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Site-directed Mutagenesis of the Dual Translational Initiation Sites of the *clpB* Gene of *Escherichia coli* and Characterization of Its Gene Products*

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The heat shock protein ClpB in *Escherichia coli* is a protein-activated ATPase and consists of two proteins with sizes of 93 and 79 kDa. By polymerase chain reaction-aided site-directed mutagenesis, both the proteins have been shown to be encoded by the same reading frame of the *clpB* gene, the 93-kDa protein (ClpB93) from the 5′-end AUG translational initiation site and the 79-kDa protein (ClpB79) from the 149th codon (an internal GUG start site). Both the purified ClpB93 and ClpB79 proteins behave as tetrameric complexes with a very similar size of about 350 kDa upon gel filtration on a Superose-6 column. Both appear to be exclusively localized to the cytosol of *E. coli*. Both show inherent ATPase activities and have an identical K_m of 1.1 mM for ATP. The ATPase activity of ClpB93 is as markedly stimulated by proteins, including casein and insulin, as that of wild-type ClpB, but the same proteins show little or no effect on ClpB79. Because ClpB79 lacks the 148 N-terminal sequence of ClpB93 but retains the two consensus sequences for adenine nucleotide binding, the N-terminal portion appears to contain a site(s) or domain(s) responsible for protein binding. Furthermore, ClpB79 is capable of inhibiting the casein-activated ATPase activity of ClpB93 in a dose-dependent manner but without any effect on its inherent ATPase activity. In addition, ClpB93 mixed with differing amounts of ClpB79 behave as tetrameric molecules, although its protein-activated ATPase activity is gradually reduced. These results suggest that tetramer formation between ClpB93 and ClpB79 may be responsible for the inhibition of the activity.

Escherichia coli contain at least two distinct ATP-dependent proteases, named proteases La and Ti. Protease La, the *lon* gene product (1, 2), is a heat shock protein (3, 4) and plays an essential role in the degradation of most abnormal proteins (1–6) and certain short-lived regulatory proteins (6). Protease La is composed of four identical subunits, each of which contains sites for ATP binding and hydrolysis (7–9). One intriguing feature of protease La is that protein substrates stimulate ATP-hydrolyzing activity of the enzyme (7). 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, component P (ClpP), which also is a heat shock protein (14), contains a serine-active site for proteolysis. The isolated ClpA also shows protein-activated ATPase activity, which in the reconstituted enzyme is linked to protein breakdown.

ClpA is a member of a family of highly conserved proteins with no previously described function; this family includes the product of a second *E. coli clpB* gene (15, 16). The sequences of this family have two regions of particularly high homology, each of which contains a consensus sequence for an adenine nucleotide binding site. ClpB, which also is a heat shock protein (17, 18), has recently been purified and shown to be a protein-activated ATPase (19). When isolated, this protein, unlike dimeric ClpA (13), behaves as a tetramer of 93-kDa subunits. In addition, ClpB does not interact with antibody raised against ClpA nor can replace ClpA in supporting the ATP-dependent casein-degrading activity of ClpP (19). Therefore, the physiological role of ClpB remains unknown.

An additional protein with 79-kDa size, which also is a heat shock protein, has been shown to be copurified with ClpB (18, 19). A number of lines of evidences have suggested that the 79-kDa protein is derived from the same reading frame as ClpB, probably by an internal translational initiation (18, 19). Perhaps the most compelling evidence is that a segment of the internal amino acid sequence, VNDQGAEDQ, of ClpB can also be found in the 79-kDa protein as its N-terminal amino acid sequence (19–21). Because the 79-kDa protein lacks 148 N-terminal amino acids of ClpB but retains the consensus sequences for adenine nucleotide binding within two regions of high homology of the ClpA family, it has been suggested to be also a functional protein.

In addition, it has been suggested that the presence of two forms of ClpB in *E. coli* cells may provide a means of targeting the proteins to different subcellular locations (18, 20) by an analogy to the *MOD5*-encoded isozymes in *Saccharomyces cerevisiae* that are partitioned to cytoplasm and mitochondria (22). However, the interrelationship and action of both forms of ClpB are unknown. In the present study, mutational analyses of the dual translational start sites of the *clpB* gene were performed to examine whether the mutations can exclusively produce the 93- and 79-kDa ClpB proteins and therefore to show both proteins can indeed be generated from a single *clpB* mRNA. In addition, both ClpB proteins were purified for their functional and structural analyses.

