Mutational analysis of the two ATP-binding sites in ClpB, a heat shock protein with protein-activated ATPase activity in *Escherichia coli*

Keun Il KIM¹, Kee Min WOO¹, Ihn Sik SEONG, Zee-Won LEE, Sung Hee BAEK and Chin Ha CHUNG²

Department of Molecular Biology and Research Center for Cell Differentiation, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea

The 93 kDa ClpB (ClpB93) is a heat shock protein and has a protein-activated ATPase activity. To define the role of the two ATP-binding sites in ClpB93, site-directed mutagenesis was performed to replace Lys²¹² or Lys⁶¹¹ with Thr or Glu. All of the mutant proteins hydrolysed ATP at a higher rate than that seen with ClpB93 at ATP concentrations up to 2 mM. However, ClpB93 carrying mutations in both of the ATP-binding sites could not cleave ATP. Thus any of the two ATP-binding sites seems to be capable of supporting the ATPase activity of ClpB93. The ATPase activities of both ClpB93/K212T and ClpB93/K212E were gradually decreased when ATP concentrations were increased above 2 mM, unlike those of ClpB93, ClpB93/K611T and ClpB93/K611E, which showed a typical saturation curve. Furthermore ADP inhibited ATP hydrolysis by ClpB93/K212T and ClpB93/K212E more effectively than that by the latter proteins, suggesting that the mutations in the first ATP-binding site result in an increase in the affinity of ADP for the second

site in ClpB93. In addition, all of the purified ClpB93 and its mutant forms behaved as an oligomer of 400–450 kDa on a Sephacryl S-300 gel-filtration column, whether or not ATP was present. Thus the binding of ATP to either of the two sites seems not to be essential for oligomerization of ClpB93. Although a low-copy plasmid carrying *clpB93* could rescue the sensitivity of a *clpB*-null mutant cell at 52 °C, none of the plasmids carrying the mutations in the ATP-binding sites could. Furthermore, incubation at 52 °C resulted in a gradual loss of the ATPase activity of ClpB93 carrying the mutations in either of the two ATP-binding sites, but not of the parental ClpB93, indicating that the mutant proteins have a greater tendency to denature at this temperature than the parental ClpB93. These results suggest that both of the ATP-binding sites in ClpB have an important role in maintaining the thermotolerance of the protein and hence in the survival of *Escherichia coli* at high temperatures.

INTRODUCTION

Protease Ti (caseinolytic protease; Clp) in *Escherichia coli* is an ATP-dependent protease consisting of two different polypeptide subunits, both of which are required for proteolysis [1–5]. ClpA is a protein-activated ATPase, whereas ClpP contains a serine active site for proteolysis. The isolated ClpA shows protein-activated ATPase activity, which in the reconstituted ClpA/ClpP complex is linked to protein breakdown [6,7]. ClpA is a member of the Clp/Hsp100 family of highly conserved proteins that have one or two regions of particularly high similarity, which contain a consensus sequence for ATP-binding [8,9]. Members of the family include a second *E. coli* gene called *clpB* and genes from other bacteria, trypanosomes, yeasts and plants. Because of the sequence similarity of ClpB with that of ClpA [8], ClpB has been suggested to have an important role in ATP-dependent proteolytic processes in *E. coli* [10]. However, it has been shown that ClpB cannot replace ClpA in supporting the ATP-dependent proteolysis by ClpP [11]. Therefore the physiological function of ClpB remains unknown.

ClpB is a heat shock protein [12,13] and has a protein-activated ATPase activity [11]. In addition, *clpB* has been shown to contain dual initiation sites for translation and therefore encodes two polypeptides with different size, the 93 and 79 kDa forms of ClpB (referred to as ClpB93 and ClpB79 respectively) [14]. Similarly to the wild-type ClpB, the purified ClpB93 protein has an inherent ATPase activity that can be stimulated by proteins, such as casein and insulin. However, ClpB79, which also is an inherent ATPase, cannot be activated by the proteins, despite the fact that ClpB79 retains the two consensus sequences for adenine nucleotide binding [14]. Thus the N-terminal portion

of ClpB93 has been suggested to contain one or more sites or domains responsible for protein binding.

