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Distinctive Roles of the Two ATP-binding Sites in ClpA, the ATPase Component of Protease Ti in *Escherichia coli**

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ClpA is the ATPase component of the ATP-dependent protease Ti (Clp) in *Escherichia coli* and contains two ATP-binding sites. A ClpA variant (referred to as ClpAT) carrying threonine in place of the 169th methionine has recently been shown to be highly soluble but indistinguishable from the wild-type, 84-kDa ClpA in its ability to hydrolyze ATP and to support the casein-degrading activity of ClpP. Therefore, site-directed mutagenesis was performed to generate mutations in either of the two ATP-binding sites of ClpAT (*i.e.* to replace the Lys^{220} or Lys^{501} with Thr). ClpAT/K220T hydrolyzed ATP and supported the ClpP-mediated proteolysis 10–50% as well as ClpAT depending on ATP concentration, while ClpAT/K501T was unable to cleave ATP or to support the proteolysis. Without ATP, ClpAT and both of its mutant forms behaved as trimeric molecules as analyzed by gel filtration on a Sephacryl S-300 column. With 0.5 mM ATP, ClpAT and ClpAT/K501T became hexamers, but ClpAT/K220T remained trimeric. With 2 mM ATP, however, ClpAT/K220T also behaved as a hexamer. These results suggest that the first ATP-binding site of ClpA is responsible for hexamer formation, while the second is essential for ATP hydrolysis. When trimeric ClpAT/K220T was incubated with the same amount of hexameric ClpAT/K501T (*i.e.* at 0.5 mM ATP) and then subjected to gel filtration as above, a majority of ClpAT/K220T ran together with ClpAT/K501T as hexameric molecules. Furthermore, ClpAT/K501T in the mixture strongly inhibited the ability of ClpAT/K220T to cleave ATP and to support the ClpP-mediated proteolysis. Similar results were obtained in the presence of 2 mM ATP and also with the mixture with ClpAT. On the other hand, the ATPase activity of the mixture of ClpAT and ClpAT/K220T was significantly higher than the sum of that of each protein, particularly in the presence of 2 mM ATP, although its ability to support the proteolysis by ClpP remained unchanged. These results suggest that a rapid exchange of the subunits, possibly as a trimeric unit, occurs between the ClpAT proteins in the presence of ATP and leads to the formation of mixed hexameric molecules.

Protease Ti, also called Clp, consists of two different multimeric components, both of which are required for ATP-dependent proteolysis in *Escherichia coli* (1–4). While component A (ClpA) contains the ATP-hydrolyzing sites, component P (ClpP), which is a heat shock protein (5), contains the serine active sites for proteolysis. The isolated ClpA shows protein-activated ATPase activity, which in the reconstituted enzyme is linked to protein breakdown.

The *clpA* gene has recently been shown to contain dual initiation sites for translation and therefore to encode two polypeptides with different sizes (*i.e.* 84- and 65-kDa subunits), of which the smaller polypeptide is derived from the internal start site (6). Accordingly, mutagenesis of the 5′-end AUG codon results in an exclusive synthesis of the 65-kDa protein, while mutation at the internal 169th AUG codon (Met) to ACG (Thr) produces only the 84-kDa protein (henceforth referred to as ClpAT). In addition, the purified ClpAT has been found to be highly soluble and to show little or no nonspecific interaction with gel filtration matrices, unlike the wild-type, 84-kDa ClpA (7, 8). Nevertheless, ClpAT is indistinguishable from the 84-kDa ClpA in its ability to cleave ATP and to support the ClpP-mediated protein degradation (6). The isolated ClpAT behaves as a trimer in the absence of ATP but as a hexameric complex in its presence (6, 9).

ClpA is a member of a family of highly conserved proteins that have two regions of particularly high homology, each of which contains a consensus sequence for an adenine nucleotide binding (10, 11). Both of the ATP-binding regions are characterized by the presence of Gly-X_2-Gly-*X*-Gly-Lys-Thr elements, of which the lysine residue interacts with one of the phosphoryl group of the bound nucleotide (12, 13). hsp104 is also a member of the highly conserved protein family and contains the two consensus ATP-binding sites (14). The isolated hsp104 exists predominantly as monomer or dimer in the absence of ATP but oligomerizes to form a hexameric complex in its presence (15). In addition, mutational analysis has revealed that both of the ATP-binding sites of hsp104 are necessary for its *in vivo* function, such as tolerance to high temperatures and high concentrations of ethanol (14). It has also been demonstrated that the second ATP-binding site is primarily responsible for the oligomerization of hsp104 (15).

In an attempt to determine the structural and functional relationship of ClpA, we generated two mutant forms of ClpAT, in which the Lys^{220} or Lys^{501} residue in the ATP-binding sites was replaced with Thr, and examined the effects of the mutations on the ATPase activity of ClpAT and on its ability to support the ATP-dependent proteolysis by ClpP. We also examined whether any of the mutations affected the hexamer formation of ClpAT in the presence of ATP. In addition, we examined the effects of the ClpAT mutant proteins on the structure and function of ClpAT.

