Vol. 217, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 5, 1995 Pages 41-51

REQUIREMENT OF ATP HYDROLYSIS FOR ASSEMBLY OF ClpA/ClpP COMPLEX, THE ATP-DEPENDENT PROTEASE Ti IN *ESCHERICHIA COLI*

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Received October 15, 1995

Summary: The ATP-dependent protease Ti (Clp) consists of two distinct components, ClpP containing the serine active sites for proteolysis and ClpA having two ATP-binding sites. A ClpA variant (ClpAT) carrying Thr in place of Met^{169} is highly soluble but indistinguishable from the wild-type ClpA in its ability to hydrolyze ATP and to support the ClpP-mediated proteolysis. Here we show that ATP hydrolysis is essential for assembly of ClpAT/ClpP complex upon analysis of the mixture of its components by gel filtration followed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Either ADP or adenosine 5'-(β,γ-imido)-triphosphate could not support the complex formation. Furthermore, ClpAT/K501T which carries a mutation in the second ATP-binding site and therefore is unable to cleave ATP could not interact with ClpP. On the other hand, ClpAT/K220T carrying a mutation in the first site and ClpP could be assembled into a complex at 2 mM ATP but not at 0.5 mM, at which concentration the trimeric mutant protein can not form a hexamer. These results indicate that assembly of protease Ti requires hydrolysis of ATP by ClpA in addition to its binding for hexamer formation.

Protease Ti, also called Clp, consists of two different components, both of which are required for ATP-dependent proteolysis in *E. coli* (1-4). While ClpA contains the ATP-hydrolyzing sites in 84-kDa subunits, ClpP consisting of 12-14 subunits of 21 kDa has the serine active sites for proteolysis. ClpA shows protein-activated ATPase activity, which in the reconstituted enzyme is linked to protein breakdown. In addition, it has recently been shown that a variant of ClpA, called ClpAT in which Met^{169} is replaced with Thr, is much more soluble in low salt buffers than the wild-type ClpA but is indistinguishable from the latter protein in its ability to cleave ATP and to support the ClpP-mediated protein degradation. The isolated ClpAT behaves as a trimer in the absence of ATP but as a hexameric complex in its presence (5,6).

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0006-291X/95 $12.00

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 (7,8). Both the ATP-binding regions are characterized by the presence of Gly-X_2-Gly-X-Gly-Lys-Thr elements, of which the Lys residue interacts with one of the phosphoryl group of the bound nucleotide (9,10). Upon site-directed mutagenesis of the Lys residues, the two ATP-binding sites in ClpA have been demonstrated to play distinctive roles: the first site being responsible for hexamer formation and the second site being essential for ATP hydrolysis (11,12). ClpAT/K220T, in which Lys^{220} in the first ATP-binding site is substituted by Thr, hydrolyzes ATP and supports the ClpP-mediated proteolysis 10 to 50% as well as ClpAT depending on ATP concentration, while ClpAT/K501T, in which Lys^{501} in the second site is replaced with Thr, is unable to cleave ATP or to support the proteolysis. Without ATP, ClpAT and both of its mutant forms behave as trimeric molecules. With 0.5 mM ATP, ClpAT and ClpAT/K501T form hexamers but ClpAT/K220T remains trimeric. With 2 mM ATP, however, ClpAT/K220T also behaves as a hexamer. Thus, interaction of ATP to the first site for hexamer formation of ClpA and its hydrolysis at the second site seem to be requisite for the ATP-dependent proteolysis by ClpP (12).

In order to clarify further the mechanism underlying the ATP-dependency for the proteolytic function of protease Ti, we examined in the present studies the role of ATP and its hydrolysis on the interaction between ClpA and ClpP.

MATERIALS AND METHODS

ClpAT and its mutant forms (*i.e.*, ClpAT/K220T and ClpAT/K501T) were purified as described previously (12). ClpP was purified as described (13). Hydrolysis of ATP and [^{3}H]casein was assayed as described (14). Proteins were assayed by the dye-binding method of Bradford (15) using bovine serum albumin as a standard.