By replacement of the Lys residue in either of the two ATP-binding sites in ClpA with Thr, it has been demonstrated that the first ATP-binding site is responsible for its oligomerization and the second site is essential for ATPase function [15,16]. To determine the role of the ATP-binding sites in ClpB we generated the ClpB93 mutant proteins, in which the Lys²¹² and/or the Lys⁶¹¹ residue in the ATP-binding sites was replaced by Thr or Glu, and examined the effects of the mutations on the ATPase activity and oligomeric nature of ClpB93. In addition we examined the effects of the mutations on the thermotolerance of ClpB93 and on the survival of *E. coli* at high temperatures.

EXPERIMENTAL

Materials

E. coli strains MC1009/pClpB and MC1000Δ*clpB*::Km^r were kindly provided by Dr. Catherine Squires (Tufts University, Boston, MA, U.S.A.). All reagents for the site-directed mutagenesis were obtained from Promega. Restriction endonucleases and other DNA-modifying enzymes were purchased from New England BioLabs and Poscochem (Korea). Oligonucleotide primers were synthesized with an automated DNA synthesizer (Applied Biosystem, model 384A). All other chemicals were from Sigma unless indicated otherwise.

Site-directed mutagenesis

The recombinant plasmids pBS-ClpB and pBS-ClpB93, carrying *clpB* and *clpB93* respectively, were constructed as described

Abbreviation used: Clp, caseinolytic protease.

¹ These authors contributed equally to this work.

² To whom correspondence should be addressed (e-mail chchung@plaza.snu.ac.kr).

Figure 1 Schematic diagram of two ATP-binding regions in ClpB93 (A) and the primers used for site-directed mutagenesis (B)

The underlines in (B) indicate the mutated nucleotides.

previously [14]. The 2.9 kb *NruI/PstI* fragments from the plasmids were inserted into the pALTER-1 mutagenesis vector (Promega). Site-directed mutagenesis was then performed with the Altered Sites II *in vitro* Mutagenesis System (Promega). Mutagenic oligonucleotides were designed to replace Lys²¹² or Lys⁶¹¹ by Thr or Glu in the ATP-binding sites of ClpB93 (Figure 1). The mutated nucleotides are indicated by underlining. The *NruI/PstI* fragments carrying the mutations were then ligated back to pBS vectors that had been digested with the same restriction enzymes. To generate ClpB93 carrying mutations in both ATP-binding sites (i.e. to generate a K212T/K611T double mutant), an *NcoI/SacI* fragment carrying the mutations for Thr in the first site was transferred to the plasmid carrying the mutations for the same amino acid in the second site. The resulting plasmids were transformed into a *clpB*-null mutant, MC1000Δ*clpB*::Km^r. The mutations were confirmed by DNA sequence analyses (results not shown). The plasmids pHSG-ClpB and pHSG-ClpB93 were also constructed by inserting the 2.9 kb *NruI/HindIII* fragments from pBS-ClpB and pBS-ClpB93 respectively into the low-copy plasmid pHSG575 [17] that had been cut with *SmaI/HindIII*. They were then transformed into the *clpB*-null mutant strain. *clpB93* carrying the mutations in the ATP-binding sites was also inserted into the low-copy plasmid.

Purification of ClpB proteins

E. coli cells harbouring the *clpB* plasmids were grown in Luria broth at 35 °C until reaching an attenuation (*D*₆₀₀) of 1.0 and were then shifted to 45 °C. After being cultured for 2 h at this temperature, the cells were harvested and resuspended in 50 ml of 20 mM Tris/HCl, pH 7.8, containing 5 mM MgCl₂, 0.5 mM EDTA, 1 mM dithiothreitol and 10 % (v/v) glycerol. The cells were then disrupted with a French press at 97 MPa and centrifuged at 100 000 *g* for 3 h. The supernatant was dialysed against the same buffer and subjected to purification of the ClpB93 proteins as described previously [11]. The purity of the proteins

was analysed by SDS/PAGE in the presence of 2-mercaptoethanol as described [18].

