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The 65-kDa Protein Derived from the Internal Translational Initiation Site of the *clpA* Gene Inhibits the ATP-dependent Protease Ti in *Escherichia coli**

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The *clpA* gene that encodes the ATPase subunit of the ATP-dependent protease Ti (Clp) in *Escherichia coli* contains a putative internal translational initiation site. Here we show that mutagenesis of its 5′-end AUG codon resulted in an exclusive synthesis of the 65-kDa protein (ClpA65), while mutation at the internal 169th AUG codon (Met) to ACG (Thr) produced only the 84-kDa protein (ClpA84T). On the other hand, the cells carrying the wild-type *clpA* gene produced both the 84- and 65-kDa proteins (ClpA84/65). While the purified ClpA84T and ClpA84/65 hydrolyzed ATP nearly as well as the 84-kDa ClpA alone (ClpA84), ClpA65 cleaved ATP at a rate less than 5% of that by ClpA84. Unlike ClpA84 and ClpA84T, ClpA65 could not support the casein-degrading activity of ClpP. Furthermore, ClpA65 inhibited the proteolysis by the mixture of ClpP with ClpA84 or ClpA84T but not that with ClpA84/65, which could support the proteolytic activity of ClpP only about 40% as well as ClpA84. Nevertheless, ClpA65 showed little or no effect on the basal or protein-activated ATPase activity of ClpA84, ClpA84T, or ClpA84/65 alone or in the presence of ClpP. These results suggest that ClpA65 may interfere the interaction of ClpA84 or ClpA84T with ClpP and, hence, impair their assembly into an active form of the ATP-dependent protease Ti.

One of the most intriguing features of intracellular protein breakdown is the requirement of metabolic energy. A number of proteolytic machinery responsible for the ATP-dependent process in *Escherichia coli* have been identified (1–4). Of these, the ATP-dependent protease La (Lon) is a heat shock protein and plays an essential role in the hydrolysis of most abnormal proteins and certain short-lived regulatory proteins (5–9). Protease La, a tetramer of 87-kDa subunits, has an inherent ATPase activity, which can be stimulated by protein substrates. Protease Ti (Clp), unlike protease La, consists of two different multimeric components, both of which are required for proteolysis (10–13). While component A (ClpA) contains the ATP-hydrolyzing sites in 84-kDa subunits, component P (ClpP), which is a heat shock protein (14), contains a serine-active site for proteolysis in 21-kDa subunits. The isolated ClpA shows protein-activated ATPase activity, which in the reconstituted enzyme is linked to protein breakdown. In addition, it has recently been shown that ClpX having limited homology to regions of ClpA can form a new type of ATP-dependent protease with ClpP in the hydrolysis of λ O-protein (15, 16).

ClpB, a sequence homolog of ClpA, is a heat shock protein and also a protein-activated ATPase (17–20). This protein, however, cannot replace ClpA in supporting the ATP-dependent proteolysis by ClpP. Therefore, the physiological role of ClpB remains unknown. One unusual structural feature of ClpB is that the purified protein contains not only the 93-kDa subunit but also the 79-kDa subunit, which also is a heat shock protein (19–21). Moreover, the *clpB* gene has been found to contain a sequence for an internal translational initiation in addition to the 5′-end start site. Subsequently, site-directed mutagenesis at the dual start sites has revealed that the smaller polypeptide is derived from the internal initiation site of the *clpB* gene (22).

Similar to the wild-type ClpB, the purified 79-kDa protein behaves as a tetrameric complex and has an inherent ATPase activity. The smaller protein, however, cannot be activated by proteins such as casein, unlike the wild-type ClpB or the 93-kDa tetramers. Furthermore, the 79-kDa protein is capable of inhibiting the protein-activated ATPase activity of the 93-kDa protein but not its basal ATPase activity, and the mixtures of the 79- and 93-kDa proteins remain behaving as tetrameric complexes. Therefore, it has been suggested that the two types of ClpB proteins form heterotetrameric complexes in which the 79-kDa subunits may control the unknown function(s) of the protein-activated ATPase activity of the 93-kDa subunits (22).

Upon comparison of the nucleotide sequence of the *clpA* gene to that of the *clpB* gene, we found that the *clpA* gene also contains a putative sequence for an internal translational initiation. In the present study, therefore, mutational analyses of the dual translational sites of the *clpA* gene were performed to determine whether the mutations can exclusively produce the 84-kDa ClpA and an abbreviated 65-kDa protein. In addition, we purified both the ClpA proteins for determination of their functional and structural properties.