EXPERIMENTAL PROCEDURES

Materials—Construction of the Bluescript KS^+ plasmid carrying the *clpA* gene and replacement of the 169th AUG codon (methionine) with ACG codon (threonine) in the gene were performed as described previously (6). The resulting recombinant plasmid was referred to as pClpAT. An *E. coli clpA* null mutant SG21118 (*clpA*319::Δ*kan*) that was

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originally constructed by Drs. S. Gottesman and M. R. Maurizi (National Institutes of Health) was obtained from Dr. A. L. Goldberg (Harvard Medical School). Oligonucleotide primers were synthesized using an automated DNA synthesizer (Applied Biosystems model 384A). [^{3}H]Methyl casein was prepared as described (16).

Site-directed Mutagenesis—Single-stranded, uracil-containing phagemids of pClpAT were prepared by infecting *E. coli* strain CJ236 (*dut$^-$ ung$^-$*) with helper phage R408 (17). Mutagenic oligonucleotides were designed to replace Lys^{220} of ClpAT with Thr (primer 1, 5′-GGTGTCGGTACCACCGCGATTG-3′ or Lys^{501} with Thr (primer 2, GGGGTCGGGACCACAGAGGTGA) and to generate new restriction sites for facilitating mutant isolation. The mutated nucleotides are indicated by boldface letters, and the newly created restriction sites for *Kpn*I and *Ava*II, respectively, in primers 1 and 2, are underlined. The primers were annealed to the template DNA and extended (18). The double-stranded mismatch plasmids were then transformed, and the mutated vectors were confirmed by restriction and DNA sequence analysis (data not shown).

Protein Purification—ClpP was purified as described previously (8). For purification of ClpAT and its mutant forms, appropriate *E. coli* cells (10 g) in 50 ml of 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 6.5) containing 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, and 20% (w/v) glycerol were disrupted by French press at 14,000 p.s.i. and centrifuged for 2 h at 120,000 × *g* (6). The supernatants were dialyzed against the same buffer and loaded onto a phosphocellulose column (1.5 × 5 cm). After extensively washing the column with the buffer, proteins were eluted with a linear gradient of 0.1–0.4 M phosphate. Aliquots of the fractions were electrophoresed in 10% (w/v) polyacrylamide slab gels containing SDS, and proteins in the gels were visualized by staining with Coomassie Blue R-250. The fractions containing the 84-kDa proteins were pooled and dialyzed against 20 mM Tris-HCl buffer (pH 7.8) containing 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 0.2 M NaCl, and 20% glycerol. Each of the protein samples was then chromatographed on a heparin-agarose column (1 × 8 cm) equilibrated with the Tris buffer. Proteins bound to the column were eluted with a linear gradient of 0.2–0.4 M NaCl, and the fractions containing the 84-kDa proteins were pooled and kept frozen at −70 °C for further use.

Assays—ATPase activity was assayed by incubating the reaction mixtures (0.1 ml) containing appropriate amounts of the mutated ClpA proteins and ATP, 100 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM EDTA (19). After incubating the mixtures at 37 °C for 30 min, the reaction was terminated by adding 0.1 ml of 1% SDS. The inorganic phosphates released were then determined as described (20). Protein degradation was assayed by incubating similar reaction mixtures (0.1 ml) containing [^{3}H]casein for 1 h at 37 °C (8). After the incubation, radioactivity released into trichloroacetic acid-soluble products was counted using a scintillation counter. Proteins were assayed by their absorbance at 280 nm or by the dye-binding method (21) using bovine serum albumin (BSA)[1] as a standard.

RESULTS

ATP Hydrolysis by ClpAT Mutants—To investigate the importance of the two ATP-binding sites for ClpA function, either Lys^{220} or Lys^{501} in ClpAT was substituted with threonine by site-directed mutagenesis. The pClpAT plasmids carrying the mutations were transformed into *E. coli clpA* null mutant cells, and two mutant forms of ClpAT (ClpAT/K220T and ClpAT/K501T) were purified from the cells to apparent homogeneity (Fig. 1).

To determine the effects of the mutations on ATP hydrolysis, the purified proteins were incubated in the presence of increasing concentrations of ATP. As shown in Fig. 2, *A* and *B*, ClpAT/K220T hydrolyzed ATP 10–50% as well as ClpAT, depending on ATP concentration. However, casein was still capable of stimulating the ATPase activity of ClpAT/K220T by about 2-fold, similar to that of ClpAT, indicating that the mutation shows little or no effect on the interaction of casein with the mutant protein. Upon double-reciprocal plot of the data obtained in the presence of casein, the K_m for ATP was estimated to be 0.21 and 1.1 mM for ClpAT and ClpAT/K220T, respectively. In addition, the V_{max} for ATP cleavage by ClpAT was estimated to be 0.51 nmol/min, and that by ClpAT/K220T was

[1] The abbreviation used is: BSA, bovine serum albumin.