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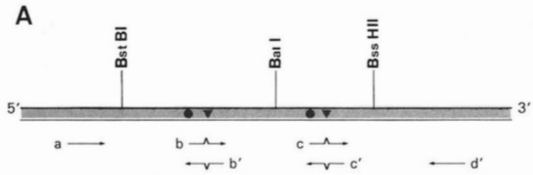

B

Primers	Nucleotide sequences	Base substitution
a	5′-TTCTCGCCTGGTTAGGGCCG-3′	
b	5′-GGAGGAGTTACGCGTCTGGAT-3′	T → C
b′	5′-ATCCAGACGCGTAACTCCTCC-3′	A → G
c	5′-GTGAAAGCGTTAACGATCAAG-3′	G → T
c′	5′-CTTGATCGTTAACGCTTTCAC-3′	C → A
d′	5′-CAAGATCGTTAAGCACGCC-3′	

Wild-type b region: --- ATGGGAGGAGTTATGCGTCTGGAT ---
Wild-type c region: --- GGAGGTGAAAGCGTTGAACGATCAA ---

FIG. 1. **The restriction map of the *clpB* gene (*A*) and the oligonucleotide primers used for site-directed mutagenesis (*B*).** In *A*, the *arrows* indicate the primers used for PCR. In *B*, the *shaded letters* indicate the mutated nucleotides, and the *underlines* show the sequences subsequently created for restriction by the mutations. The wild-type nucleotide sequences of regions *b* and *c* are also shown. The *dots* show possible Shine-Dalgarno sequences, and the *triangles* indicate the translational initiation sites.

EXPERIMENTAL PROCEDURES

Materials—*E. coli* strains, MC1009/pClpB and MC1000Δ*clpB*::Km^r (18), were kindly provided by Dr. Catherine Squires (Columbia University). The *clpB* gene product was purified as described previously (19). The antibody against the 93-kDa protein of the purified ClpB was obtained as described (19). All reagents for the polymerase chain reaction (PCR),[1] including *Taq* polymerase, were obtained from Boehringer Mannheim. Restriction endonucleases and other DNA-modifying enzymes were purchased from New England BioLabs and KOSCO Biotech. (Korea). Oligonucleotide primers were synthesized using an automated DNA synthesizer (Applied Biosystem, model 380A) and purified by gel electrophoresis as described by the manufacturer. DEAE-Sepharose and Superose-6 were purchased from Pharmacia LKB Biotechnology Inc., heparin-agarose from Bethesda Research Laboratories, and ^{125}I-labeled protein A from Du Pont-New England Nuclear. All other reagents were obtained from Sigma.

Mutagenesis and Subcloning—Site-directed mutations were created by the PCR method, which consists of two sequential PCRs (23). pClpB (18) was digested by *Pst*I, and the 4.2-kilobase fragment containing the entire *clpB* gene was ligated into the *Pst*I site of Bluescript KS$^+$ plasmid (pBS). The resulting recombinant plasmid (pBS-ClpB) was linearized by digestion with *Sma*I and used as the template for the primary PCRs. Reaction mixtures (100 μl each) contained 50 ng of the template, 0.5 units of *Taq* polymerase, 1 × PCR buffer (Boehringer Mannheim), 200 μM each of deoxynucleotide triphosphate (dNTP), and 50 pmol of appropriate primers (see below). The reactions were carried out for 30 cycles using a DNA thermal cycler (Ericomp, Inc.). The primary PCRs generated three different fragments as follows: fragment I by primers *a* and *b′*, fragment II by *b* and *c′*, and fragment III by *c* and *d′* (Fig. 1).

Prior to the secondary PCRs, the same amounts (200 ng each) of fragments I and II and of II and III were mixed and subjected to annealing between the fragments by denaturation-renaturation reactions. The DNAs with recessed 3′-OH ends were extended by Klenow to produce complete duplex fragments. The extended DNAs were then used as the templates for the secondary PCRs. The reactions were performed for 30 cycles as above but using a different combination of primers. Fragment IV was synthesized using primers *a* and *c′*, while fragment V was made using primers *b* and *d′*.