EXPERIMENTAL PROCEDURES

Materials—The DNA clones producing ClpA were obtained by immunoscreening of an *E. coli* genomic library in λgt11 (Clontech). Screening of the library was carried out using anti-84-kDa ClpA antiserum as described (23). The 3.4-kilobase *Bam*HI-*Pst*I fragment from one of the positive clones was ligated into Bluescript KS$^+$ plasmid, and the resulting recombinant plasmid was referred to as pClpA. An *E. coli clpA* null mutant SG21118 (*clpA319*::Δ*kan*), which was 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 Biosystem, model 384A) and purified by gel electrophoresis as recommended by the manufacturer. All reagents for the polymerase chain reaction (PCR),[1] including *Taq* polymerase, were

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[1] The abbreviations used are: PCR, polymerase chain reaction; DTT, dithiothreitol.

purchased from Boehringer Mannheim. Restriction endonucleases and other DNA modifying enzymes were obtained from New England BioLabs and KOSCO Biotech (Korea). [^{3}H]casein was prepared as described (24). 8-N_3-[α-^{32}P]ATP (6.7 Ci/mmol) was obtained from ICN. All other reagents were purchased from Sigma.

Site-directed Mutagenesis—Site-directed mutations were created by the PCR method, which consists of two sequential PCRs (25). The primers used for the elimination of the first start site were 5′-GAGGTGCCTGAGCTCAATCAA-3′ (primer a) and 5′-TTGATTGAGCTCAGGCACCTC-3′ (primer a′), in which the boldface letters indicate the mutated nucleotides and the underlines show the sequences subsequently created for restriction by the mutations. The primers used for removal of the second site were 5′-GAGGAACGTACGGAGAATTTC-3′ (primer b) and 5′-GAAATTCTCCGTACGTTCCTC-3′ (primer b′). Thus, the mutagenesis using the latter two primers (*i.e.* b and b′) should result in the replacement of Met at the second start site by Thr. Two other primers necessary for PCRs were 5′-GACTTTGATCAACTGGCGGAA-3′ (primer c) and 5′-AGTCGCCGCGATATTTTGTGC-3′ (primer d), of which primer c is identical to the sequence from 310-base upstream of the first start site, and d is identical to that from 260-base downstream of the second site.

The PCR reactions were carried out using a DNA thermal cycler (Ericomp, Inc.) and generated four different fragments: Fragment I by primers c and a′, Fragment II by a and d, Fragment III by c and b′, and Fragment IV by b and d. Prior to the secondary PCRs, the same amounts (400 ng each) of Fragments I and II and of III and IV were mixed and subjected to annealing between the fragments by denaturation-renaturation reactions. The DNAs with recessed 3′-OH ends were extended by Klenow and used as the templates for the secondary PCRs. The PCR reactions were performed to generate two different fragments, Fragments V and VI by c and d.

Fragments V and VI were digested by *Nru*I and *Eco*RV and ligated into pClpA, from which the same restriction fragments had been eliminated prior to use. The resulting plasmid containing Fragment V was referred to as pClpA65, and that containing Fragment VI was referred to as pClpA84T. The plasmids were maintained in a *clpA* null mutant cell (SG21118).

Purification—The *E. coli* SG21118 cells harboring pClpA, pClpA84T, and pClpA65 were grown in Luria broth at 37 °C overnight and kept frozen at −70 °C until use. The frozen cells (10–15 g each) were thawed and resuspended in 50 ml of 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 6.5) containing 1 mM ATP, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol (DTT), and 20% (v/v) glycerol. The cells were then disrupted with a French press at 14,000 psi and centrifuged at 100,000 × *g* for 3 h. The supernatants were dialyzed against the same buffer and referred to as crude extracts.

Each of the extracts was then loaded onto a phosphocellulose column (1.5 × 5 cm) equilibrated with the same buffer. After extensively washing the column, proteins were eluted with a linear gradient of 0.1–0.4 M phosphate. Fractions of 2 ml were collected at a flow rate of 20 ml/h. Aliquots of the fractions were electrophoresed on 10% (w/v) polyacrylamide gels in the presence of SDS, and proteins were visualized by staining with Coomassie Blue R250. The fractions containing 84-, 65-kDa protein, or both were pooled and dialyzed against 20 mM Tris-HCl buffer (pH 7.8) containing 1 mM ATP, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 0.2 M NaCl, and 20% glycerol. Individual protein sample was then subjected to chromatography on a heparin-agarose column (1 × 8 cm) equilibrated with the Tris buffer. The columns were then developed with a linear gradient of 0.2–0.4 M NaCl. Fractions containing the ClpA proteins were pooled and kept frozen at −70 °C for further use.