RESULTS

ATP hydrolysis is essential for ClpA/ClpP complex formation

In order to access the role of ATP in assembly of ClpA/ClpP complex, each of the purified ClpAT and ClpP was subjected to gel filtration on a Sephacryl S-300 column in the presence and absence of ATP. The fractions obtained from the column were then electrophoresed on polyacrylamide slab gels containing SDS (16). After the electrophoresis, proteins in the gels were visualized by silver staining (17). In accord with our earlier report (12), the trimeric ClpAT (approximately 250 kDa) became a hexameric form (500 kDa) upon treatment of ATP (Fig. 1A and 1B). As also expected, the size of ClpP (250 kDa) remained

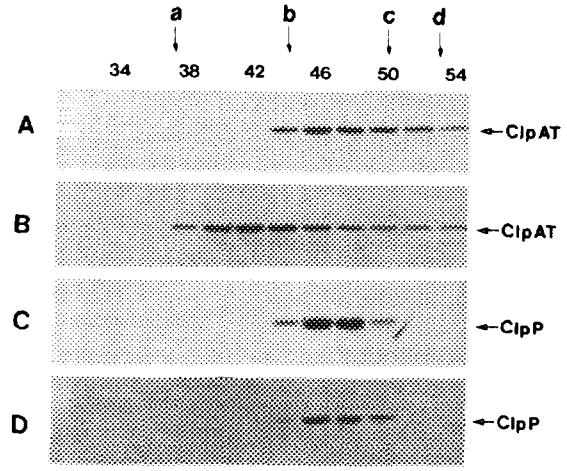

Fig. 1. Elution of ClpAT (A and B) and ClpP (C and D) from a Sephacryl S-300 column and gel electrophoretic analysis of the column fractions. Each of the purified ClpAT (200 μg) and ClpP (50 μg) was incubated at 4 °C for 15 min in the absence (A and C) and presence of 2 mM ATP (B and D) and chromatographed on the gel filtration column (1 x 40 cm) that had been equilibrated with 20 mM Tris-HCl buffer (pH 7.8) containing 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, and 0.1 M NaCl and 20% (v/v) glycerol. For the samples incubated with ATP, the column buffer was also added with the nucleotide. Fractions of 0.5 ml were collected at a flow rate of 6 ml/h. Aliquots of the fractions were subjected to electrophoresis on 13% (w/v) polyacrylamide slab gels containing SDS and 2-mercaptoethanol. The proteins in the gels were visualized by silver-staining. The size markers used were a, thyroglobulin (669 kDa); b, apoferritin (443 kDa); c, alcohol dehydrogenase (150 kDa); d, bovine serum albumin (66 kDa). The numerals on the top of the gels indicate the fraction numbers.

unchanged whether ATP was present or not (Fig. 1C and 1D), since ClpP is known not to interact with ATP (18).

When ClpAT and ClpP were incubated in the presence of 2 mM ATP and then analyzed as above, a significant portion of both the proteins was eluted in the fractions corresponding to sizes much larger than 500 kDa (Fig. 2B). However, when the same experiments were performed in the absence of ATP, both the proteins ran as if each was chromatographed separately without ATP (Fig. 2A). When the proteins were incubated with ADP, ClpAT ran as a hexamer whereas the size of ClpP remained to be about 250 kDa (Fig. 2C). These results suggest that ATP is essential for interaction between ClpA and ClpP and thus for formation of a complex, although a large portion of the proteins were also recovered in the column fractions corresponding to sizes smaller than 500 kDa. However, the recovery of the ClpAT and ClpP proteins in the low molecular weight fractions was not due to the presence of one protein in excess over the other, because a maximal activity for casein degradation was seen with the amounts of the proteins used for these studies (*i.e.*, at a mass ratio of 4:1 for trimeric ClpAT to 250-kDa ClpP) and because changes in the amount of one protein over the other did not alter their chromatographic behavior (data not shown).