Fragments IV and V were gap-filled by Klenow and ligated into pBS, and the recombinant plasmids were maintained in *E. coli* strain JM109 for further use. *Bst*BI/*Bal*I and *Bal*I/*Bss*HII fragments were electro-purified from the plasmids containing fragments IV and V, respectively. The restriction segments were then ligated into pBS-ClpB, from which the same restriction fragments had been eliminated prior to use. The resulting plasmid containing the mutated *Bst*BI/*Bal*I fragment was referred to as pBS-ClpB79 and that containing the mutated *Bal*I/*Bss*HII as pBS-ClpB93. The plasmids were maintained in a *clpB* null mutant, MC1000Δ*clpB*::Km^r.

Immunochemical Analysis—*E. coli* cells were grown at 35 °C in Luria broth to reach an optical density (600 nm) of 1.0 and shifted to 45 °C. After culturing for 2 h at this temperature, appropriate amounts of the cells were harvested, boiled, and electrophoresed in 8% (w/v) polyacrylamide slab gels in the presence of SDS (24). Proteins in the gels were transferred to nitrocellulose membranes and incubated with anti-ClpB antiserum and then with ^{125}I-labeled protein A solution (0.1 μCi/ml). After incubation, the membranes were dried and autoradiographed (25).

Assays—ATPase activity was assayed by incubating the reaction mixtures (0.1 ml) containing appropriate amounts of the mutated ClpB proteins, 5 mM ATP, 100 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM EDTA (19). ATP was titrated to pH 7.5 with 0.1 N NaOH just prior to the incubation. After incubating the mixtures at 37 °C for 30 min, the reaction was terminated by adding 0.1 ml of 1% SDS. The phosphate released was then determined as described by Ames (26). Proteins were assayed by their absorbance at 280 nm or by the method of Bradford (27) using serum albumin as a standard.

Preparation of E. coli Crude Extract—*E. coli* cells were grown as above and kept frozen at −70 °C until use. The frozen cells (15 g) were thawed and resuspended in 30 ml of 20 mM Tris-HCl (pH 8) containing 100 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM EDTA, and 10% (v/v) glycerol. The cells were then disrupted with a French press at 14,000 psi and centrifuged 100,000 × *g* for 3 h. The supernatant was dialyzed against the same buffer and referred to as crude extract.

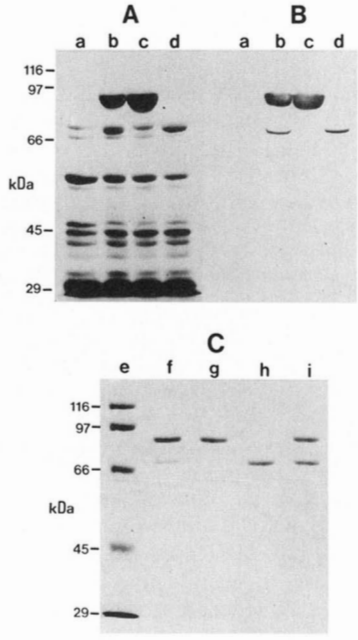

FIG. 2. **Electrophoretic and immunoblot analyses of ClpB93 and ClpB79.** *E. coli* cells were grown at 35 °C in Luria broth to reach an optical density (600 nm) of 1.0 and shifted to 45 °C. After culturing for 2 h at this temperature, the same aliquots of the cells were harvested, boiled, and electrophoresed as described under "Experimental Procedures." The gels were then stained with Coomassie R250 (*A*) or subjected to immunoblot analysis (*B*). *Lanes a*, the *clpB* mutant (MC1000Δ*clpB*::Km^r); *b*, the cell harboring pBS-ClpB; *c*, pBS-ClpB93 (MC1093); *d*, pBS-ClpB79 (MC1079). From the cells containing the mutated plasmids, ClpB93 and ClpB79 were purified by the same protocol used for the purification of wild-type ClpB (19). The purified proteins (5 μg each) were electrophoresed, and the resulting gels were stained as described above (*C*). *Lane e*, size markers; *f*, purified ClpB; *g*, ClpB93; *h*, ClpB79; *i*, ClpB93 plus ClpB79.

