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Oligomeric Characterization of GroESLx Chaperonin from Symbiotic X-Bacteria in *Amoeba proteus*

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GroESLx proteins of symbiotic X-bacteria were overproduced in *Escherichia coli* and their structural characteristics were assayed after simple purification. The GroESx and GroELx were heat-stable at 80℃ and 50℃, respectively. After heat-treatment, GroESx was purified by DEAE Sephadex A-50 chromatography and GroELx was purified by step- and linear sucrose density gradient ultracentrifugation. Molecular masses of GroESx and GroELx were 50-80 kDa and 800 kDa, respectively, as estimated by sucrose density gradient ultracentrifugation. In chemical cross-linking analysis, subunits of GroESx were mostly cross-linked by incubation for 3 h in 0.4% glutaraldehyde and GroESx was found to be composed of homo-heptamer subunits. Those of GroELx were cross-linked within 10 min in 0.3% glutaraldehyde and GroELx was in two stacks of homo-heptamer subunits. On the other hand, GroESx and GroELx proteins in a solution could not be cross-linked even after incubation for 3 h in 0.5% glutaraldehyde. GroELx was stable at 4-37℃. In the presence of both GroESx and ATP, $GroELx_{14}$ was stable at 37℃ but not at 4℃ or 24℃. Thus, we confirmed the oligomeric properties of $GroESx_7$ and $GroELx_{14}$ and their stability to heat and in the interaction with GroESx.

Among molecular chaperonins, the GroESL chaperonins are universally conserved, and *groE* genes encoding the chaperonins have been characterized in many free-living, pathogenic and symbiotic microorganisms (Ahn et al., 1994). The GroESL chaperonins maintain some polypeptides in unfolded state, thus facilitating their translocation across membranes, and/or accelerating proper folding and assembly by preventing misfolding. They are necessary for λ head-tail assembly and T5 tail assembly, assembly and disassembly of oligomeric proteins, and essential for cellular growth. The GroEL chaperonins are identical to the common antigen in bacterial infections (Georgopoulos et al., 1994).

Functional and structural characteristics of GroESL of *Escherichia coli* have been well affirmed (Braig et al., 1994; Hunt et al., 1996). GroES is a homo-heptamer arranged as a single ring in a dome-shaped structure (Hunt et al., 1996). The cavity within the dome is continuous with the polypeptide binding chamber of GroEL in the chaperonin complex. As a cochaperonin GroES has a regulatory role and is required for successful refolding of polypeptides by GroEL. The crystal structure of *Escherichia coli* GroEL shows a porous cylinder of 14 subunits made of two nearly 7-fold rotationally symmetrical rings stacked back-to-back with dyad symmetry (Braig et al., 1994). The GroEL with its cochaperonin GroES facilitates protein folding with an ATP-dependent mechanism. Structural analysis suggests that various modes of molecular plasticity are responsible for binding of nonnative substrates and their release into the shielded *cis* assembly (Chen and Sigler, 1999).

The GroESL proteins are required for the survival of various symbiotic organisms (Fisher et al., 1993; Rusanganwa and Gupta, 1993; Dohra et al., 1998; Morin et al, 1999). In pathogenic or endosymbiotic bacteria GroEL analogues are overproduced, while other stress proteins, like hsp70, are not (Charles et al., 1997; Morioka and Ishikawa, 1998). The GroEL proteins in parasitic or symbiotic bacteria have been suggested to play an additional protective role for microorganisms in the initial infection and in the maintenance of organismic interaction with the host (Choi et al., 1991; Morioka et al., 1993; Fossati et al., 1995). However, these assumptions and native structure of the GroESL analogues in these organisms have not been critically analyzed due to the difficulty in culturing them *in vitro*.

The *groEx* gene cloned from symbiotic X-bacteria in *A. proteus* is an analogue of the *groE* gene of *E. coli* and complemented $groE^-$ mutations in *E. coli* (Ahn et al., 1991; Lee et al., 2001). The gene has a potent promoter within the coding region of *groES* in addition to the heat shock consensus promoter at its 5′-end (Ahn et al., 1994) and a Rho-dependent transcription terminator (*Tx*) having 97% termination efficiency at its

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3′-end (Lee et al., 2002). By using the two promoters and *Tx*, the GroELx and GroESx proteins of symbiotic X-bacteria were overproduced in *E. coli* and purified by simple procedures (Jung and Ahn, 2001).