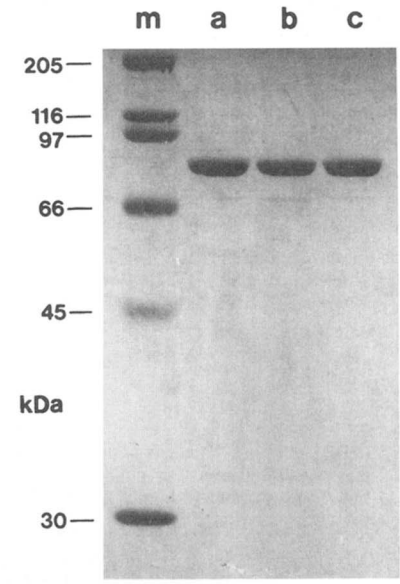

FIG. 1. **Polyacrylamide gel electrophoresis of the purified mutant forms of ClpAT.** ClpAT (*lane a*) and its mutant forms, ClpAT/K220T (*lane b*) and ClpAT/K501T (*lane c*), were purified as described under "Experimental Procedures." 10 μg of each were then electrophoresed on a 10% slab gel containing SDS (22). After electrophoresis, the gel was stained with Coomassie Blue R-250. *Lane m* indicates the size markers (from *top* to *bottom*): myosin heavy chain, β-galactosidase, phosphorylase *b*, BSA, ovalbumin, and carbonic anhydrase.

0.26 nmol/min. In the absence of casein, the V_{max} for each of the proteins was reduced to about one-half of the values seen in its presence while the K_m values remained unchanged. Therefore, the mutation at the first of the two ATP-binding sites reduces not only the affinity of ClpAT to ATP but also its V_{max}. On the other hand, ClpAT/K501T showed little or no ATPase activity regardless of whether casein was present (Fig. 2*C*). Thus, it appears that the ATPase activity of ClpA depends more strictly on the second ATP-binding site than the first.

Ability of ClpAT Mutants in Supporting ClpP-mediated Proteolysis—To determine the effects of the mutations on the ability of ClpAT in supporting the proteolytic activity of ClpP, the mutant proteins were incubated in the presence of increasing concentrations of ATP. Similar to the effects on ATP hydrolysis, ClpAT/K220T supported the ClpP-mediated casein hydrolysis 10–50% as well as ClpAT, depending on ATP concentration (Fig. 3). On the other hand, ClpAT/K501T, which is almost completely devoid of ATPase activity, was not at all able to support the casein hydrolysis by ClpP. These results support the previous finding that ATP hydrolysis is essential for protein degradation by protease Ti (7).

Hexamer Formation of ClpAT Mutants—Since ATP has been shown to induce hexamer formation of ClpA (6, 9), we examined which of the two ATP-binding sites is involved in the oligomerization. The purified ClpAT and its mutant forms were each subjected to gel filtration on a Sephacryl S-300 column in the presence and absence of ATP. Because extents of the decrease in ATP cleavage by the mutation in the first site were dependent on ATP concentration (see Fig. 2), the gel filtration analysis was performed in the absence and presence of about one-half and twice the K_m for ATP of ClpAT/K220T (*i.e.* 0.5 and 2 mM, respectively). As shown in Fig. 4 (*open symbols*), all of the ClpAT and its mutant forms ran as trimeric molecules without ATP. In the presence of 0.5 mM ATP, ClpAT/K501T as well as ClpAT behaved as hexameric molecules, while ClpAT/K220T remained trimeric. Both ClpAT/K501T lacking ATPase activity and ClpAT also behaved as hexamers in the presence of ADP

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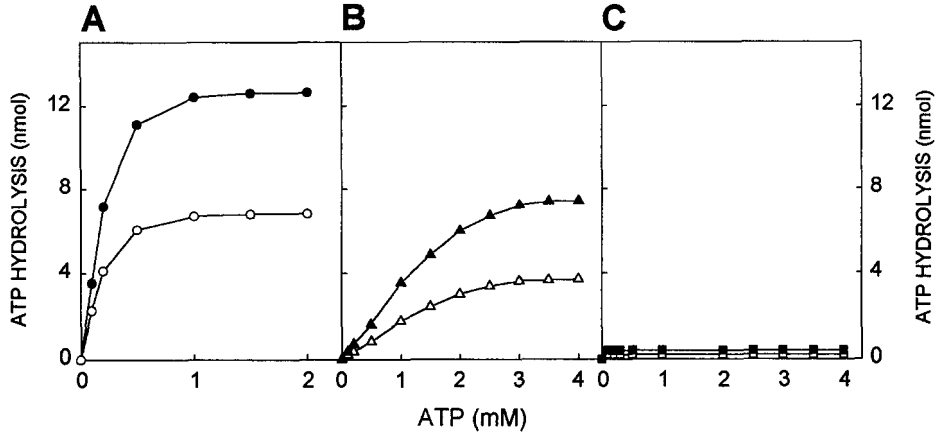

FIG. 2. **ATP hydrolysis by ClpAT and its mutant forms.** The ATPase activities of ClpAT (*A*), ClpAT/K220T (*B*), and ClpAT/K501T (*C*) were assayed in the absence (*open symbols*) and presence of 10 μg of casein (*closed symbols*). Reaction mixtures containing 0.2 μg of the purified proteins and various amounts of ATP were incubated at 37 °C for 30 min, and the inorganic phosphates released were determined.