[1] The abbreviation used is: PCR, polymerase chain reaction.

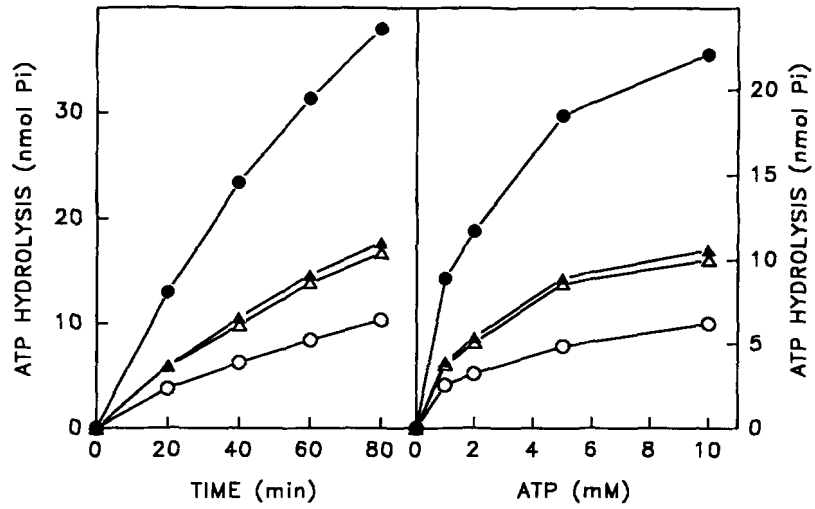

FIG. 3. **Effect of casein and increasing concentrations of ATP on the ATPase activities of ClpB93 and ClpB79.** *Left panel*, the purified proteins (3 μg each) were incubated at 37 °C with 5 mM ATP for various periods in the presence and absence of 10 μg of casein. After incubation, assays were performed as described under "Experimental Procedures." *Right panel*, ATP hydrolysis was assayed as above but in the presence of varying amounts of ATP. Incubations were at 37 °C for 30 min. *Circles* are for ClpB93, and *triangles* are for ClpB79. The *closed symbols* represent the ATPase activity in the presence of casein, while the *open symbols* show the activity in its absence.

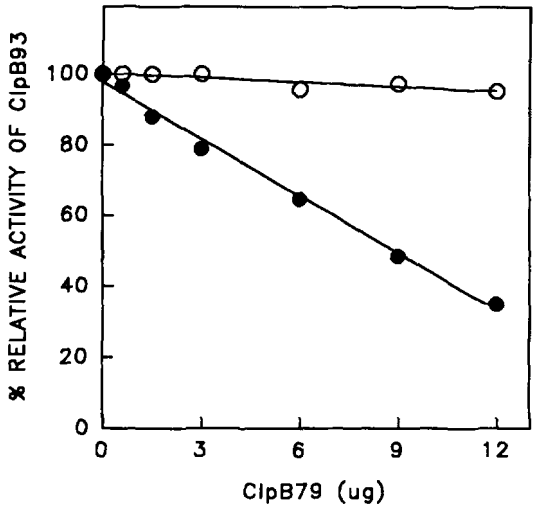

FIG. 4. **Effect of increasing amounts of ClpB79 on the ATPase activity of ClpB93.** ClpB93 (3 μg) was incubated with increasing amounts of ClpB79 at 37 °C for 10 min. ATP hydrolysis by the mixtures was then assayed as in Fig. 3. The activities seen with differing amounts of ClpB79 alone were subtracted from those with the fixed amount of ClpB93, and the differences in the activities with (●) or without (○) casein were expressed as the percents of the activity by ClpB93 only. The ATPase activity of ClpB93 in the presence or absence of casein was set as 100%.