GroESx showed over 90.6% identity with its analogue of *Legionella micdadei* in amino acid sequences, while only 44.8% identity was found with that of *E. coli* (Ahn et al., 1991). GroELx showed a 91.2% identity with its analogue of *L. micdadei*, while it had a 73.2% identity with that of *E. coli*. The pI of GroESx (5.83) was different from that of *E. coli*'s GroES (pI 4.91). GroELx was also more basic (pI 5.39) as compared with that of *E. coli* (pI 4.85). These biochemical deviations were also shared among pathogenic *L. micdadei* and *L. pneumophila*. The GroEL analogue (HSP60) of rat liver mitochondria is also basic, with a pI over 9.0 (Hartman et al., 1993). The pI of cloned GroES analogue (HSP10) of yeast mitochondria was 9.8 (Rospert et al., 1993). It is interesting to note that the pIs of chaperonins of mitochondria and pathogenic bacteria are more basic than those of *E. coli*. In terms of pI GroESLx could be an intermediate divergent between GroESL of *E. coli* and HSP10 and HSP60 of mitochondria. However, the effect of positive charges on the chaperonin structure and function is not known.

In this study, we analyzed structural characteristics of GroESx and GroELx by ultracentrifugation and chemical cross-linking. We confirmed their oligomeric properties of $GroESx_7$ and $GroELx_{14}$ in two stack of $GroELx_7$. However, GroESLx complex was not detected either in ultracentrifugation or chemical cross-linking.

Materials and Methods

Cell culture and overproduction of GroESLx proteins

GroELx and GroESx proteins were overproduced separately in *E. coli* MC4100 transformed with pAJX91 and pUXGPRM, respectively (Jung and Ahn, 2001). The pAJX91 DNA clone was one of the original clones of *groEx* lacking the P1 promoter and it over-expressed *groELx* by the specific P2 promoters located within the coding region for *groESx* (Ahn et al., 1994). The pUXGPRM contained a 0.8-kb fragment of *groEx* as an insert DNA in pUC119 vector (Lee and Ahn, 2000) and expressed *groESx* by P1 promoters composed of heat shock consensus promoter and a σ^{70}-dependent promoter. *E. coli* cells were grown overnight in 10 mL of LB medium containing ampicillin (50 μg/mL) at 37℃. The cells in 10-mL culture were then inoculated to one-litter LB medium and grown for 7 h at 37℃ in a rotary shaker at 225 rpm.

Purification of GroESLx

E. coli cells in one litter culture were harvested and suspended in 20 mL of Buffer A (50 mM Tris-HCl, pH 7.6 and 1 mM dithiothreitol). Cells were lysed by three cycles in a French pressure cell at 2000 psi. The homogenate was centrifuged for 30 min at 10,000 g at 4℃ and the supernatant was saved as the crude extract. The crude extract was heated for 10 min at various temperatures to determine optimal temperature for GroES and GroEL purification and centrifuged for 10 min at 10,000 g to remove heat-coagulated proteins.

GroESx and GroELx were purified by the procedures described in Jung and Ahn (2001). In brief, GroESx proteins in heat-coagulated supernatant were precipitated with 70% ammonium sulfate, dialyzed for 15 h against Buffer A at 4℃ and loaded on a diethylaminoethyl (DEAE)-Sephadex A-50 anion column (10 mm × 150 mm) equilibrated with Buffer A. Proteins were eluted with 0-0.5 M NaCl in Buffer A at a flow rate of 0.2 mL/min. Fractions of 2.5 mL each were collected. Proteins in 1 mL of each fraction were precipitated by 10% (v/v) trichloroacetic acid (TCA), resuspended in 10 μL of 50 mM Tris-HCl (pH 7.6) and were subjected to SDS-PAGE (15% gel) followed by Brilliant Coomassie Blue (BCB) staining. Fractions containing GroESx were pooled, dialyzed for 15 h against 50 mM Tris-HCl (pH 7.6), and concentrated by using an Ultrafree-centrifugal filter device (Millipore Co.).