Assays

ATP hydrolysis was assayed by incubating the reaction mixtures (0.1 ml) containing appropriate amounts of the ClpB93 proteins and various concentrations of [γ -³²P]ATP in 100 mM Tris/HCl (pH 8.0)/10 mM MgCl₂/1 mM dithiothreitol/1 mM EDTA [19]. ATP was titrated to pH 7.5 with 0.1 M NaOH before the incubation. After incubation of the samples at various temperatures for appropriate periods, the reaction was terminated by the successive addition of 0.1 ml of 0.1 M HCl, 0.1 ml of 2 mg/ml BSA and 0.25 ml of a 10 % (w/v) suspension of activated charcoal. The samples were stirred on a vortex mixer, incubated for 15 min on ice and then centrifuged at 15000 *g* for 5 min. The radioactivity of free phosphates released into the supernatant fractions were then counted using a liquid-scintillation counter. Proteins were assayed by their absorbance at 280 nm or by the dye-binding method with BSA as a standard [20].

Thermotolerance test

E. coli were grown at 30 °C to reach a *D*₆₀₀ of 0.5 (i.e. mid-exponential phase) in a minimal medium containing 22.2 mM glucose, 47.8 mM Na₂HPO₄, 22.0 mM KH₂PO₄, 6.6 mM NaCl and 18.7 mM NH₄Cl. The cells were then shifted to 52 °C and incubated for various periods. After incubation they were diluted in ice-cold Luria broth and plated in triplicate on agar plates containing ampicillin. The plates were incubated overnight at 30 °C and the cell colonies were counted.

RESULTS

ATP hydrolysis by ClpB mutants

To investigate the importance of the two ATP-binding sites for ClpB function, either Lys²¹² or Lys⁶¹¹ in ClpB93 was replaced

Figure 2 SDS/PAGE of the purified ClpB93 and its mutant forms

ClpB93 and its mutant forms (7 μ g of each) were subjected to electrophoresis on 10 % (w/v) polyacrylamide slab gels containing SDS and 2-mercaptoethanol. After electrophoresis the gel was stained with Coomassie Blue R-250. Lane a, ClpB93; lane b, ClpB93/K212T; lane c, ClpB93/K212E; lane d, ClpB93/K611T; lane e, ClpB93/K611E; lane f, ClpB93/K212T/K611T. Lane m (from top to bottom), protein size markers: myosin heavy chain, β -galactosidase, phosphorylase *b*, BSA, ovalbumin and carbonic anhydrase.

Figure 3 Incubation time course for ATP hydrolysis by ClpB93 and its mutant forms

ATP hydrolysis by ClpB93 (●), ClpB93/K212T (▲), ClpB93/K212E (△), ClpB93/K611T (■), ClpB93/K611E (□), or ClpB93/K212T/K611T (◆) was assayed by incubation of 3 μ g of the purified proteins with 10 μ g of casein and 2 mM [γ - 32 P]ATP for various periods at 37 °C. Radioactive free phosphates that had been released during incubation were then determined as described in the Experimental section. The results are averages of those obtained from two independent experiments.

with Thr or Glu by site-directed mutagenesis. We also generated a double mutant in which both the Lys residues were replaced with Thr. The pBS-ClpB93 plasmids carrying the mutations were transformed into a *clpB*-null mutant cell. The mutant forms of ClpB93 (referred to as ClpB93/K212T, ClpB93/K212E, ClpB93/K611T, ClpB93/K611E and ClpB93/K212T/K611T) were purified from the cells to apparent homogeneity (Figure 2).

To determine the effects of the mutations in the ATP-binding sites of ClpB93 on the casein-activated ATP hydrolysis, the purified proteins were incubated with casein and ATP (2 mM) at 37 °C for various periods. Surprisingly, ClpB93 carrying mutations in either of two ATP-binding sites hydrolysed ATP more rapidly than the parental ClpB93 (Figure 3). In contrast, ClpB93 carrying mutations in both of the ATP-binding sites (i.e. ClpB93/K212T/K611T) could not hydrolyse ATP at all.