The wild type form of ClpA was also purified by an alternate purification protocol. The SG21118/pClpA cell (15 g) was grown, and its extract was prepared as above but in 20 mM Tris-HCl buffer (pH 7.8) containing 1 mM ATP, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT and 20% glycerol (*i.e.* without NaCl). The extract was extensively dialyzed against the Tris buffer and then loaded on a DEAE-Sepharose column (1.5 × 20 cm) equilibrated with the same buffer. After washing the column, proteins were eluted with a linear gradient of 0–250 mM NaCl. Fractions of 4 ml were collected, and aliquots of them were assayed for ATP-dependent proteolysis by incubation with the purified ClpP and [^{3}H]casein (see below). Fractions containing high activity were pooled and dialyzed against 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 6.5) containing 1 mM ATP, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, and 20% (v/v) glycerol. The dialyzed sample was then subjected to chromatographies on phosphocellulose and heparin-agarose columns in order as described above. ClpP was purified as described previously (11).

Assays—Protein breakdown was measured using [^{3}H]casein as the substrate (11). Reaction mixtures (0.1 ml) contained 10 μg of casein and

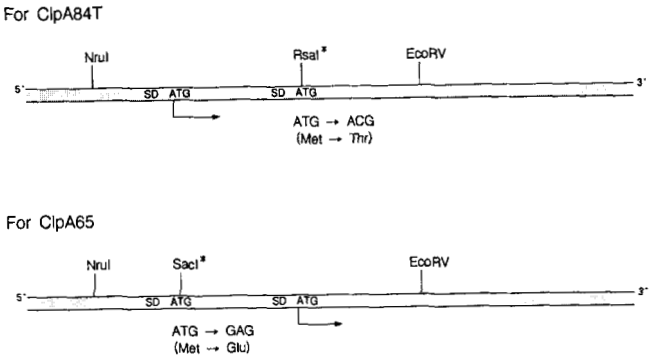

FIG. 1. **The restriction map of the *clpA* gene and the dual translational initiation sites for site-directed mutagenesis.** *SD* indicates the putative Shine-Dalgarno sequence, GGAGG; the *asterisk* show the newly created restriction sites by the mutations.

appropriate amounts of purified ClpP and ClpA or its mutated forms in 0.1 M Tris-HCl (pH 8), 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, and 1 mM EDTA. Incubations were performed for 30–60 min at 37 °C, and the production of radioactive materials soluble in 10% (w/v) trichloroacetic acid was measured. ATPase activity was assayed by incubating the similar reaction mixtures for appropriate periods at 37 °C. After the incubation, the samples were added with 0.1 ml of 1% SDS, and the phosphate released was determined as described (26). Proteins were assayed by their absorbance at 280 nm or by the method of Bradford (27) using bovine serum albumin as a standard.

Affinity Labeling with Azido-[α-^{32}P]ATP—The ClpA proteins were dialyzed against 30 mM Tris-HCl buffer (pH 7.8) containing 5 mM $MgCl_2$, 0.3 M NaCl, and 0.5 mM EDTA in order to remove ATP and DTT in the purified enzyme preparations. The dialyzed proteins (3 μg each) were incubated with 2 μCi each of 8-N_3-[α-^{32}P]ATP for 30 min on ice and irradiated with UV-light using a UVGL-58 lamp (260 nm, Ultra-Violet Products, Inc.) for 1 min at room temperature. The resulting samples were mixed with equal sample volumes of a solution containing 4% SDS and 5% (v/v) 2-mercaptoethanol. They were then electrophoresed as above, stained with Coomassie Blue R250, dried, and exposed to x-ray films.

RESULTS

Mutagenesis of the Dual Translational Start Sites in the clpA Gene—Analysis of the nucleotide sequence of the *clpA* gene revealed that a putative internal translational initiation codon AUG is located at the 169th codon downstream of 5′-end initiation AUG codon of ClpA and is preceded by a good Shine-Dalgarno sequence, GGAGG (Fig. 1). In addition, the internal AUG codon is separated from the Shine-Dalgarno sequence by 5 nucleotides, the same as the 5′-end AUG initiation codon of ClpA. Therefore, using PCR-aided site-directed mutagenesis, we examined whether the *clpA* gene can encode two polypeptides with different sizes from the same reading frame. The N-terminal codon AUG was replaced by GAG for the exclusive synthesis of an abbreviated form of ClpA from codon 169 and for the generation of a new restriction site for *Sac*I to facilitate the mutant isolation. This approach should generate a 65-kDa ClpA protein (referred to as ClpA65) if indeed internal initiation occurs. Similarly, the internal AUG codon was replaced by ACG for blocking the internal initiation and for creating a new *Rsa*I restriction site, such that the *clpA* can encode only the 84-kDa ClpA but with a replacement of the 169th amino acid Met by Thr (ClpA84T). The Bluescript KS$^+$ plasmids carrying the mutated *clpA* genes were named as pClpA65 and pClpA84T, and the mutations were confirmed by the restriction and nucleotide sequence analyses of the plasmids (data not shown). The mutated plasmids were transformed into a *E. coli clpA* null mutant, SG21118 (16).