For GroELx, a 1 mL aliquot of the heat-coagulated supernatant solution was loaded on top of a sucrose step gradient (15/25/50%) in 50 mM Tris-HCl (pH 7.6) and centrifuged for 15 h at 154,000 g at 4℃ in a P40ST rotor of Hitachi CP 100α ultracentrifuge (Hitachi Koki Co. Ltd., Tokyo). One mL fractions were collected from top of the gradient and analyzed for proteins by SDS-PAGE. Fractions containing GroELx were pooled. Proteins were then precipitated by ammonium sulfate (80%), resuspended in 50 mM Tris-HCl (pH 7.6) and dialyzed for 15 h against the same buffer at 4℃. One mL of the dialyzed sample was loaded on top of 10 - 40% sucrose linear gradient, and centrifuged for 15 h at 154,000 g at 4℃ in a P40ST rotor. Fractions containing GroELx were confirmed and pooled by the same method for step-gradient centrifugation. Purified GroELx in 50 mM Tris-HCl (pH 7.6) was used for further biochemical characterization.

Sucrose density gradient ultracentrifugation

An aliquot of purified proteins in 50 mM Tris-HCl, pH 7.6 was layered on top of 10 - 40% sucrose gradient and centrifuged in P55ST2 rotor (Hitachi Koki Co. Ltd., Tokyo) at 235,000 g at 4℃ for 10 h. For measuring sucrose density a tube having the same sucrose gradient layered with the buffer was centrifuged in parallel with the tube containing proteins. The gradients were fractionated into 0.3 mL from the top. Proteins in each fraction were precipitated by 80% ammonium sulfate and monitored by 15% SDS-PAGE. Sucrose density in fractions from protein-blank tube was measured by densitometer and the molecular masses of proteins in equivalent fractions were calculated using Hitachi centrifuge internal equation.

Chemical cross-linking

An aliquot (20 μl) of purified proteins(1-1.5 mg proteins/mL) were incubated with glutaraldehyde (GA) at 25℃ for chemical cross-linking. At time intervals the reaction was stopped by adding 0.2 volume of 2.5 M Tris-HCl (pH 7.0) and mixed with 0.25 volume of 0.2 M sodium phosphate buffer (pH 7.0) containing 2% SDS, 2% β-mercaptoethanol, 0.2% bromophenol blue and 12 M urea. It was then heated for 5 min in boiling water bath and subjected to SDS-PAGE.

Rhodanese refolding assay

A stock solution (180 μM) of highly purified mitochondrial rhodanese from the bovine liver (Sigma, Cat. No. R1756) was prepared in 0.2 M potassium phosphate (pH 7.6). The enzyme (9 μM, 0.3 mg/mL) was unfolded in 0.2 M potassium phosphate buffer (pH 7.6) containing 1 mM β-mercaptoethanol and 8 M urea for 2 h at 30℃. Refolding was initiated by diluting 2.5 μL of unfolded rhodanese into a final volume of 250 μL of 50 mM Tris-HCl (pH 7.6) buffer containing GroELx (2.5 μM protomer), GroESx (2.5 μM protomer), 200 mM β-mercaptoethanol, 50 mM sodium thiosulfate, 10 mM $MgCl_2$, 10 mM KCl, and 2 mM ATP at 37℃. For assessing the regain of rhodanese activity by following Westley (1981), an aliquot (25 μl) of the refolding mixture was withdrawn at time intervals, added to 1 mL of the rhodanese assay mixture prepared fresh by combining 0.15 M $Na_2S_2O_3$, 0.15 M KCN and 0.12 M KH_2PO_4 at 1:1:1 volume ratio, and incubated for 15 min at 37℃. The reaction was terminated by the addition of 0.5 mL of 18% formaldehyde. After adding the ferric nitrate reagent, the developed color was measured at 460 nm.

Stability of GroELx oligomer

In order to assay the stability of GroESLx oligomer at various conditions, purified GroELx proteins in 50 mM Tris-HCl, pH 7.6, were incubated alone, or with 2 mM ATP or GroESx at various temperatures for 1 h. Then, samples were mixed with loading buffer and analyzed by 5% non-denaturing polyacrylamide gel electrophoresis and stained with BCB.