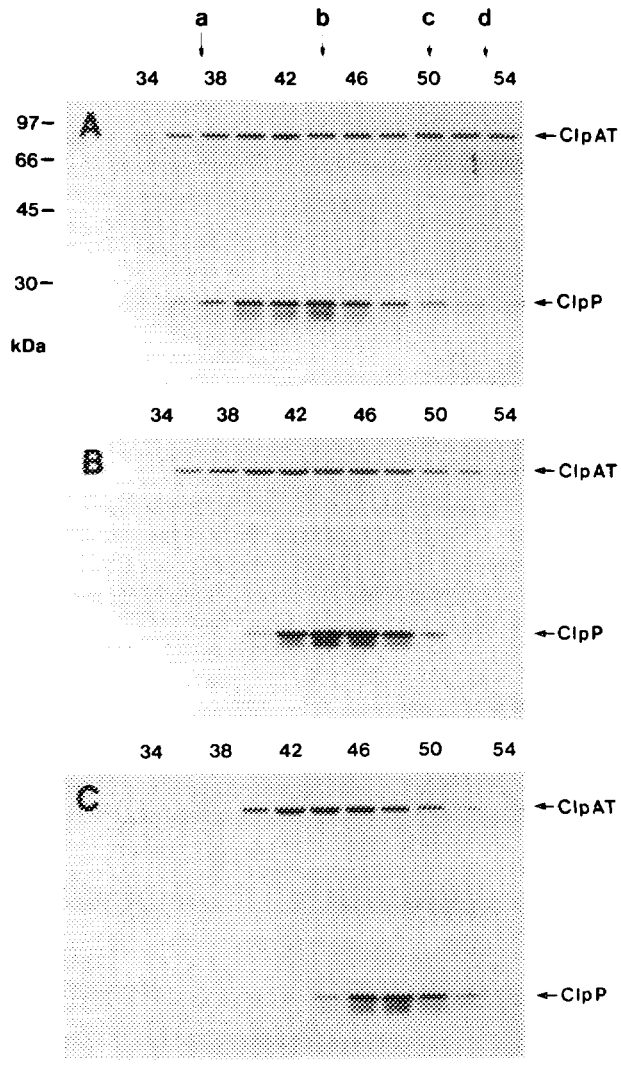

Fig. 2. Elution of the mixtures of ClpAT and ClpP from a Sephacryl S-300 column and gel electrophoretic analysis of the column fractions. ClpAT (200 μg) and ClpP (50 μg) were incubated at 4 °C for 15 min in the absence (A) and presence of 2 mM ATP (B) or ADP (C) and chromatographed on the gel filtration column. Fractions of 0.5 ml were collected and subjected to gel electrophoresis as in Fig. 1.

To clarify further the role of ATP in ClpA/ClpP complex formation, we incubated ClpP with the ClpAT protein containing a mutation in either of the two ATP-binding sites. The incubation mixtures were then analyzed by gel filtration followed by electrophoresis as above. In the presence of 0.5 mM ATP, both ClpAT/K220T and ClpP were eluted in the fractions corresponding to 250 kDa (Fig. 3A), which is the same size as that seen without ATP (see also Fig. 2A). With 2 mM ATP, however, both the proteins behaved similarly to the incubation mixture of the parental ClpAT and ClpP, although the amounts of the proteins