Thus it seems that either of the two ATP-binding sites is capable of supporting the ATPase function of ClpB93.

To examine whether the increased activity of ClpB93 carrying the mutations in either of two ATP-binding sites is due to the change in their affinity for ATP, the proteins were incubated with increasing amounts of ATP in the presence and in the absence of casein at 37 °C for 10 min, during which period the rate of ATP hydrolysis was linear with time. In the presence of ATP up to 2 mM, both the basal and casein-stimulated ATPase activities of the mutant proteins were again significantly higher than those of the parental ClpB93 (Figure 4). However, the ATPase activities of ClpB93/K212T and ClpB93/K212E decreased gradually when the ATP concentration was increased above 2 mM, unlike those of ClpB93, ClpB93/K611T and ClpB93/K611E, which showed a typical saturation curve.

We have previously shown that the rate of ATP hydrolysis by ClpB is not linear with time when incubations are performed for more than 10 min and that the affinity for ADP is at least 30-fold higher than that for ATP [11]. Therefore we have suggested that ADP molecules, generated from ATP under the assay conditions, might negatively modulate the ATPase activity of ClpB. To determine whether the mutations in the ATP-binding sites in ClpB93 might influence the affinity for ADP, ATP hydrolysis by ClpB93 and its mutant forms was assayed in the presence of increasing concentrations of ADP. As shown in Figure 5, the ATPase activities of both ClpB93/K212T and ClpB93/K212E were much more sensitive to inhibition by ADP than those of ClpB93, ClpB93/K611T and ClpB93/K611E. The ADP concentration required for 50% inhibition for ClpB93 carrying the mutations in the first ATP-binding site (120 μ M) was approx. one-third of that for the protein carrying the mutations in the second site (340 μ M). Thus it seems that the mutations in the first ATP-binding site result in an increase in the affinity of ADP for the second site in ClpB93.

Oligomeric properties of ClpB mutants

To examine whether the mutation in the ATP-binding sites of ClpB93 might influence the oligomeric nature of the protein, ClpB93 and each of its mutant forms were subjected to gel filtration on a Sephacryl S-300 column in the presence and in the absence of ATP. Under typical gel-filtration chromatographic

Figure 4 Effects of increasing concentrations of ATP on the ATPase activities of ClpB93 and its mutant forms

The ATPase activities of ClpB93 and its mutant forms were assayed in the absence (open symbols) and in the presence of 10 μ g of casein (filled symbols). Reaction mixtures containing 3 μ g of the purified proteins and increasing amounts of [γ - 32 P]ATP were incubated for 10 min at 37 °C. Similar results were obtained from at least two independent experiments. (A) ATP hydrolysis by ClpB93 (○, ●); (B) ATP hydrolysis by ClpB93/K212T (△, ▲) and ClpB93/K212E (□, ■); (C) ATP hydrolysis by ClpB93/K611T (▽, ▼) and ClpB93/K611E (◇, ◆).

Figure 5 Effect of ADP on ATP hydrolysis by ClpB93 and its mutant forms

ATP hydrolysis was assayed by incubating 3 μ g of ClpB93 (●), ClpB93/K212T (▲), ClpB93/K212E (△), ClpB93/K611T (■) or ClpB93/K611E (□) with 10 μ g of casein and 2 mM [γ - 32 P]ATP in the absence and in the presence of increasing amounts of ADP for 10 min at 37 °C. The activities in the absence of ADP were defined as 100% and the others are expressed as values relative to this. The results are averages of those obtained from two independent experiments.

conditions with the proteins at 1 mg/ml in the presence of 0.1 M NaCl, ClpB93 and all of the purified mutant forms ran as an oligomer of 400–450 kDa whether or not ATP was present (results not shown). However, the size of the ClpB93 proteins varied markedly from 100 to 650 kDa depending on the salt concentration (K. M. Woo, K. I. Kim and C. H. Chung, unpublished work). Nevertheless all of the mutant proteins behaved similarly to the parental ClpB93 at any protein and/or salt concentration tested and whether or not ATP was present (results not shown). Thus it seems that binding of ATP to either of the nucleotide-binding sites is not essential for the oligomerization of ClpB93.