We then examined whether the *E. coli* cells are capable of producing either ClpA65 or ClpA84T using their single translational start site of the mutated *clpA* gene. The cells were

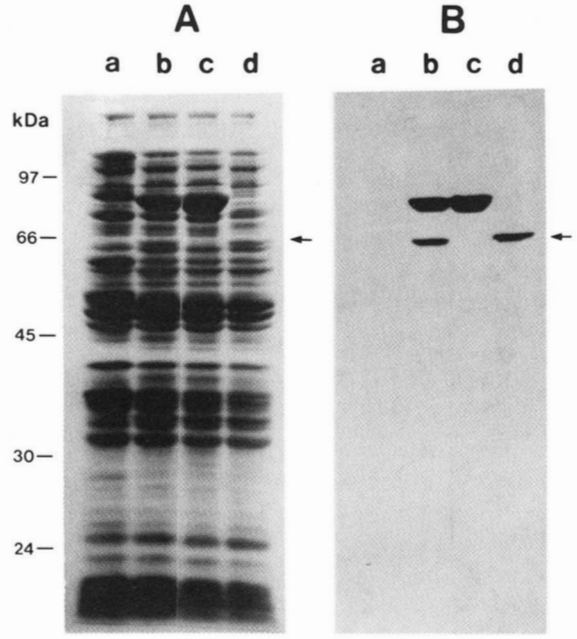

FIG. 2. **Electrophoretic and immunoblot analyses of ClpA84/65, ClpA84T, and ClpA65.** The *E. coli* SG21118 cells (*lane a*) and the cells harboring pClpA (*lane b*), pClpA84T (*lane c*), and pClpA65 (*lane d*) were grown in Luria broth at 37 °C to mid log phase. After the culture, the same amounts of the cells were harvested, boiled, and electrophoresed on 10% polyacrylamide gels in the presence of SDS. The gels were then stained with Coomassie Blue R250 (*A*) or subjected to immunoblot analyses (*B*) as described (31). The *arrows* indicate the positions where ClpA65 migrated.

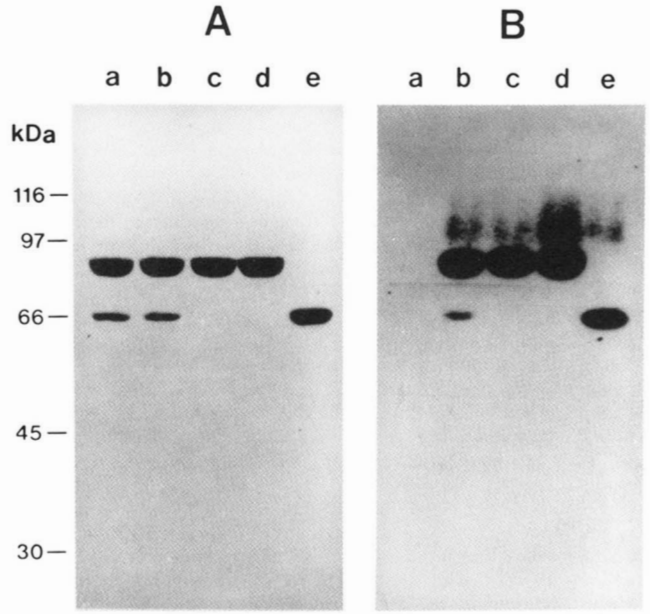

FIG. 3. **Gel electrophoretic patterns of the purified ClpA proteins labeled with azido-[α-^{32}P]ATP.** ClpA84/65 (*lanes a* and *b*), ClpA84 (*lane c*), ClpA84T (*lane d*), and ClpA65 (*lane e*) were purified, labeled with 8-N_3-[α-^{32}P]ATP, and electrophoresed in the presence of SDS as described under "Experimental Procedures." The radiolabeled proteins in the gels were then visualized by staining with Coomassie Blue R250 (*A*) and also by exposure to x-ray films (*B*). The protein sample in *lane a* was not irradiated with UV-light but otherwise treated the same as the others.

grown to the same optical density and subjected to electrophoretic and immunoblot analyses. As shown in Fig. 2, the cells carrying pClpA65 overproduced only ClpA65, as those carrying pClpA84T exclusively did ClpA84T. On the other hand, the cells carrying the wild-type *clpA* gene in Bluescript KS$^+$ plasmid (pClpA) produced both 84- and 65-kDa proteins. Moreover, the antibody raised against the 84-kDa protein of the wild-type ClpA strongly interacted with ClpA65 as well as ClpA84T. These results indicate that the *clpA* gene can encode two polypeptides with different sizes from the same reading frame.

