexes may function in vivo in both protein remodeling and degradation pathways.

ClpA has two ATP-binding consensus sequences, and mutational studies have indicated that both sites have ATPase activity and are required for full expression of activity in stimulating protein degradation by ClpP (*12*). The assembly defect of ClpA-K220Q suggested that domain I plays the major role in formation of ClpA hexamers (*12*). Our data showing the faster exchange rate for ClpA-K220R and ClpA-K220V at low ATP concentrations further support this conclusion. Domain I activity is also required for expression of molecular chaperone activity (*10*). Since proteins would be expected to interact with the chaperone component first, it is likely that the distal tier seen in electron micrographs of ClpA is the N-terminal domain. The domain II site has a high affinity for nucleotide and may be responsible for 80−90% of the ATP hydrolysis. Domain II is required for the major energy-dependent step in protein degradation (*12*), which presumably involves translocation of unfolded regions of protein substrates into the ClpP proteolytic active sites. Data presented here indicate that ATPase activity in domain II is required to allow dissociation of ClpA from ClpP,

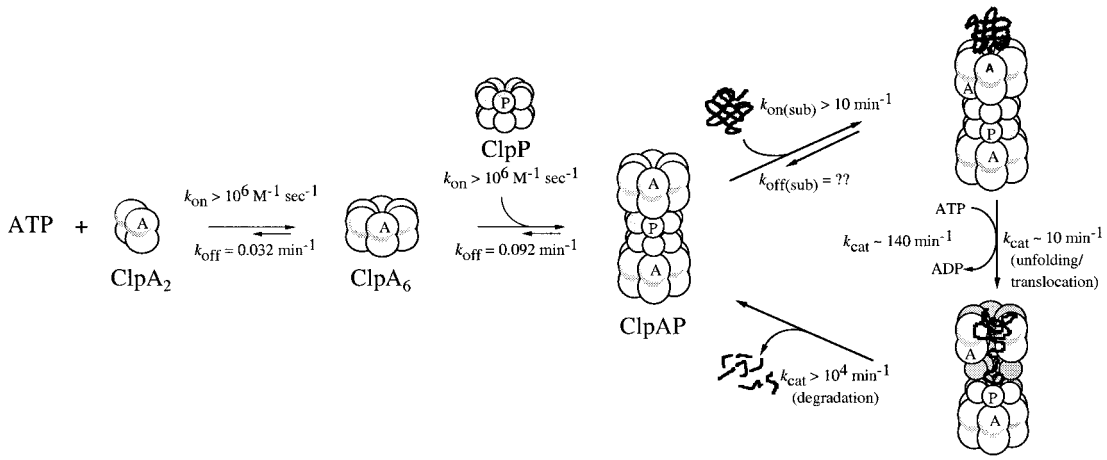

FIGURE 8: Kinetic constants for ClpAP assembly and reaction pathway. The k_{on} for formation of ClpA hexamers and ClpAP complexes are estimated to be $>10^6$ because assembly occurs within 10 s of mixing at submicromolar concentrations (this study). Second-order rate constants are used for ClpA assembly assuming that addition of a dimer to rapidly formed tetramers is rate-limiting. Assembly of ClpAP is given a second-order rate constant, because 1:1 complexes are fully active (*2*). Off rates from ClpA hexamers and ClpAP complexes were calculated in this study. The k_{cat} for ATP hydrolysis and peptide bond cleavage are from published data (*11*). The rate-limiting step in degradation is assumed to reflect unfolding and/or translocation; k_{cat} is estimated from the turnover number for α-casein monomers (*15*). The k_{on} for substrate is a pseudo-first-order constant (k'[E]) reflecting binding of substrate present in molar excess over enzyme and is estimated only to be greater than that for the rate-limiting step. Substrate dissociation rates have not been measured and may depend on the nucleotide state of the enzyme (*10*, *18*).

suggesting that ATP hydrolysis at this site has direct effects on contacts between them. We believe that these data are consistent with direct interaction between domain II and ClpP.

A summary of our current kinetics data on assembly of ClpAP and the catalytic steps is presented in Figure 8. The association/dissociation properties of other ATP-dependent proteases, such as the proteasome and Lon families, which also undergo nucleotide-dependent oligomerization, would be expected to parallel those of ClpAP. Protein unfolding and translocation should occur within the intact complexes, which would help explain the high degree of processivity observed for protein degradation by ClpAP and other proteases.

Since the rates of peptide bond cleavage and ATP hydrolysis are both more than 100 times faster than protein turnover (*11*), the rate-limiting step in degradation is either substrate binding, unfolding, or translocation. Data obtained with preassembled substrate complexes still leave the question open. Pak and Wickner (*18*) showed that RepA binding to ClpA involves at least two stages, initial binding and conversion to a committed complex from which dissociation is much slower. The time course for conversion to the committed state is in the range expected for a rate-limiting step in the unfolding/degradation reaction pathway. Whether conversion to the more stable complex is a pure binding step or involves some degree of unfolding of the substrate is not known. Even from committed complexes, only a fraction (<30%) of bound substrate is degraded, suggesting that translocation to ClpP may be slower than release when ATP hydrolysis is allowed (9). It is possible that the rate-limiting step will change depending on the number and nature of structural elements in the substrate protein.

Clp ATPases have significant sequence and structural similarities to other ATPases. In addition to ClpA, ATPases that tend to oligomerize to form hexameric rings include NSF (*19*), yeast Vps4p (*20*), the *E. coli* Rho transcription terminator (*21*), polyoma large T antigen (*22*), F1 ATPase (*23*), and presumably, the base complex of the yeast 26S protease (*24*). Some of these proteins are members of the AAA family, which, like ClpA, mediate protein conformational changes resulting in protein unfolding, protein−protein interactions, or changes in domain interactions within complex protein structures. A common property among these proteins is that assembly of the hexamer (e.g., Vsp4p) or association of the hexamer with other proteins in the functional complexes (e.g., the 26S proteasome) is dependent on ATP binding. As we have shown with ClpAP, complete disassembly may not occur frequently once the complex is assembled. However, with many similar complexes, loosening of subunit interactions during the cycles of ATP hydrolysis may be an important aspect of the conformational changes catalyzed by ATP hydrolysis. Such changes may be needed to allow expansion of channels or interior chambers in the complexes or to accommodate protein substrates during transfer from one component to another. Subunit dissociation could also contribute to regulation of activity by affecting the turnover of dissociated subunits in vivo. ClpA is rapidly degraded both in vivo and in vitro (*25*), especially in the absence of other good substrates. It is possible that other ATPases with association properties similar to those of ClpA also tend to be degraded in vivo. Since substrate binding did not appear to affect the association constant for ClpA, the exact mechanism of autodegradation of ClpA and its inhibition by substrates is unclear. These effects are currently under investigation.

NOTE ADDED IN PROOF

The ability of *E. coli* ClpA to promote ATP-dependent global unfolding of proteins was recently demonstrated in the laboratory of A. Horwich (*26*).

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