Polyacrylamide gel electrophoresis (PAGE)

Protein concentrations were determined by Lowry et al. (1951) with bovine serum albumin as the standard. SDS-PAGE was carried out by the method of Laemmli (1972). For the non-denaturing PAGE, protein samples in 50 mM Tris-HCl (pH 7.6) were diluted with sample buffer (X5) without SDS or β-mercaptoethanol and loaded without heat treatment. Prior to loading samples, gels containing 5% glycerol were pre-run for 2 h. Band densities of BCB stained proteins were scanned and quantified by using the Scion Image Software (Scion Corp. MD). Molecular masses of the proteins were calculated by Weber et al. (1972).

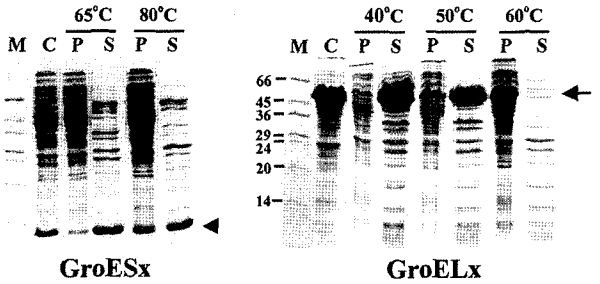

Fig. 1. SDS PAGE of heat-treated GroESx and GroELx proteins produced in *E. coli*. Lanes M; molecular mass markers (kDa), C; crude extract, P and S; pellet and supernatant fraction after heat treatment and centrifugation, respectively. The arrowhead and arrow indicate GroESx and GroELx, respectively.

Results and Discussion

The GroESx proteins were heat-stable at 80℃ for 10 min (Fig. 1) and could be purified by elution with 0.1 M NaCl in DEAE Sephadex A-50 chromatography (Fig. 2). The unusual stability of GroESx at such a high temperature was applied in the purification of GroESx from *E. coli*. The yield and purity of GroESx was 62.2% and 91%, respectively. We obtained 14.8 mg GroESx proteins from 1 liter culture. GroELx was less stable than GroESx at high temperature. After a step- and a linear sucrose density gradient ultracentrifugation, the purity of GroELx was about 90% as determined by scanning of the density of BCB-stained SDS gels (Fig. 3). The final yield of GroELx was 55.8% and 27.8 mg proteins from 1 liter culture.

In ultracentrifugation on sucrose density gradient, GroESx was resolved between 3.24*S* and 4.61*S*. GroELx was resolved at 22.15*S*. Native molecular mass of GroES and GroELx were estimated to be 50-70 kDa and 820 kDa, respectively (Fig. 4). In SDS PAGE GroESx and GroELx had a subunit molecular mass of 10 kDa and 60 kDa, respectively. Thus, both proteins were in homo-oligomeric complex under the condition of purification steps.

In order to clarify the oligomeric properties, purified GroESLx proteins were cross-linked with glutaralde-

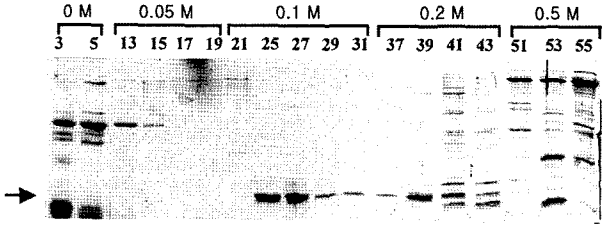

Fig. 2. SDS-PAGE of DEAE Sephadex A-50 column fractions for the purification of GroESx proteins. Lane numbers correspond to fraction numbers eluted by step gradients of 0-0.5 M NaCl. The arrow indicates GroESx eluted in 0.1 M NaCl.

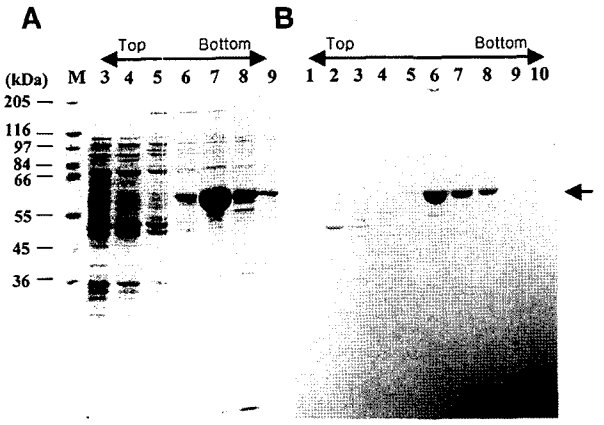

Fig. 3. SDS-PAGE of fractions from step (A; 15-25-50%) and linear (B; 10-40%) sucrose density ultracentrifugation of heat-coagulated supernatant in the purification of GroELx. Lane M; molecular mass markers. Lane numbers correspond to fraction numbers. In A, GroELx (arrow) was mostly accumulated at the boundary between 25% and 50% sucrose (fraction 7). Fractions 6-9 in A were pooled and centrifuged in linear sucrose gradient (B).