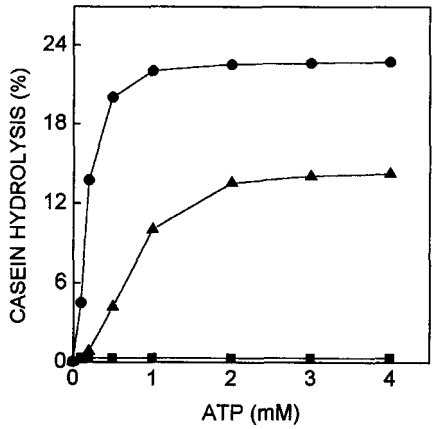

FIG. 3. **Ability of ClpAT and its mutant forms to support the ClpP-mediated proteolysis.** Casein hydrolysis was measured by incubating 0.2 μg of ClpAT (●), ClpAT/K220T (▲), or ClpAT/K501T (■), 0.1 μg of ClpP, and 10 μg of [^{3}H]casein in the presence of increasing amounts of ATP. Incubations were performed for 1 h at 37 °C, and the radioactivities released into acid-soluble products were determined using a scintillation counter.

(data not shown). Thus, hexamer formation of ClpA requires binding of the adenine nucleotides but not their hydrolysis. In the presence of 2 mM ATP, however, ClpAT/K220T also became a hexameric molecule. These results suggest that the first of the two ATP-binding sites in ClpAT is responsible for the hexamer formation and that the inability of ClpAT/K220T in hexamer formation at the low ATP concentration may be due to its reduced affinity to ATP.

Interaction of ClpAT/K220T with ClpP—In order to determine whether the mutation in the first ATP-binding site may also influence the interaction of ClpAT with ClpP, ATP hydrolysis by ClpAT/K220T was assayed in the presence of ClpP, casein, or both, and 2 mM ATP. In the absence of casein, ClpP increased the extent of ATP hydrolysis by ClpAT/K220T by about 20%, while the same protein reduced that by ClpAT by about 30% (Table I). In the presence of casein, however, ClpP increased the ATPase activities of both ClpAT/K220T and ClpAT to a greater extent than without casein. To clarify further this differential effect of ClpP, the ATPase activities of ClpAT and ClpAT/K220T were measured in the presence of increasing amounts of ClpP. In the absence of casein, ClpP increased the ATPase activity of K220T in a dose-dependent manner until a maximal effect was reached, unlike that of ClpAT (Fig. 5*A*). In the presence of casein, however, ClpP increased both of the activities (Fig. 5*B*). Moreover, the magnitude of the increase in the casein-activated ATPase activity of ClpAT/K220T was significantly higher than that of ClpAT. Similar effects of ClpP were observed when the same experiments were performed in the presence of 0.5 mM ATP (data not shown). Thus, it appears likely that the mutation in the first ATP-binding site also somehow influences the interaction of ClpAT with ClpP.

Interaction between ClpAT Mutants—Since it appears clear that the two ATP-binding sites play distinctive roles in the structure and function of ClpAT, we examined whether the ClpAT mutants can complement their defective roles with each other, such as in hexamer formation. When equal amounts of the ClpAT mutant proteins were mixed together in the presence of 0.5 mM ATP and subjected to gel filtration analysis as in Fig. 4, a majority of ClpAT/K220T, which alone behaves as a trimer at the ATP concentration, eluted together with ClpAT/K501T in the column fractions corresponding to the size of hexameric molecules (Fig. 6). Nearly identical results were obtained when the same experiments were performed with the mixture of ClpAT and ClpAT/K220T (data not shown). These results suggest that the hexameric ClpAT/K501T or ClpAT protein can interact with the trimeric ClpAT/K220T molecules and form a mixed hexameric complex.

We then examined the effect of ClpAT/K501T on the casein-activated ATPase activity of ClpAT or ClpAT/K220T in the presence of ClpP. As shown in Table II (experiment A), ClpAT/K501T strongly inhibited the ATPase activities of the latter proteins. Similar inhibitory effects were observed in the presence of 2 mM ATP, at which concentration all of the ClpAT proteins by themselves behave as hexameric molecules. On the other hand, the ATPase activity of the mixture of ClpAT and ClpAT/K220T was higher than the sum of that of each protein. Particularly in the presence of 2 mM ATP, ATP hydrolysis by the mixture of ClpAT and ClpAT/K220T was enhanced to an extent that could be seen by the doubled amount of ClpAT alone. Similar stimulatory or inhibitory effects of ClpAT/K220T or ClpAT/K501T, respectively, on the ATPase activity of ClpAT were observed when the same experiments were performed in the absence of casein, ClpP, or both (data not shown). Furthermore, the mixture of any of the two ClpAT proteins eluted in the fractions corresponding to a hexameric size from the Sephacryl S-300 column (data not shown). These results clearly suggest that the hexameric ClpAT proteins can also interact with each other and form hybrid molecules.