Purification and Physical Properties of ClpA Proteins—Unlike the present finding that the *E. coli* cells carrying the pClpA plasmid are capable of producing both the 84- and 65-kDa proteins, ClpA purified from wild-type *E. coli* strains has been shown to be a single protein of about 80 kDa (11, 13). To clarify the discrepancy in the composition of ClpA, we purified each of ClpA65, ClpA84T, and the wild-type form of ClpA, but using a phosphocellulose column as the first step for purification instead of a DEAE-cellulose column or polyethyleneimine precipitation step that was used in the previous reports (11, 13). As shown in Fig. 3*A*, the wild-type ClpA consisted of two proteins with sizes of 84 and 65 kDa (ClpA84/65), while ClpA65 or ClpA84T was comprised of a single polypeptide, indicating that these proteins were purified to apparent homogeneity. During the purification, however, we noticed that ClpA65 has much higher tendency to precipitate in low salt solutions (*e.g.* below 0.4 M NaCl) than the wild-type 84-kDa ClpA, although the latter protein also slowly aggregates upon prolonged dialysis under the same conditions (data not shown). Therefore, we suspected that the 65-kDa polypeptide in ClpA84/65 may be selectively removed by precipitation during purification by our previously reported protocol. To test this possibility, ClpA was again subjected to purification from the *E. coli* carrying pClpA but by an alternate procedure involving dialysis of the cell extract in low salt buffer followed by DEAE-cellulose chromatography (see "Experimental Procedures"). Fig. 3*A* (*lane c*) shows that the ClpA protein purified by this alternate approach contains the 84-kDa protein (ClpA84) but not the 65-kDa protein. Thus, under low salt conditions, the 65-kDa polypeptide must be selectively removed by precipitation from the wild-type ClpA84/65.

The isolated ClpA84 has been reported to behave as a monomeric or dimeric molecule in the absence of ATP (10, 28) but form a hexameric complex when incubated with ATP (28). However, the size estimation was performed under conditions with a recovery of the enzyme activity less than 20% of the total. In addition, we found in the present study that none of the ClpA65 protein could be recovered upon analyses by gel filtration on chromatographic columns, such as Sephacryl S-300 and Superose-6, even in the presence of high salt and/or ATP (data not shown). Furthermore, all of the ClpA65, ClpA84, and ClpA84/65 proteins spread over the entire fractions obtained from the glycerol density gradient centrifugation that were performed in the presence of 0.3 M NaCl and 2 mM ATP (data not shown). On the other hand, ClpA84T, in which only the 169th amino acid residue (*i.e.* Thr) is different from that of ClpA84 (*i.e.* Met), was found to be highly soluble in both low and high salt conditions. Therefore, ClpA84T was subjected to gel filtration analysis on a Sephacryl S-300 column for determination of its size under nondenaturing conditions. As shown in Fig. 4, the peak of the ClpA84T protein appeared in the fractions corresponding to about 240 kDa in the absence of ATP but to fractions corresponding to about 500 kDa in its presence. Thus, the isolated ClpA84T appears to behave as a hexameric complex of the 84-kDa subunits in the presence of ATP, in accordance with the report by Maurizi (28), but as a trimer in its absence. These results suggest that the N-terminal domain and the 169th amino acid residue of ClpA84 may play a role in conferring the solubility of the protein and oligomerization into an active form of the ATPase. However, it remains unclear why ClpA84T behaves as a larger molecule (*i.e.* as a trimeric com-

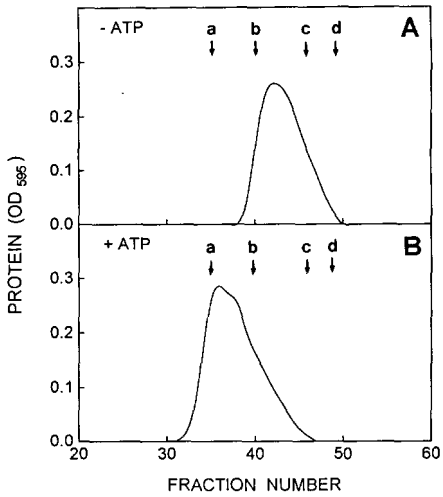

FIG. 4. **Estimation of the size of ClpA84T under nondenaturing conditions.** An aliquot (0.2 mg) of the purified ClpA84T was loaded on a Sephacryl S-300 column (1 × 46 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.8) containing 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 0.1 M NaCl, and 20% glycerol. Fractions of 0.5 ml were collected at a flow rate of 10 ml/h. Elution of ClpA84T was then monitored by assaying proteins in the fractions by the dye-binding method (*A*). The same experiment was repeated but in the presence of 1 mM ATP (*B*). The size markers used were as follows: *lane a*, thyroglobulin (669 kDa); *lane b*, apoferritin (440 kDa); *lane c*, alcohol dehydrogenase (150 kDa); *lane d*, bovine albumin (66 kDa).

plex) than ClpA84 (*i.e.* monomer or dimer) in the absence of ATP, despite the finding that ClpA84 has a higher tendency to aggregate than ClpA84T under the same condition.