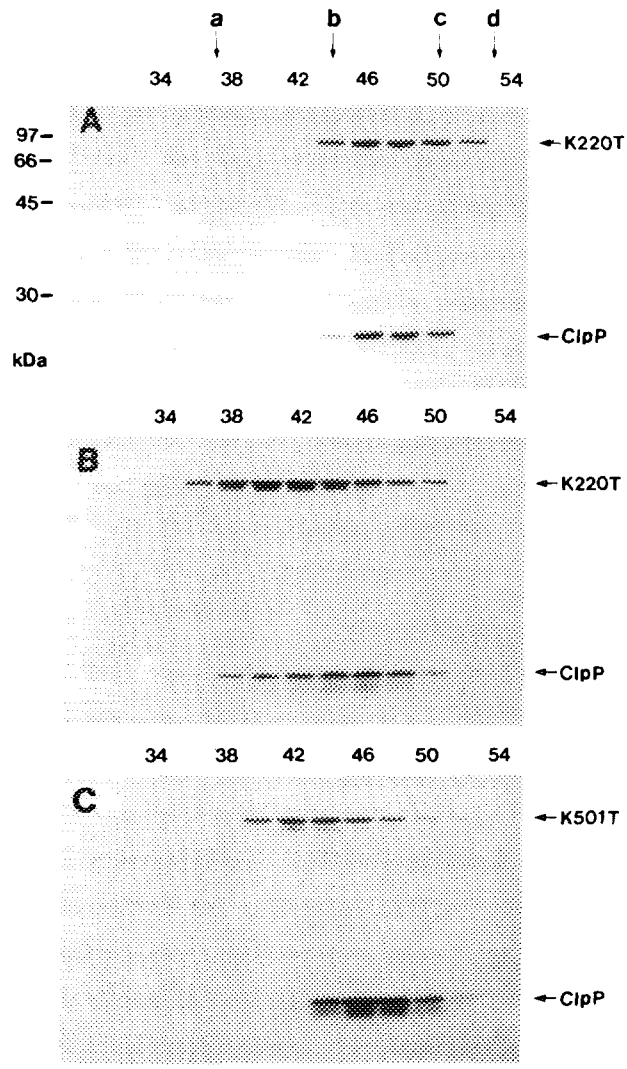

Fig. 3. Elution of the mixtures of ClpP and ClpAT/K220T (A and B) or ClpAT/K501T (C) from a Sephacryl S-300 column and gel electrophoretic analysis of the column fractions. ClpAT/K220T or ClpAT/K501T (200 μg) and ClpP (50 μg) were incubated at 4 °C for 15 min in the presence of 0.5 mM (A) or 2 mM ATP (B and C) and chromatographed on the gel filtration column. Fractions were collected and subjected to gel electrophoresis as in Fig. 1.

recovered in the fractions corresponding to sizes larger than 500 kDa were consistently less than those obtained with the latter mixture (Fig. 3B). ClpAT/K220T carrying the mutation in the first ATP-binding site has been shown to form a hexamer in the presence of 2 mM ATP but not with 0.5 mM ATP (12). It has also been shown that, even in the presence of 2 mM ATP, ClpAT/K220T hydrolyzes ATP and supports the ATP-dependent casein degradation by ClpP at about one-half of the rates seen with ClpAT (12). These results suggest that the hexameric form of ClpA, but not its trimeric form, interacts with ClpP for assembly of

ClpA/ClpP complex. These results also suggest that the reduced ability of ClpAT/K220T in formation of a complex with ClpP is due to the decrease in its ability to cleave ATP and consequently results in the reduction of its capacity to support the ClpP-mediated proteolysis.

However, when the incubation mixture of ClpP and ClpAT/K501T containing the mutation in the second ATP-binding site, that is essential for ATP hydrolysis, was analyzed as above, the ClpAT mutant protein formed a hexamer but ClpP was recovered as 250-kDa proteins (Fig. 3C). Furthermore, when the same experiments were carried out with the mixture of the parental ClpAT and ClpP but in the presence of adenosine 5'-(β,γ-imido)-triphosphate, a non-hydrolyzable ATP analog, nearly the same results were obtained (data not shown). Therefore, it seems clear that assembly of ClpA/ClpP complex requires not only the ClpA hexamer formation that is due to the first ATP-binding site but also the second site-mediated ATP hydrolysis.

ADP blocks ClpA/ClpP complex formation

Noteworthy was the finding that both the ClpAT and ClpP proteins that had been incubated with ATP for 15 min at 4 °C were spreaded out in the Sephacryl S-300 column fractions corresponding to sizes ranging from 200 to 900 kDa (see Fig. 2B), instead of forming a discrete peak of ClpAT/ClpP complex with a definitive size. Because ADP can induce ClpAT hexamer formation but is unable to support the ability of ClpAT to interact with ClpP (see Fig. 2C), we suspected if ADP molecules that were generated by ATP hydrolysis during the incubation and gel filtration chromatography may interfere with the formation of ClpAT/ClpP complexes, such as by inhibiting the ATPase activity of ClpAT, and therefore cause a rapid dissociation of the complex into ClpAT hexamer and 250-kDa ClpP.