RESULTS

ClpB79 Is Derived by an Internal Translational Initiation—Codon 149 of ClpB is GUG and is preceded by a good Shine-Dalgarno sequence (GGAGG) separated from this codon by 7 nucleotides, similarly to the 5′-end AUG initiation codon of ClpB (18). To determine whether the 79-kDa protein, copurified with the 93-kDa ClpB protein, is indeed derived from the possible internal translational initiation site, PCR-aided site-directed mutagenesis of the *clpB* gene was performed. In the construction of mutagenic primers, codon GUG was replaced by GUU for blocking the internal initiation (*i.e.* to prevent synthesis of the 79-kDa protein), for allowing the synthesis of the 93-kDa ClpB protein (ClpB93) without substitution of the amino acid at the site of mutation since both the codons are for Val, and for creating a new restriction site for *Hpa*I to facilitate the mutant isolation. Similarly, codon AUG at the N terminus of ClpB was replaced by ACG for the exclusive synthesis of the 79-kDa protein (ClpB79) and for the generation of a new *Mlu*I restriction site. The mutagenesis using the primers yielded two mutated plasmids, named pBS-ClpB79 and pBS-ClpB93, and the mutations were confirmed by the restriction and nucleotide sequence analyses of the plasmids (data not shown). The plasmids, pBS-ClpB79 and pBS-ClpB93, were maintained in a *E. coli clpB* null mutant (MC1000Δ*clpB*::Km^r), and the resulting cells were referred to as MC1079 and MC1093, respectively.

To examine whether the *E. coli* cells are capable of producing either of ClpB93 or ClpB79 using their single start site of the mutated *clpB* gene, they were grown to the same optical density under a heat-stressed condition and subjected to electrophoretic analysis. As shown in Fig. 2*A*, MC1093 overproduced only ClpB93, as MC1079 exclusively did ClpB79. In addition, the antibody raised against the 93-kDa protein of the wild-type ClpB strongly interacted with ClpB79 as well as ClpB93 (Fig. 2*B*). These results clearly demonstrate that the 79-kDa protein of ClpB is derived from the same reading frame as ClpB93 by an internal translational initiation.

Structure and ATPase Activities of ClpB79 and ClpB93—To determine the structural organization of ClpB79 and ClpB93, each of the proteins was purified from the crude extracts of MC1079 and MC1093, respectively, using the same purification protocol used for the wild-type ClpB (19). Analyses of the purified ClpB93 and ClpB79 by polyacrylamide gel electrophoresis in the presence of SDS reveal that both proteins are apparently homogeneous (Fig. 2*C*). We then chromatographed the purified proteins on a Superose-6 column under nondenaturing conditions for estimation of their native sizes. Both proteins eluted as single symmetric peaks with an apparent size of about 350 kDa (see below). Therefore, it appears likely that both ClpB79 and ClpB93 behave as tetrameric complexes.

When isolated, the wild-type ClpB consists of the major 93-kDa protein and the minor 79-kDa protein and is a protein-activated ATPase (19). To determine whether ClpB93 and/or ClpB79 also are functional proteins, ATP hydrolysis by the purified proteins was assayed in the presence and absence of casein. As shown in Fig. 3, casein dramatically stimulated the ATPase activity of ClpB93 as it did for the wild-type ClpB (19). On the other hand, little or no effect of casein was observed for ClpB79. No other protein so far tested, including insulin, which also stimulates the ATPase activity of ClpB93, was capable of activating the ATP hydrolysis by ClpB79 (data not shown). These results suggest that the 148 N-terminal leader sequence of ClpB may contain a casein-binding domain(s), which confers the protein-activation of the ATPase. Noteworthy is the finding that, in the absence of casein, the