Effect of *clpB* mutations on thermotolerance

ClpB is a heat shock protein and is required for the survival of *E. coli* at high temperatures [12,13]. Therefore we examined whether the mutations in the ATP-binding sites in ClpB93 might influence thermotolerance of the cells. Because the transformation of *E. coli* with high-copy pBS plasmids significantly decreased the rate of cell proliferation (results not shown), *clpB93* and its mutant forms were transferred into a low-copy vector called pHSG575 [17]. *clpB*-null mutant cells were then transformed with the pHSG plasmids carrying the mutations in the ATP-binding sites. The cells were grown at 30 °C until reaching a mid-exponential phase and were then shifted to 52 °C. In accord with a previous report [12], the *clpB*-null mutant cells were much more sensitive to incubation at 52 °C than were the wild-type cells (Figure 6A). Nearly identical results were obtained when the same experiments were performed with the cells carrying the pHSG vector only (Figure 6B). In addition, transformation with pHSG-ClpB93 could rescue the heat sensitivity of the null mutant cells as well as that with pHSG carrying the wild-type *clpB*. However, none of the plasmids carrying the mutations in the first or second ATP-binding site could rescue the heat sensitivity. These results show clearly that both the ATP-binding sites of ClpB93 are required for the cell's survival at high temperatures, despite the findings that all of the ClpB93 proteins carrying a mutation in either of the two ATP-binding sites were capable of hydrolysing ATP to an extent even greater than that

Figure 6 Effect of incubation at 52 °C on the viability of *E. coli* cells harbouring low-copy plasmids carrying *clpB93* and its mutant forms

Cells grown at 30 °C to a D_{600} of 0.5 were shifted to 52 °C, at which temperature they were incubated for the indicated periods. After incubation the cells were plated on agar plates and their abilities to form colonies were determined. Similar results were obtained from at least five independent experiments. (A) Surviving fractions of the wild-type *E. coli* MC1000 (●) and a *clpB*-null mutant MC1000 Δ clpB::Km^r (i.e. MC1009) (○); (B) surviving fractions of the mutant cells harbouring pHSG vector alone (○), pHSG-ClpB93 (●), pHSG-ClpB93/K212T (▲), pHSG-pClpB93/K212E (△), pHSG-ClpB93/K611T (■) and pHSG-ClpB93/K611E (□).

seen with the parental ClpB93 at ATP concentrations near or below 2 mM.

Because the heat sensitivity of the *clpB*-null mutant cells could not be rescued by transformation of the pHSG plasmids carrying the mutations in either of the two ATP-binding sites, we wondered whether the mutant ClpB93 proteins might not be functional at high temperatures. To test this possibility, we first monitored the changes in the ATPase activity of the parental ClpB93 at temperatures ranging from 37 to 57 °C. Figure 7 shows that the rate of ATP hydrolysis by ClpB93 gradually increased on raising the temperature to 52 °C. When incubations were performed at 57 °C for up to approx. 10 min, the enzyme activity was also higher than that at 52 °C. However, no further increase in ATP

Figure 7 Effects of incubation temperatures on ATP hydrolysis by ClpB93

ATP hydrolysis was assayed by incubating 3 μ g of ClpB93 with 10 μ g of casein and 2 mM [γ - 32 P]ATP for the indicated periods at 37 °C (●), 42 °C (○), 47 °C (▲), 52 °C (△), and 57 °C (■). The results are averages of those obtained from two independent experiments.

Figure 8 Effect of incubation at 52 °C on ATP hydrolysis by ClpB93 and its mutant forms

ATP hydrolysis was assayed by incubating 2 mM [γ - 32 P]ATP with 10 μ g of casein and 3 μ g of ClpB93 (●), ClpB93/K212T (▲), ClpB93/K212E (△), ClpB93/K611T (■) or ClpB93/K611E (□). Incubations were performed for the indicated periods at 52 °C. The results are averages of those obtained from two independent experiments.

hydrolysis was observed on prolonged incubation, indicating that ClpB93 was gradually denatured on incubation at 57 °C.