To test this possibility, we first examined whether ADP can indeed be produced by the mixture of ClpAT and ClpP under the similar condition used for their incubation and gel filtration analysis at 4 °C. As shown in Table I, more than 20% of the total ATP in the reaction mixture were converted to ADP and inorganic phosphate upon incubation at 4 °C for 4 h. Since gel filtration chromatography of the incubation mixture on the Sephacryl S-300 column usually took more than 5 h, ADP generated during the period may be sufficient for inhibiting the ATPase activity of ClpAT. And this possibility is supported by the finding that the increase in ATP hydrolysis under the assay condition is not linear with time.

To clarify further the effect of ADP, ATP hydrolysis was assayed by incubating ClpAT and increasing amounts of ADP in the presence and absence of ClpP at 37 °C. As shown in Fig. 4A, ADP inhibited ATP hydrolysis in a dose-dependent manner. Similar results were obtained when the same experiments were performed in the presence of casein, which is

Table I. Time-dependent generation of ADP by the mixture of ClpAT and ClpP at 4 °C

Incubation time (h)	ATP-hydrolyzed (nmol)	ADP-generated (%)
0.5	7.5	9
1	10.9	14
2	14.1	18
4	18.1	23

ATP hydrolysis was assayed by incubating the reaction mixtures (0.5 ml) containing ClpAT (200 μg), ClpP (50 μg) and 2 mM ATP at 4 °C. At the indicated time, 40 μl aliquots in duplicates of the incubation mixtures were taken out and added with 0.16 ml of 1% SDS. The samples were then subjected to analysis for the production of inorganic phosphate (14). The ADP-generated indicates percentage of ATP converted to ADP and inorganic phosphate.

known to stimulate the ATPase activity of ClpA (18). In addition, ADP similarly inhibited the ATP-dependent casein hydrolysis by the mixture of ClpAT and ClpP (Fig. 4B). We also determined the K_i value for ADP by incubating ClpAT with increasing amounts of ATP. Upon double reciprocal plot of the data, K_m and K_i values were calculated to be 210 and 95 μ M for ATP and ADP, respectively (*i.e.*, the affinity of ClpAT to ADP is about 2-fold higher than that to ATP). Addition of ClpP in the assay mixture did not change the kinetic

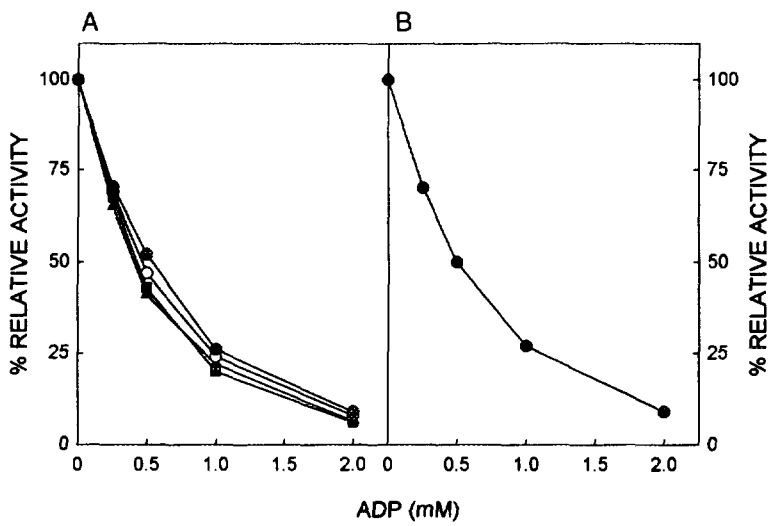

Fig. 4. Effect of increasing concentration of ADP on the ability of ClpAT in hydrolyzing ATP and in supporting the ClpP-mediated casein hydrolysis. (A) ATP hydrolysis was assayed by incubating the reaction mixtures containing ClpAT (0.4 μg), 0.5 mM ATP and increasing amounts of ADP (○) at 37 °C for 45 min. The reaction mixtures were also added with 0.1 μg of ClpP (●), 10 μg of casein (▲) or both (■) and increasing amounts of ADP. (B) Hydrolysis of [^{3}H]casein was determined by incubating 0.4 μg of ClpAT, 0.1 μg of ClpP, 10 μg of [^{3}H]casein and 0.5 mM ATP at 37 °C for 1 h in the presence of increasing amounts of ADP (●). The activities seen without ADP were expressed as 100% and the others were as their relative values.