ATPase Activity of ClpA Proteins—ClpA84 is known to be an inherent ATPase that can be stimulated by protein substrates for ClpP (11). Therefore, we examined whether the mutations in ClpA show any alterations in the protein-activated ATPase activity. As shown in Table I, ClpA84T hydrolyzed ATP nearly at the same rate as ClpA84 and casein stimulated their ATPase activities by about 2-fold. Thus, replacement of the 169th Met residue by Thr in ClpA84 appears to be without any effect on the casein-stimulated ATPase activity. In contrast, ClpA65 cleaved ATP at a rate less than 5% of that seen by ClpA84. Because ClpA65 lacking the N-terminal 168 amino acids still retains the consensus sequences for ATP-binding within the high homology of the ClpA family, we examined whether the dramatic decrease in the ATPase activity of ClpA65 is due to its reduced ability to bind to ATP. Analysis by photoaffinity labeling experiment using azido-[α-^{32}P]ATP demonstrated that ClpA65 could be covalently labeled with the ATP analog as well as ClpA84 or ClpA84T upon irradiation with UV-light (Fig. 3*B*). In addition, both the 65- and 84-kDa proteins in ClpA84/65 could be radiolabeled under the same conditions. Because the concentration of azido-[α-^{32}P]ATP (*i.e.* 10 μM) used for the labeling experiment was much lower than its K_m (*i.e.* 210 μM) (11) and because the intensities of the labeled ClpA proteins were more or less proportional to the Coomassie-stained protein bands, it appears that ClpA65 is capable of binding to ATP equally as well as ClpA84. Furthermore, the ATPase activity of ClpA65, although markedly reduced, could be stimulated to about 2-fold by casein but not by proteins that are not substrate for ClpP and was precipitable upon incubation with the antibody raised against ClpA84 (data not shown). Therefore, the low ATP-cleaving activity seen by the ClpA65 preparation is not due to any other contaminating ATPase.

Ability of ClpA Proteins in Supporting ATP-dependent Proteolysis by ClpP—In order to determine whether the mutated forms of ClpA are capable of supporting the ATP-dependent

TABLE I
Ability of the ClpA proteins in ATP hydrolysis

ClpA proteins	ATP hydrolysis	
	−Casein	+Casein
	nmol	
ClpA84	11.9	20.3
ClpA84T	12.0	19.4
ClpA65	1.1	2.1
ClpA84/65	9.5	17.4

ATP hydrolysis was assayed by incubating the reaction mixtures containing 1 mM ATP, 0.3 μg of the purified ClpA84, ClpA84T, or ClpA65 or 0.39 μg of ClpA84/65 in the presence of and absence of 10 μg of casein. Incubations were performed at 37 °C for 30 min. The ratio of the 84-kDa protein to the 65-kDa polypeptide in the purified ClpA84/65 was 3.4:1 as estimated by densitometric scanning of the gel shown in lane a of Fig. 3*A*. Therefore, 0.39 μg of ClpA84/65 should contain 0.3 μg of the 84-kDa protein.

protein breakdown by ClpP, each of the purified ClpA84, ClpA84T, ClpA84/65, and ClpA65 proteins was incubated with ClpP, [^{3}H]casein, and ATP for various periods. While ClpA84T could support the casein hydrolysis as well as ClpA84, ClpA65 was not at all able to support it (Fig. 5*A*). On the other hand, ClpA84/65 supported the proteolysis only about 40% as well as ClpA84. Furthermore, the reduced ability of ClpA84/65 in supporting the proteolysis was not affected by increasing its protein concentration (Fig. 5*B*). These results raised a possibility that the 65-kDa polypeptides in the wild-type ClpA84/65 may down-regulate the ATP-dependent proteolytic function of the enzyme assembled with the 84-kDa ATPase and ClpP containing the serine active site.