To determine whether the interaction between the ClpAT proteins may also influence their ability to support the ClpP-mediated proteolysis, casein hydrolysis was assayed by incubation of their mixtures in the presence of 0.5 and 2 mM ATP. Table II (experiment B) shows that ClpAT/K501T strongly inhibited the ability of ClpAT or ClpAT/K220T to support the proteolytic activity of ClpP, similar to its effect on ATP cleav-

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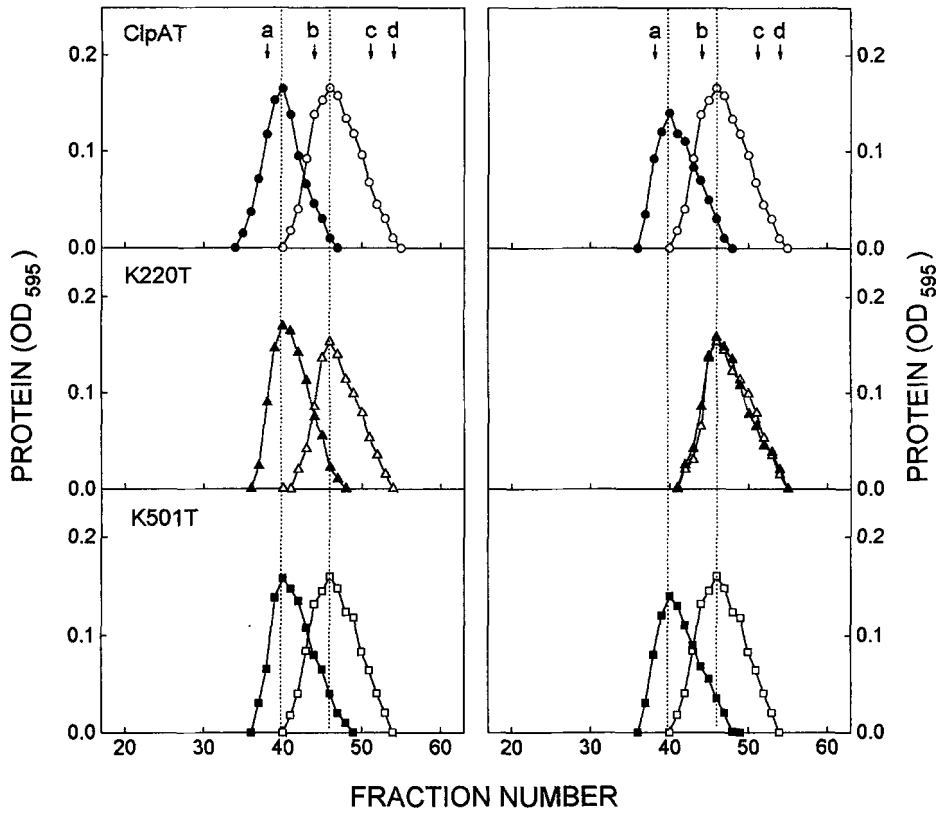

FIG. 4. **Size estimation of ClpAT and its mutant forms using a Sephacryl S-300 column.** Aliquots (0.2 mg each) of the purified ClpAT (●), ClpAT/K220T (▲), and ClpAT/K501T (■) were incubated with 0.5 (*right panel*) or 2 mM ATP (*left panel*) for 15 min at 4 °C. Each was then loaded onto the gel filtration column (1 × 40 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8) containing 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 0.1 M NaCl, and 0.5 or 2 mM ATP. *Open symbols* are for the proteins incubated and chromatographed without ATP. Fractions of 0.5 ml were collected at a flow rate of 7 ml/min, and aliquots of them were assayed for protein concentration. The *dotted lines* indicate the positions where the protein peaks eluted. The *arrows* show the size markers: *lane a*, thyroglobulin (669 kDa); *lane b*, apoferritin (443 kDa); *lane c*, alcohol dehydrogenase (150 kDa); *lane d*, BSA (66 kDa).

TABLE I
Effect of casein, ClpP, or both, on ATP hydrolysis by ClpAT and ClpAT/K220T

Reaction mixtures contained 2 mM ATP; 0.2 μg of ClpAT or ClpAT/K220T; and 0.1 μg of the purified ClpP, 10 μg of casein, or both. Incubations were performed for 30 min at 37 °C.

Addition	ATP hydrolyzed by	
	ClpAT	ClpAT/K220T
	nmol	
None	6.3	3.2
ClpP	4.7	4.5
Casein	12.3	6.8
Casein + ClpP	15.9	12.5

age. Furthermore, the inhibitory effects of ClpAT/K501T occurred in a dose-dependent manner on the ability of ClpAT or ClpAT/K220T in supporting the ClpP-mediated proteolysis as well as in ATP hydrolysis (Fig. 7), again indicating that ATP hydrolysis is tightly coupled to protein breakdown by protease Ti. On the other hand, ClpAT/K220T showed little or no effect on the ability of ClpAT in supporting the proteolysis by ClpP at both of the ATP concentrations (*i.e.* the casein-degrading activity of ClpP in the presence of their mixture was nearly identical to the sum of that seen with each protein) (Table II, experiment B). To clarify further the differential effect of ClpAT/K220T on casein hydrolysis from that on ATP cleavage, the same assays were performed as in Table II (experiment B) but by varying the incubation period. As shown in Fig. 8, nearly the same results were obtained at all time points for ATP hydrolysis and for casein degradation. These results suggest that the mutation in the first ATP-binding site may indeed influence the interaction of ClpA with ClpP, and therefore the enhanced ATP hydrolysis by the mixture of ClpAT and ClpAT/K220T could not increase its ability to support the ClpP-mediated casein degradation.