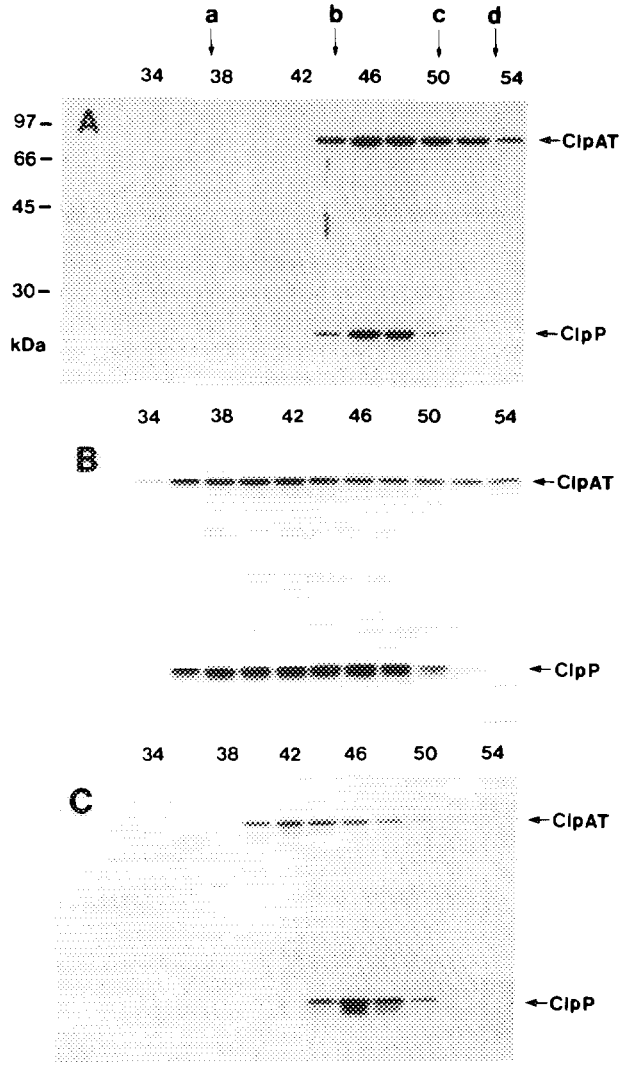

Fig. 5. Effect of ADP on the formation of ClpA/ClpP complex. ClpAT (200 μg) and ClpP (50 μg) were incubated with 2 mM ATP at 4 °C for 15 min in the absence (A) and presence of 0.4 mM (B) or 2 mM ADP (C) and chromatographed on a Sephacryl S-300 column followed by electrophoretic analysis as in Fig. 1. When examined the effect of ADP, the column buffer contained the indicated concentration of ADP as well as 2 mM ATP.

parameters (data not shown). In addition, we have previously shown that casein neither changes the K_m for ATP (13). These results suggest that ADP competes with ATP for binding to the second ATP-binding site in ClpA.

To determine whether ADP is indeed capable of blocking the formation of ClpA/ClpP complex, ClpAT and ClpP were incubated with ATP in the presence of increasing amounts of ADP for 15 min at 4 °C and then subjected to analysis by gel filtration on the Sephacryl S-300 column followed by electrophoresis. Fig. 5 shows that ADP inhibits the ClpAT/ClpP complex

formation in a dose-dependent manner. These results strongly suggest that ADP generated by ATP hydrolysis involves in rapid dissociation of ClpA/ClpP complex.

DISCUSSION

The present studies have demonstrated that ATP hydrolysis is essential for assembly of the ATP-dependent protease Ti. ADP or adenosine 5'-(β,γ-imido)-triphosphate could not support the ClpA/ClpP complex formation. Furthermore, ClpAT/K501T carrying the mutation in the second ATP-binding site was not capable of forming a complex with ClpP. We have previously shown that ClpAT/K501T is unable to hydrolyze ATP and to support the ClpP-mediated proteolysis (12). In addition, it has been reported that the chemically inactivated ClpP (*i.e.*, ClpP treated with diisopropylfluorophosphate) can associate with ClpA but is unable to support the casein-mediated activation of the ATPase activity of ClpA, unlike normal ClpP (6). Therefore, ATP hydrolysis seems to be required not only for the cleavage of peptide bonds in protein substrates but also for the ClpA/ClpP complex formation.