We then compared the ATPase activity of the parental ClpB93 with that of ClpB93 carrying mutations in either of two ATP-binding sites by incubation at 52 °C for various periods. When incubations were performed for up to 10 min, the activities of the mutant proteins were significantly lower than that of the parental ClpB93 (Figure 8), unlike their activity at 37 °C (see Figure 3). Furthermore ATP hydrolysis by the mutant proteins did not increase any further on prolonged incubation at 52 °C, unlike that by the parental ClpB93. These results indicate that the ClpB93 proteins carrying mutations in either of two ATP-binding sites have a much higher tendency towards denaturation at 52 °C than the parental ClpB93. Thus it seems that both of the ATP-binding sites have an important role in maintaining the thermotolerance of ClpB93 and hence in the survival of *E. coli* cells at high temperatures.

DISCUSSION

The two ATP-binding sites of ClpA in *E. coli* and Hsp104 in *Saccharomyces cerevisiae*, which is a homologue of ClpB, have been shown to have distinctive roles in the ATPase function and oligomerization of the proteins [15,16,21]. In ClpA, replacement of the Lys²²⁰ in the first ATP-binding site by Thr prevents oligomerization and that of Lys⁵⁰¹ in the second site by Thr impairs the ATP activity. In contrast, replacement of Lys²¹⁸ in the first ATP-binding site of Hsp104 with Thr decreases the ATPase activity and that of Lys⁶²⁰ in the second site with Thr blocks oligomerization. Thus the contributions to ATPase activity and oligomerization of each of the two ATP-binding sites are reversed in ClpA and Hsp104, although the same amino acid substitutions were made. Nevertheless it seemed that each of the ATP-binding sites in the Clp/Hsp100 family containing two ATP-binding sites might have a distinctive role in either ATP hydrolysis or oligomerization.

In the present studies we therefore initially generated mutations by replacing the Lys residues in either of the two ATP-binding sites in ClpB93 with Thr only, to compare the effects of the mutations with those of ClpA and Hsp104. Unlike the mutant forms of ClpA and Hsp104 with the same amino acid substitutions, both ClpB93/K212T and ClpB93/K611T were capable

of hydrolysing ATP at a higher rate than the parental ClpB93 (although such an increase in the rate for ClpB93/K212T was observed only at ATP concentrations near or below 2 mM). In ClpA the replacement of the Lys residue with Thr in the first ATP-binding site increased the K_m for ATP but did not abolish its binding; hence trimeric ClpA could form a hexamer in the presence of high concentrations of ATP [15]. These results indicate that the Thr residue can still somehow interact with ATP although with decreased affinity. Therefore we generated another form of the ClpB93 mutants by replacing the positively charged Lys residue in either of the two ATP-binding sites with a negatively charged Glu. Because the Lys residue in the Gly-Xaa-Xaa-Gly-Xaa-Gly-Lys-Thr motifs in the Walker A-type regions has been suggested to interact with at least one of the phosphoryl groups of the bound nucleotide [22,23], we expected that introduction of a strong negative charge in the motifs would completely impair the binding of ATP and hence its cleavage. Nevertheless, both ClpB93/K212E and ClpB93/K611E were also capable of hydrolysing ATP to extents similar to those seen by ClpB93/K212T and ClpB93/K611T. These surprising findings led us to generate a double mutant protein carrying Thr in place of the Lys residues in both of the ATP-binding sites. ClpB93/K212T/K611T was completely incapable of hydrolysing ATP, regardless of whether or not casein was present, even in the presence of 5 mM ATP. Therefore it seems clear that either of the two ATP-binding sites is capable of supporting the ATPase function of ClpB93.