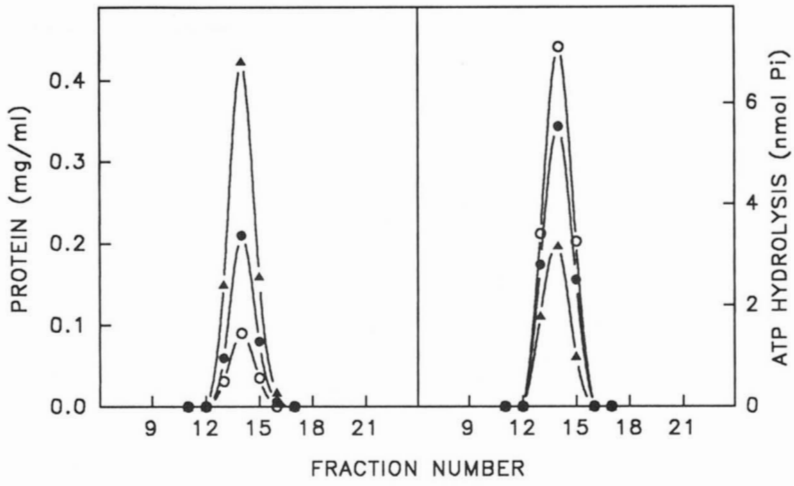

FIG. 5. **Elution profiles of ClpB93 and its mixture with increasing amounts of ClpB79 from a Superose-6 column.** ClpB93 (0.2 mg) was incubated at 37 °C for 10 min with 0 (○), 0.2 (●), or 0.6 (▲) mg of ClpB79. Each of the mixtures in a final volume of 0.2 ml was then loaded on a Superose-6 column (1 × 30 cm) equilibrated with 20 mM Tris-HCl (pH 8) buffer containing 5 mM $MgCl_2$, 1 mM dithiothreitol, and 0.5 mM EDTA. Proteins were eluted at a flow rate of 18 ml/h, and aliquots of each fraction were assayed for protein (*left panel*). The fractions were also assayed for ATP hydrolysis in the presence and absence of 10 μg of casein as in Fig. 4, and the activities seen in the absence of casein were subtracted from those seen in its presence (*right panel*).

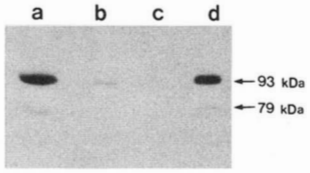

FIG. 6. **Subcellular fractionation of ClpB.** *E. coli* strain, MC1009, was grown to mid-log phase as described in the text, and its subcellular fractions were prepared by the method of Nossal and Heppel (28). After the osmotic shock step, the cells were suspended in 50 mM Tris-HCl (pH 7.8) containing 10 mM $MgCl_2$ and 0.2 M KCl and were disrupted by a French press at 14,000 psi. The unbroken cells were removed by centrifugation at 2,000 × *g* for 10 min, and the supernatant was again centrifuged at 30,000 × *g* for 1 h. The resulting supernatant represents cytosolic fraction (*lane a*). The pellet, which represents membrane fraction (*lane b*), was washed twice with the same buffer. All the subcellular fractions including the shock fluid, periplasmic fraction (*lane c*), were dialyzed overnight against 10 mM Tris-HCl (pH 7.4) at 4 °C. The same amounts of the fractions (50 μg each) were electrophoresed and subjected to immunoblot analysis. Relative distributions of the β-galactosidase activity were 97.8, 1.9, and 0.3% in the same cytosolic, membrane and periplasmic fractions, respectively. *Lane d* indicates the purified ClpB.

ATPase activity of ClpB79 is about 2-fold higher than that of ClpB93. However, the affinity of ATP to both proteins was estimated to be identical (K_m = 1.1 mM). In addition, neither ClpB93 nor ClpB79 was capable of replacing ClpA in supporting the ATP-dependent casein-hydrolysis by ClpP (data not shown), similarly to the wild-type ClpB (19).

Molecular Interaction of ClpB93 with ClpB79—In our previous report (19), we have suggested that the 93-kDa ClpB may be able to form a hetero-oligomeric complex with the 79-kDa protein. To examine the possible molecular interaction between the proteins, ClpB93 was mixed with increasing concentrations of ClpB79 and assayed for ATP hydrolysis. As shown in Fig. 4, ClpB79 decreased the casein-stimulated ATPase activity of ClpB93 in a dose-dependent manner (*i.e.* the activities shown by the mixtures were not additive). On the other hand, ClpB79, in the absence of casein, showed little or no effect on ATP hydrolysis by ClpB93. Because the ATPase activity of ClpB79 is not activated by proteins, the inhibition of the protein-activated ATPase activity of ClpB93 is likely to be mediated by interaction of ClpB79 with ClpB93.