In addition to the second ATP-binding site that is essential for ATP hydrolysis, the first site responsible for formation of hexameric ClpA must also be involved in assembly of ClpA/ClpP complex. ClpAT/K220T carrying the mutation in the first ATP-binding site was unable to form a complex with ClpP at 0.5 mM ATP but did at 2 mM ATP, despite that fact that the hexamer formation requires just binding of ATP but not its hydrolysis (12). We have previously shown that ClpAT/K220T has a reduced affinity to ATP and therefore, at low concentrations of ATP (*e.g.*, 0.5 mM), is unable to form a hexamer and to support the ClpP-mediated proteolysis (12). These results strongly suggest that ClpP interacts with hexameric ClpA but not its trimeric form for assembly of ClpA/ClpP complex. Taken together, both the ATP-binding sites in ClpA play a critical role in assembly of the ATP-dependent protease Ti.

ATP hydrolysis seems to involve not only in assembly of ClpA/ClpP complex but also in its rapid dissociation. When the incubation mixture of ClpAT and ClpP in the presence of ATP were subjected to gel filtration analysis, both the proteins were recovered in the fractions corresponding to sizes ranging from 200 to 900 kDa, instead of generating a discrete peak of ClpAT/ClpP complex with a definitive size. Lines of evidence suggest that the rapid dissociation of ClpA/ClpP complex is due to ADP that were generated by ATP hydrolysis: (1) ADP inhibits both the ATPase activity of ClpAT and the ClpP-mediated proteolysis; (2) it shows a higher affinity to ClpAT than ATP; and (3) it blocks the ClpAT/ClpP complex formation.

Although a distinct peak of ClpA/ClpP complex could not be recovered from gel filtration analysis, we suspect that the protein species larger than hexameric ClpAT (*i.e.*, those with

sizes ranging from 700 to 900 kDa) represent the complex of ClpAT and ClpP (see Fig. 2B). Based on the size estimation, we suggest that ClpA/ClpP complex consists of one molecule each of ClpA and ClpP. However, it seems also possible that ClpA/ClpP complex consists of two molecules of ClpA and one ClpP, when considering the fact that the mixture of ClpAT and ClpP at a mass ratio of 4:1 shows a maximal activity for casein degradation.

An unanswered question is why ClpA and ClpP make a futile cycle by continuous, rapid association and dissociation from each other at the expense of ATP. We initially thought that degradable protein substrates, like casein, may reduce the affinity of ADP by binding to ClpA and hence help in maintaining the structure of ClpA/ClpP complex for proteolysis. However, for the ATP-cleaving activity of ClpAT, ADP was found to compete with ATP in the presence of casein equally as well as in its absence. In addition, treatment of casein showed little or no effect on the K_i for ADP (data not shown). Nevertheless, it has been shown that casein and other protein substrates, but not nonhydrolyzed proteins, stimulate 2- to 4-fold the ATPase activity of this enzyme (13). In the absence of casein, ClpP reduces the rate of ATP hydrolysis by ClpA. Therefore, it seems possible that rapid association of ClpA and ClpP to form an active complex and its dissociation into individual components at the expense of ATP may help ensure against inappropriate or excessive proteolysis in the cytosol.

The 26S proteasome in eukaryotic cells is an ATP-dependent protease that degrades ubiquitin-conjugated protein substrates (19). This enzyme is comprised of three different multimeric enzyme components, called CF-1, CF-2 and CF-3 (20). Of these, CF-3 has been identified as the multicatalytic 20S proteasome, which is the proteolytic core of the 26S proteasome (21,22). Assembly of the 26S enzyme complex from CF-1, CF-2 and CF-3 specifically requires ATP and is accompanied by formation of ATPase activity (23). In this respect, protease Ti in *E. coli* resembles the eukaryotic 26S proteasome, in addition to the similarity in their quaternary structure proposed by Rechsteiner (24).

Acknowledgments: This work was supported by the grants from Korea Science and Engineering Foundation through SRC for Cell Differentiation and the Ministry of Education.

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