To test this possibility, casein hydrolysis by the mixture of ClpP and ClpA84 or ClpA84T was assayed in the presence of increasing amounts of ClpA65. Fig. 6*A* shows that ClpA65 inhibits the casein-degrading activity of the reconstituted enzyme in a dose-dependent manner, although the inhibitory effect of ClpA65 does not reach to completion even in the presence of excess ClpA65 over ClpA84 or ClpA84T. However, at all tested concentrations, ClpA65 showed relatively little effect on casein hydrolysis by the mixture ClpP and ClpA84/65. Therefore, the reduced ability of ClpA84/65 to support the proteolysis by ClpP is due to the presence of the inhibitory 65-kDa polypeptides already in ClpA84/65, although it is presently unknown whether ClpA84/65 exists as a mixed oligomeric complex of the 84- and 65-kDa proteins or as a simple mixture of ClpA84 and ClpA65.

Since the protein-degrading activity of isolated ClpP is known to be tightly linked to the ATP-hydrolysis by ClpA (11), we suspected that the inhibitory effect of ClpA65 on the casein hydrolysis might be due to its inhibition of the ATPase activity of ClpA84 or ClpA84T in the reconstituted enzyme. Therefore, the reaction mixtures were prepared the same as in Fig. 6*A* and assayed for ATP hydrolysis. As shown in Fig. 6*B*, ClpA65 showed little or no effect on the casein-activated ATPase activity of the reconstituted enzyme. In addition, ClpA65 did not show any effect on the basal ATPase activity of ClpA84 alone or in the presence of ClpP (data not shown). Thus, ClpA65 may interfere the interaction of ClpA84 with ClpP and, hence, can inhibit the reconstitution into an active ATP-dependent protease but without any effect on the inherent or protein-activated ATPase activity of ClpA84.

DISCUSSION

The present studies demonstrate that the *clpA* gene contains dual translational initiation sites and therefore can encode two polypeptides with different sizes of 84 and 65 kDa (*i.e.* ClpA84 and ClpA65) from the same reading frame. We have previously reported that the *clpB* gene also containing dual translation

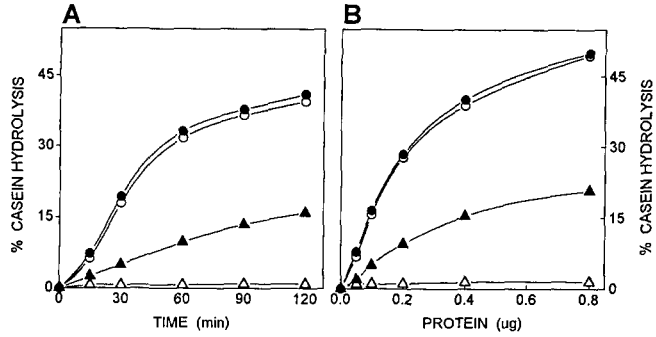

FIG. 5. **Ability of the purified ClpA proteins in supporting the ATP-dependent casein hydrolysis by ClpP.** *A*, reaction mixtures contained 0.1 μg of ClpP, 10 μg of [^{3}H]casein, and 0.3 μg of ClpA84 (●), ClpA84T (○), ClpA65 (△), or 0.39 μg of ClpA84/65 (▲). Proteolysis was then assayed by incubating them at 37 °C for various periods as described under "Experimental Procedures." *B*, proteolysis was also assayed as above but for 30 min in the presence of increasing amounts of the ClpA proteins and 0.5 μg of ClpP.

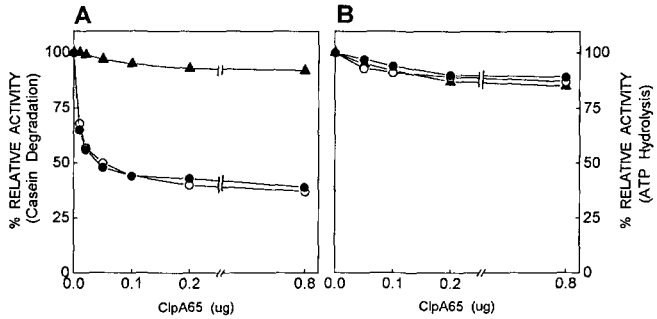

FIG. 6. **Effect of increasing concentrations of ClpA65 on the ATP-dependent casein degradation (*A*) and casein-activated ATP hydrolysis (*B*) by the mixture of ClpA84 (●), ClpA84T (○), or ClpA84/65 (▲) with ClpP.** Incubations were performed as in Fig. 5 but for 30 min and in the presence of increasing amounts of ClpA65. After the incubation, the extents of casein and ATP hydrolysis were determined as described under "Experimental Procedures." The casein- and ATP-degrading activities seen in the absence of ClpA65 were expressed as 100% and the others as their relative values.

start sites can encode 93- and 79-kDa polypeptides (22). In addition to these genes, the *clpC* gene in *Mycobacterium leprae* has also been found to contain a putative internal translation initiation site, GTG, that is preceded by a good Shine-Dalgarno sequence, AGGGAGAGAG (data not shown and Ref. 29). Therefore, all of the ClpA family so far identified in bacteria appear to contain dual translational start sites that can generate two polypeptides with different sizes from the same reading frames of their genes.