DISCUSSION

The present studies have demonstrated that the first of the two ATP-binding sites in ClpA is responsible for the ATP-mediated hexamer formation, while the second site is critical for the ATPase function. The mutation in the first site (K220T) prevents hexamer formation of ClpAT at 0.5 mM ATP but not at 2 mM. Of interest is the finding that the mutation results in the reduction of not only the affinity of ClpAT to ATP but also the V_{max} value for ATP hydrolysis and hence also decreases the ability of ClpAT to support the proteolytic activity of ClpP. It has been reported that the kinetics of proteolysis by protease Ti shows positive cooperativity with respect to ATP with a Hill coefficient of 1.6 (9). Therefore, it appears that binding of ATP to the first site influences the efficiency of its binding to the second site and thus the ability of ClpA to support the ClpP-mediated proteolysis.

Noteworthy was the finding that the mode of interaction of ClpP with ClpAT/K220T appears to differ from that with ClpAT. We have previously shown that ClpP reduces the rate of ATP hydrolysis by ClpA in the absence of casein but increases the ATPase activity in its presence (8). However, the ATPase activity of the mutant form of ClpAT was found to be increased by ClpP whether or not casein was present. Furthermore, the extent of the increase in ATP hydrolysis by ClpAT/K220T was significantly greater than that by ClpAT. Therefore, it appears that the reduced ability of the mutant form of ClpAT in supporting the ClpP-mediated proteolysis may also be attributed to the change in the mode of its interaction with ClpP.

On the other hand, the mutation in the second site (K501T) almost completely eliminated the ATPase activity of ClpAT but with little or no effect on the hexamer formation in the presence of 0.5 or 2 mM ATP. Furthermore, ClpAT/K501T can form a hexameric complex even in the presence of ADP, indicating that hexamer formation requires the binding but not the hydrolysis of the adenine nucleotides. Therefore, the mutation in the second ATP-binding site does not seem to exert any influ-

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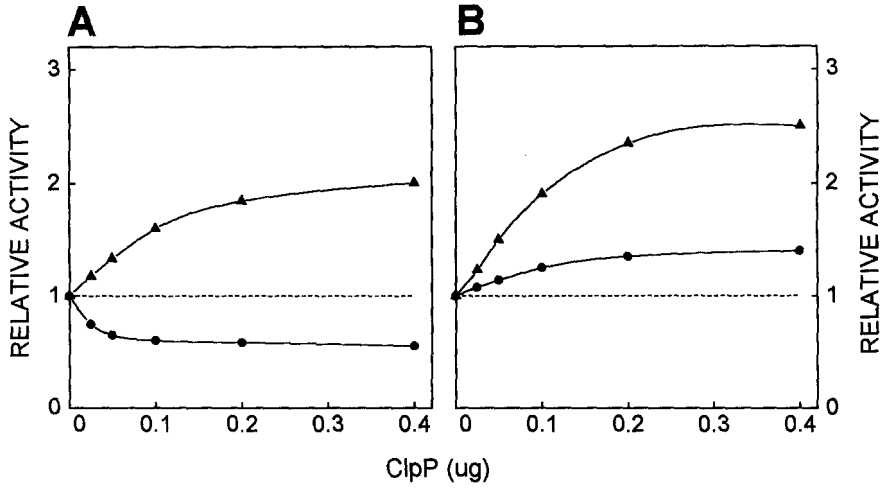

FIG. 5. **Effects of increasing concentrations of ClpP on the ATPase activities of ClpAT and ClpAT/K220T.** The ATPase activities of ClpAT (●) and ClpAT/K220T (▲) were assayed without (*A*) and with 10 μg of casein (*B*) as in Table I but in the presence of increasing amounts of ClpP. The assays were also performed with increasing amounts of BSA (*dotted lines*) instead of ClpP. However, the amounts of BSA added were in 50-fold excess of the indicated amounts of ClpP. The activities seen in the absence of ClpP are expressed as 1.0, and the others are expressed as their relative values.