In order to examine whether ClpB79 and ClpB93 interact in a tetrameric complex, in a larger oligomeric complex, or as heterodimers, ClpB93 was incubated with different amounts of ClpB79, and the mixtures were run on a Superose-6 column. Under all conditions, the proteins eluted as a sharp peak indistinguishable in size from the ATPase activity (Fig. 5). Furthermore, the ClpB93 appeared to be in tetramers, even when they were mixed with increasing amounts of ClpB79 such that the casein-activated ATPase activity was gradually inhibited. Thus, it appears that ClpB79 does not inhibit by causing dissociation of tetrameric ClpB93 into dimers and monomers or by causing aggregation of the complex.

Subcellular Location of ClpB79 and ClpB93—The N-terminal leader sequence of ClpB has been suggested to be involved in mobilizing the protein for transport (18, 20). To examine whether the sequence plays a role in differential partitioning of ClpB93 and ClpB79, *E. coli* cells (MC1009) were grown to mid-log phase under heat-stressed conditions and subjected to subcellular fractionation. Both the 93- and 79-kDa proteins were found to locate almost exclusively in the cytosolic fraction (Fig. 6). Nearly identical results were obtained when MC1093 or MC1079 was used for the preparation of subcellular fractions (data not shown). Thus, it appears clear that the N-terminal portion of ClpB is not related with protein targeting.

DISCUSSION

The present studies demonstrate that the *clpB* gene encodes two proteins with different sizes, ClpB93 and ClpB79, from the same reading frame but using its dual translational initiation sites. Mutagenesis of the 5′-end initiation site eliminated the synthesis of ClpB93 but resulted in an exclusive production of ClpB79, while mutation at the internal initiation site produced only ClpB93. In addition, ClpB79 is found to have the same affinity to ATP as ClpB93 and also be an inherent ATPase. These results are in accord with the fact that ClpB79 retains the intact, two highly conserved sequences (N1 and N2) for adenine nucleotide binding (16, 18). Of interest, however, is the finding that the ATPase activity of ClpB79, lacking the 148 N-terminal amino acids of ClpB93, is not activated by proteins. Therefore, it is possible that the N-terminal portion of ClpB93 contains a protein-binding site(s) that is responsible for the activation of the ATPase.

Of particular interest is the demonstration that ClpB79 is capable of inhibiting the casein-activated ATPase activity of ClpB93 but not its inherent ability to cleave ATP. Because ClpB79 is unable to dissociate the tetrameric ClpB93 into dimers and monomers or to aggregate the complex, inhibition appears to be mediated by direct interaction between ClpB79 and ClpB93, such as by forming hetero-tetrameric complexes. However, our attempts to separate inactive molecules containing both ClpB79 and ClpB93 subunits from the homo-

tetrameric forms of the proteins were unsuccessful, probably because of their very similar chromatographic behavior and similar sizes. Therefore, direct evidence about the molecular interaction of ClpB79 with ClpB93 is lacking. Nevertheless, we were able to show that the inhibited form of ClpB93 and its fully active form were almost the same size. In addition, we have previously shown that the wild-type ClpB, which ran as a single band in polyacrylamide gels under nondenaturing conditions, still consisted of both the 93- and 79-kDa proteins (19). The most likely explanation of these findings is that the ClpB79 and ClpB93 subunits interact to form mixed tetramers, which lack or have reduced casein-activated ATPase activity.

The wild-type ClpB cells thus must contain both the protein-activated ATPase activity (the 93-kDa protein) and its inhibitory activity (the 79-kDa protein) (both encoded by the same gene). Also noteworthy is the finding that in the absence of casein, the ATPase activity of ClpB79 is significantly higher than that of ClpB93. Therefore, it is unclear why these two opposing functions exist in the cells simultaneously within the same oligomers or in separate homo-tetrameric molecules. A possible explanation for the presence of two types of the ClpB proteins is that the 79-kDa protein may play a role in the control of the protein-activated ATPase activity of the 93-kDa protein and hence regulate its unknown function in cells. The factors influencing the relative proportions of these two types of ClpB proteins *in vivo*, therefore, seemed to be interesting to investigate. However, it has been reported that the 93- and 79-kDa proteins are made in coordinate amounts under a variety of conditions, including anaerobiosis, heat shock, overproduction by multicopy plasmids containing the *clpB* gene, and the combination of the plasmid and heat shock (18). And yet, other conditions, such as amino acid limitation, also need to be tested for elucidation of possible translational control of expression of the 93- and 79-kDa proteins. It would also be interesting to determine whether any *in vivo* phenotype(s) is associated with the engineered ClpB proteins. Does the construct expressing the 93-kDa protein alone, or the 79-kDa protein alone, behave differently from wild-type? These questions are presently under investigation.