ClpA65 lacking the N-terminal 168 amino acids hydrolyzed ATP at a negligible rate as compared with ClpA84 or ClpA84T. However, ClpA65 was found to have the same affinity to ATP as ClpA84 and ClpA84T upon analysis by photoaffinity labeling with azido-[α-^{32}P]ATP. This result is consistent with the fact that ClpA65 retains the intact, two highly conserved sequences for adenine nucleotide binding (30). Therefore, it appears that the N-terminal domain of ClpA84 is essential for providing a proper three-dimensional atmosphere for ATP hydrolysis at the active site, although not necessary for its binding, and for oligomerization into an active form of the ATPase. In the case of the 93-kDa ClpB protein (ClpB93) that is a close homolog of ClpA84, its N-terminal domain of 148 amino acids has previously been shown to contain a protein-binding site(s) that is responsible for the activation of the ATPase. Furthermore, ClpB79, the abbreviated form of ClpB93, still is an active ATPase and behaves as a tetrameric molecule samely as ClpB93. In addition, the N-terminal domain of ClpC in plants has been reported to contain a transit sequence for its targeting to chloroplasts (21). Thus, the apparent roles of the N-terminal portions of the ClpA family seem to be quite diverse and may also be different from one another.

Of interest is the finding that ClpA65 can inhibit the ATP-dependent proteolytic activity of the enzyme reconstituted with ClpA84 and ClpP. This finding is analogous to our previous demonstration that ClpB79 is capable of inhibiting the protein-activated ATPase activity of ClpB93 (22). And this analogy is by the criteria that the products from the internal translational start sites may down-regulate the functions of products from the 5′-end of the same reading frames. However, ClpA65 has little or no effect on the protein-activated ATPase activity of ClpA84 itself and in the reconstituted enzyme. In addition, the ClpA65-mediated inhibition of the protein breakdown is not affected by the presence of excess ClpP over ClpA84 and ClpA65 (data not shown), indicating that the inhibition is not due to simple competition between ClpA84 and ClpA65 for binding to ClpP. Therefore, we suggest that ClpA65 may interfere the interaction of ClpA84 with ClpP and hence can block their assembly into an active form of the ATP-dependent protease.

Also of interest is the finding that the extent of inhibition by ClpA65 of the casein degrading activity of the reconstituted enzyme did not reach to completion but maximally was about 60% even in the presence of excess ClpA65 over ClpA84. The inhibitory effect reached a plateau when ClpA65 was treated with ClpA84 to a molar ratio of about 0.3 and did not significantly increase upon raising of the molar ratio. In the purified ClpA84/65, the 65-kDa polypeptide and the 84-kDa protein already exist at a molar ratio of 1:3.4. Perhaps for this reason, ClpA84/65 could support the casein-degrading activity of ClpP only about 40% as well as ClpA84 or ClpA84T, and its ability to support the proteolysis was not reduced any further upon the treatment of increasing concentrations of ClpA65. These observations clearly suggest that there exists certain stoichiometric interaction between ClpA65 and the reconstituted enzyme or its components, ClpA84 and/or ClpP. However, our attempts to determine their molecular interaction were unsuccessful because of the unusual physical properties of ClpA65 itself, such as high tendency to aggregate in low salt solutions, the inability to elute from gel filtration columns, and unusual behavior on nondenaturing polyacrylamide gels and on glycerol density gradients.

By both the genetic and biochemical approaches, the present studies have demonstrated that the *E. coli* cells harboring the pClpA plasmid produce both the 84- and 65-kDa proteins (*i.e.* ClpA84/65), although we do not know whether ClpA84/65 exists as a mixed oligomeric complex of the 84- and 65-kDa protein molecules in certain stoichiometric ratios or as a simple mixture of ClpA84 and ClpA65. Therefore, the wild-type cells without the plasmid must also contain both the protein-activated 84-kDa ATPase and the inhibitory 65-kDa polypeptide. In this regard, it is puzzling why these two opposing functions for the ATP-dependent proteolysis exist in the cells simultaneously within the same complex or as separate oligomeric molecules. A possible explanation for the presence of two types of the ClpA proteins is that the 65-kDa polypeptide may play an important role in the regulation of the ATP-dependent protein breakdown in the cells. Therefore, it would be interesting to determine the factors that influence the relative proportions of these two types of ClpA *in vivo* and whether any *in vivo* phenotype(s) is associated with the engineered ClpA proteins. These studies are presently under investigation.

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