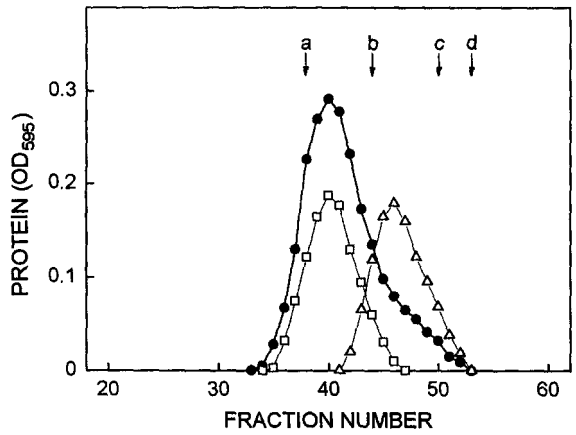

FIG. 6. **Elution of the mixture of ClpAT/K220T and ClpAT/K501T from a Sephacryl S-300 column.** The mixture of 0.2 mg each of ClpAT/220T and ClpAT/K501T (total 0.4 mg) containing 0.5 mM ATP (●) was incubated for 15 min at 4 °C and subjected to gel filtration on a Sephacryl S-300 column as described in the legend to Fig. 4 but in the presence of 0.5 mM ATP only. The same experiments were performed with 0.2 mg of ClpAT/K220T (△) or ClpAT/K501T (□) alone. The size markers (*arrows*) are the same as shown in Fig. 4.

TABLE II
Ability of the ClpAT proteins and their mixtures in ATP hydrolysis and in supporting the casein degradation by ClpP

ATP hydrolysis was assayed by incubating ClpAT, ClpAT/K220T, or ClpAT/K501T alone (0.1 μg) or their mixtures (0.2 μg) in the presence of 10 μg of casein for 30 min at 37 °C. The incubation mixtures also contained ClpP with one-half the amount of the ClpAT proteins. For casein hydrolysis, incubations were performed as above but in the presence of twice the amount of the Clp proteins.

Addition	Hydrolysis of ATP or casein in the presence of	
	0.5 mM ATP	2 mM ATP
Exp. A: ATP hydrolysis	*nmol*	
ClpAT	12.4	16.5
ClpAT/K220T	1.9	11.6
ClpAT/K501T	0	0
ClpAT + ClpAT/K220T	17.6	33.7
ClpAT + ClpAT/K501T	3.9	5.6
ClpAT/K220T + ClpAT/K501T	0.4	2.7
Exp. B: casein hydrolysis	%	
ClpAT	17.2	21.4
ClpAT/K220T	3.2	9.8
ClpAT/K501T	0	0
ClpAT + ClpAT/K220T	21.7	30.4
ClpAT + ClpAT/K501T	4.4	5.7
ClpAT/K220T + ClpAT/K501T	0.8	3.2

ence on the binding of ATP or ADP to the first site.

Of particular interest was the finding that the hexameric ClpAT or ClpAT/K501T can interact with the trimeric form of ClpAT/K220T and generate a mixed hexameric complex. Furthermore, ClpAT/K501T inhibited the ability of ClpAT or ClpAT/K220T in ATP hydrolysis and in supporting the ClpP-mediated proteolysis. These inhibitory effects of ClpAT/K501T could also be seen in the presence of 2 mM ATP, at which concentration all of the ClpAT proteins by themselves and the mixtures of any two of them behave as hexameric molecules. In addition, the ATPase activity of the mixture of ClpAT and ClpAT/K220T was found to be significantly higher than the sum of that of each protein, although its ability to support the proteolysis by ClpP remained unchanged. Therefore, it appears likely that a rapid exchange of the subunits occurs between the ClpAT proteins in the presence of ATP and results in the formation of hybrid molecules.

However, it is presently unclear whether the exchange of the subunits between the ClpAT proteins occurs at the level of a trimeric unit or otherwise. Upon the gel filtration analysis of hexameric ClpAT/K501T incubated with increasing amounts of trimeric ClpAT/K220T (*i.e.* at 0.5 mM ATP as in Fig. 6), we found that the right side shoulder of the protein peak increases much more significantly than the peak height itself (data not shown). These results suggest a possibility that each trimeric unit of hexameric ClpAT/K501T in the incubation mixture is interacting with a trimeric ClpAT/K220T to form a hybrid hexameric complex until all of the trimeric units of ClpAT/K501T are replaced and occupied by trimeric ClpAT/K220T molecules, and therefore the excess of ClpAT/K220T remains trimeric. This possibility is in part supported by the finding that the inhibitory effect of ClpAT/K501T on the ATPase activity of ClpAT or ClpAT/K220T does not reach completion even in the presence of a 2–6-fold excess of ClpAT/K501T (see Fig. 7*A* and data not shown). However, this possibility is based on the assumption that the hybrid molecule containing one of each trimeric unit of ClpAT and ClpAT/K501T should be partially active in ATP hydrolysis and in supporting the ClpP-mediated proteolysis. In addition another possibility, that the exchange of the subunits occurs at the level of a monomeric or dimeric unit and results in the formation of various kinds of hybrid molecules, cannot be totally excluded.