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REFERENCES

1. Chung, C. H., and Goldberg, A. L. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 4931–4935
2. Charette, M. F., Henderson, G. W., and Markovitz, A. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 4728–4732
3. Phillips, T. A., VanBogelen, R. A., and Neidhardt, F. C. (1984) *J. Bacteriol.* **159,** 283–287
4. Goff, S. A., Casson, L. P., and Goldberg, A. L. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 6647–6651
5. Goff, S. A., and Goldberg, A. L. (1985) *Cell* **41,** 587–595
6. Gottesman, S. (1989) *Annu. Rev. Genet.* **23,** 163–198
7. Waxman, L., and Goldberg, A. L. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 4883–4887
8. Chin, D. T., Goff, S. A., Webster, T., Smith, T., and Goldberg, A. L. (1988) *J. Biol. Chem.* **263,** 11718–11728
9. Menon, A. S., and Goldberg, A. L. (1987) *J. Biol. Chem.* **262,** 14921–14928
10. Katayama-Fujimura, Y., Gottesman, S., and Maurizi, M. R. (1987) *J. Biol. Chem.* **262,** 4477–4485
11. Katayama, Y., Gottesman, S., Pumphrey, J., Rudikoff, S., Clark, W. P., and Maurizi, M. R. (1988) *J. Biol. Chem.* **263,** 15226–15236
12. Hwang, B. J., Park, W. J., Chung, C. H., and Goldberg, A. L. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 5550–5554
13. Hwang, B. J., Woo, K. M., Goldberg, A. L., and Chung, C. H. (1988) *J. Biol. Chem.* **263,** 8727–8734
14. Kroh, H. E., and Simon, L. D. (1990) *J. Bacteriol.* **172,** 6026–6034
15. Gottesman, S., Clark, W. P., and Maurizi, M. R. (1990) *J. Biol. Chem.* **265,** 7886–7893
16. Gottesman, S., Squires, C., Pichersky, E., Carrington, M., Hobbs, M., Mattick, J. S., Dalryple, B., Kuramitsu, H., Shioza, T. Foster, T., Clark, W. C., Ross, B., Squires, C. L., and Maurizi, M. R. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 3513–3517
17. Kitagawa, M., Wada, C., Yoshioka, S., and Yura, T. (1991) *J. Bacteriol.* **173,** 4247–4253
18. Squires, C. L., Pedersen, S., Ross B. M., and Squires, C. (1991) *J. Bacteriol.* **173,** 4254–4262
19. Woo, K. M., Kim, K. I., Goldberg, A. L., Ha, D. B., and Chung, C. H. (1992) *J. Biol. Chem.* **267,** 20429–20434
20. Squires, C., and Squires, C. L. (1992) *J. Bacteriol.* **174,** 1081–1085
21. Pontis, E., Sun, X., Jornvall, H., Krook, M., and Reichard, P. (1991) *Biochem. Biophys. Res. Commun.* **180,** 1222–1226
22. Gillman, E. C., Slusher, L. B., Martin, N. C., Hopper, A. K. (1991) *Mol. Cell. Biol.* **11,** 2382–2390
23. Higuchi, R., Krummel, B., and Saiki, R. K. (1988) *Nucleic Acids Res.* **16,** 7351–7367
24. Laemmli, U. K. (1970) *Nature* **227,** 680–685
25. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
26. Ames, B. (1966) *Methods Enzymol.* **8,** 115–118
27. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
28. Nossal, N. G., and Heppel, L. A. (1966) *J. Biol. Chem.* **241,** 3055–3062