Of interest was the finding that ADP inhibited the ATPase activities of both ClpB93/K212T and ClpB93/K212E more effectively than those of ClpB93, ClpB93/K611T and ClpB93/K611E. Thus it seems that the mutations in the first ATP-binding site might induce a conformational change in ClpB93, which in turn might increase the affinity of ADP for the second ATP-binding site. However, it is unclear why the mutation in the second ATP-binding site does not show such an effect of ADP on the first site. Neither is it clear how a mutation in one of the two ATP-binding sites causes an increase in the ability of the adjacent site to support ATP hydrolysis. However, it is at least unlikely that occupancy of ATP in one of the two sites could down-regulate ATP binding at the other site, because the affinity of ATP for ClpB93 was found to be very similar to that for ClpB93/K611T or ClpB93/K611E on analysis of the results in Figures 4(A) and 4(C) with a double-reciprocal plot (results not shown). Because either of the two ATP-binding sites is capable of supporting ATP hydrolysis at the catalytic site in ClpB93, the single catalytic site must somehow make a choice to interact with either of the two ATP-binding sites. In this respect, two ATP molecules bound to the ATP-binding sites might compete with each other for interaction with the single catalytic site and a mutation in either of the two ATP-binding sites might eliminate such competition.

Of particular interest was the finding that ATP hydrolysis by both ClpB93/K212T and ClpB93/K212E, but not by ClpB93, ClpB93/K611T or ClpB93/K611E, gradually decreased on raising the ATP concentration above 2 mM. Similar inhibitory effects of high ATP concentrations were observed for the hexameric RuvB ATPase from *E. coli*, which has a single ATP-binding domain [24]. It has been suggested that inhibition by high substrate concentrations can occur when an enzyme has several groups, each combining with a particular part of the substrate molecule ([25], pp. 126–137). In the effective enzyme–substrate complex, one substrate molecule is combined with all these groups. However, in the presence of high concentrations of the substrate, where the substrate molecules tend to crowd on the enzyme, the chance of formation of an ineffective complex in

which some of the binding groups are combined with one substrate molecule and the other groups with an additional substrate molecule is likely to increase. The ATP-binding domains in ClpB have been shown to contain at least two distinct regions, which might interact specifically with the adenosine and phosphoryl groups of ATP respectively [8,9,22,23]. Possibly the mutations in the first ATP-binding site in ClpB93 induce a conformational change, which might allow ClpB93/K212T or ClpB93/K212E to form an ineffective complex with ATP, in which one region of the second ATP-binding domain is combined with one ATP molecule and the other region with another ATP molecule when ATP is present at high concentrations. However, it is again unclear why the mutations in the second ATP-binding site do not show such effects of high ATP concentrations on the first site.

Purified ClpA behaves as a trimer of 84 kDa subunits in the absence of ATP but becomes a hexameric complex in its presence [26]. Similarly, isolated Hsp104 exists predominantly as a monomer or dimer in the absence of ATP but oligomerizes into a hexamer in its presence [21]. Unlike these two proteins, the behaviour of the purified ClpB93 protein on a Sephacryl S-300 column remained similar whether or not ATP was present. Furthermore, all of the mutant proteins behaved in a similar manner to the parental ClpB93 on the gel-filtration column under all conditions tested. Thus it seems that ATP binding is not essential for the oligomerization of ClpB93 subunits, unlike that of ClpA or Hsp104, despite the fact that the sequences of the ATP-binding regions in these proteins are known to be highly similar to each other [8,9,27].

ClpB in *E. coli* and Hsp104 in yeast have been shown to be required for the survival of the cells at high temperatures, such as 50 °C [12,27,28]. In addition, the ATP-binding sites in Hsp104 have been revealed to be necessary for the thermotolerance. The present studies have also demonstrated that both of the two ATP-binding sites in ClpB93 are required for the survival of *E. coli* at high temperatures. None of the pHSG-ClpB93 plasmids carrying mutations at the first or second ATP-binding site could rescue the heat sensitivity of *clpB*-null mutant cells. Furthermore all of the ClpB93 mutant proteins were found to be much more sensitive to heating at 52 °C, unlike the parental ClpB93. Thus it seems likely that the binding of ATP to both of the ATP-binding sites is required for maintaining the thermotolerance of the ClpB93 protein, which in turn is essential for the survival of *E. coli* under heat shock conditions.