In contrast to the present findings that the first ATP-binding site is responsible for hexamer formation of ClpA and the second is for ATPase activity, Lindquist and co-workers (15) have recently demonstrated that a single amino acid substitution in the second ATP-binding site eliminates hexamer forma-

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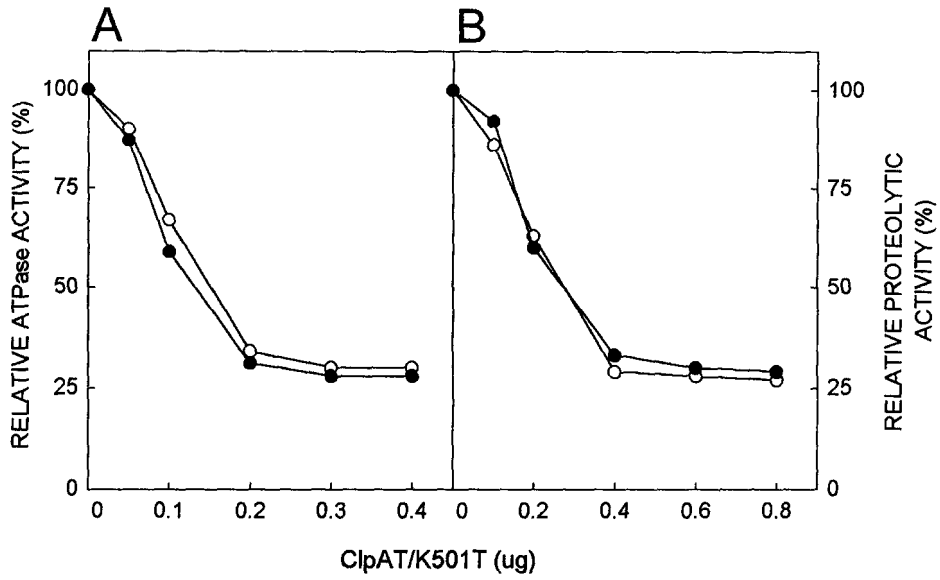

FIG. 7. **Effects of increasing concentrations of ClpAT/K501T on the ability of ClpAT or ClpAT/K220T in ATP hydrolysis (*A*) and in supporting the casein hydrolysis by ClpP (*B*).** ATP hydrolysis was assayed by incubating 10 μg of casein and 0.2 μg of ClpAT (●) or ClpAT/K220T (○) for 30 min at 37 °C in the presence of various amounts of ClpAT/K501T. The incubation mixtures also contained ClpP with one-half the amount of the ClpAT proteins. For casein hydrolysis, incubations were performed as above but in the presence of twice the amount of the ClpAT proteins.

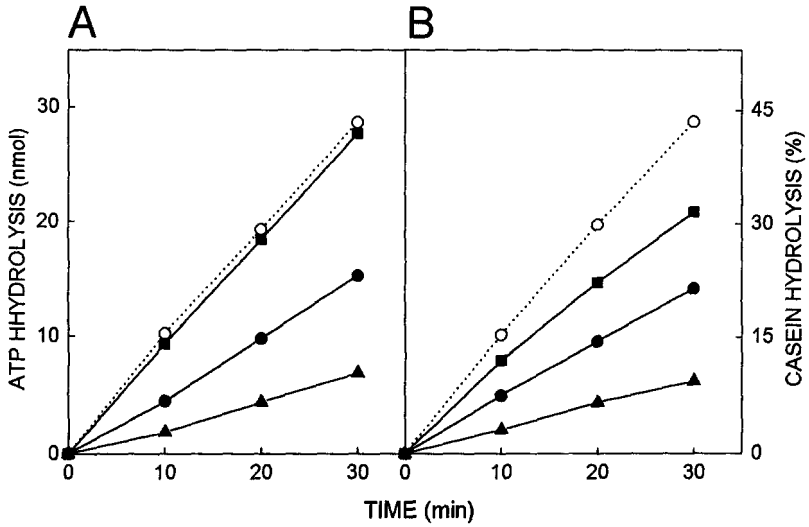

FIG. 8. **Effects of ClpAT/K220T on the ability of ClpAT in ATP hydrolysis (*A*) and in supporting the casein hydrolysis by ClpP (*B*).** ATP hydrolysis was assayed by incubating 10 μg of casein and 0.2 μg of ClpAT (●), 0.2 μg of ClpAT/K220T (▲), 0.2 μg each of both (■), or 0.4 μg of ClpAT (○) for various periods at 37 °C in the presence of various amounts of ClpAT/K501T. The incubation mixtures also contained ClpP with one-half the amount of the ClpAT proteins. For casein hydrolysis, incubations were performed as above but in the presence of twice the amount of the Clp proteins.

tion of hsp104 with only a slight defect in ATP hydrolysis. Furthermore, a mutation in the first site completely eliminates the ATPase activity of hsp104 without much influence on oligomerization. These reversed functions of the two ATP-binding sites are rather striking, because the sequences of each ATP-binding region containing the Gly-X_2-Gly-*X*-Gly-Lys-Thr elements in ClpA and hsp104 are known to be highly homologous with each other (14) and because in both studies the same lysine residue that interacts with one of the phosphoryl group of the bound nucleotide (12, 13) was substituted with threonine. Therefore, it appears that the distinctive functions (*i.e.* ATP hydrolysis and oligomerization) of the two ATP-binding sites may be determined by the tertiary structures of ClpA and hsp104, such as the proximity of any one of the two sites to the catalytic site for ATP hydrolysis or to certain unknown sequence(s) that is involved in oligomerization, but not by the sequences of the homologous ATP-binding regions themselves.

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