We thank Dr. Catherine Squires (Tufts University, Boston, MA, U.S.A.) for providing the pClpB plasmid and the *clpB*-null mutant strain. This work was supported by grants from Korea Science and Engineering Foundation through the Research Center for Cell Differentiation, the Ministry of Education and the Lotte Foundation.

REFERENCES

- Goldberg, A. L. (1992) *Eur. J. Biochem.* **203**, 9–23
- Gottesman, S. and Maurizi, M. R. (1992) *Microbiol. Rev.* **56**, 592–621
- Maurizi, M. R. (1992) *Experientia* **48**, 178–201
- Chung, C. H. (1993) *Science* **262**, 372–374
- Chung, C. H., Seol, J. H. and Kang, M. S. (1996) *Biol. Chem. Hoppe-Seyler* **377**, 549–554
- Hwang, B. J., Woo, K. M., Goldberg, A. L. and Chung, C. H. (1988) *J. Biol. Chem.* **263**, 8727–8734
- Maurizi, M. R. (1991) *Biochem. Soc. Trans.* **19**, 719–723
- Gottesman, S., Squires, C., Pichersky, E., Carrington, M., Hobbs, M., Mattick, J. S., Dalrymple, B., Kuramitsu, H., Shioza, T., Foster, T. et al. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3513–3517
- Schirmer, E. C., Glover, J. R., Singer, M. A. and Lindquist, S. (1996) *Trends Biochem. Sci.* **21**, 289–296
- Squires, C. and Squires, C. L. (1992) *J. Bacteriol.* **174**, 1081–1085
- Woo, K. M., Kim, K. I., Goldberg, A. L., Ha, D. B. and Chung, C. H. (1992) *J. Biol. Chem.* **267**, 20429–20434
- Squires, C. L., Pedersen, S., Ross, B. M. and Squires, C. (1991) *J. Bacteriol.* **173**, 4257–4262
- Kitagawa, M., Wada, C., Yoshioka, S. and Yura, T. (1991) *J. Bacteriol.* **173**, 4247–4253
- Park, S. K., Kim, K. I., Woo, K. M., Seol, J. H., Tanaka, K., Ichihara, A., Ha, D. B. and Chung, C. H. (1993) *J. Biol. Chem.* **268**, 20170–20174
- Seol, J. H., Baek, S. H., Kang, M. S., Ha, D. B. and Chung, C. H. (1995) *J. Biol. Chem.* **270**, 8087–8092
- Singh, S. K. and Maurizi, M. R. (1994) *J. Biol. Chem.* **269**, 29537–29545
- Takeshita, S., Sato, M., Toba, M., Masahashi, W. and Hashimoto-Gotoh, T. (1987) *Gene* **61**, 63–74
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Armon, T., Ganeth, D. and Hershko, A. (1990) *J. Biol. Chem.* **265**, 20723–20726
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Parsell, D. A., Sanchez, Y., Stitzel, J. D. and Lindquist, S. (1994) *J. Biol. Chem.* **269**, 4480–4487
- Walker, J. E., Saraste, M., Runswick, M. J. and Gay, N. J. (1982) *EMBO J.* **1**, 945–951
- Fry, D. C., Kuby, S. A. and Mildvan, A. S. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 907–911
- Marrión, P. E. and Cox, M. M. (1995) *Biochemistry* **34**, 9809–9818
- Dixon, M. and Webb, E. C. (1979) *The Enzymes*, 3rd edn., Longmans, London
- Seol, J. H., Yoo, S. J., Kim, K. I., Kang, M. S., Ha, D. B. and Chung, C. H. (1994) *J. Biol. Chem.* **269**, 29468–29473
- Parsell, D. A., Sanchez, Y., Stitzel, J. D. and Lindquist, S. (1991) *Nature (London)* **353**, 270–273
- Sanchez, Y. and Lindquist, S. L. (1990) *Nature (London)* **248**, 1112–1115