hyde (GA) at various conditions and resolved by SDS PAGE in tube gels (Fig. 5). When GroESx was incubated for 1 h in 0.2% GA, a ladder of cross-linked dimeric to heptameric homologous subunits were revealed. After incubation for 3 h in 0.4% GA most of GroESx subunits were cross-linked to 7-mer. This confirmed that GroESx was composed of homo-7-mer subunits.

On the other hand, subunits of GroELx were cross-linked rapidly (5-10 min) in 0.2% GA to show dimer to heptamer. By incubation for 10 min in 0.3% GA, most of GroELx subunits were cross-linked to 14-mer. The intermediates between 7- and 14-mer could not be observed. This could be due to the cross-linking among subunits in the same toroid being apparently faster than among subunits in adjacent toroidal ring of GroELx. In other cases, cross-linking among the subunits in the same toroid stabilize the structure for another round of

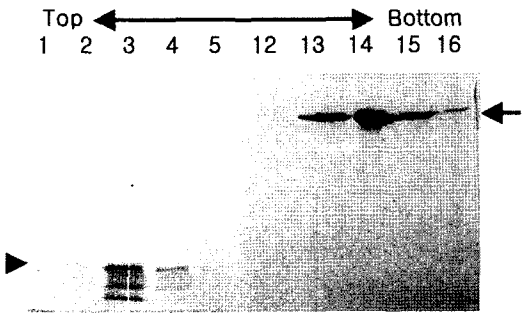

Fig. 4. SDS PAGE for the estimation of molecular mass of GroESx and GroELx by sucrose density gradient ultracentrifugation. Lane numbers correspond to fraction numbers from top to bottom of centrifuged sample. The arrow and arrowhead indicate GroELx and GroESx protein, respectively. Sucrose densities (%) for fraction 3 and 4 were 17.0% (3.24S) and 18.5% (4.61S) having molecular masses of 46.4 kDa and 78.7 kDa, respectively. The sucrose density for fraction 14 was 30.0% (22.15S) having a molecular mass of 818.1 kDa.

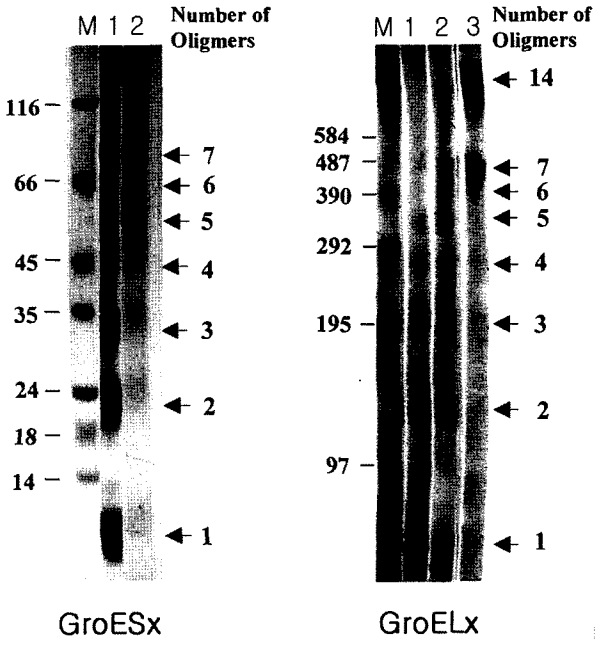

Fig. 5. SDS PAGE of cross-linked GroESx and GroELx. Purified GroESx and GroELx proteins were cross-linked with glutaraldehyde (GA) at 25℃ and analyzed in 7.5% and 3.3% tube gels, respectively. The GroESx was cross-linked for 1 h with 0.2% GA (lane 1) and 3 h with 0.4% GA (lane 2). The GroELx was incubated with 0.2% GA for 5 min (lane 1), 10 min (lane 2) or with 0.3% GA for 10 min (lane 3). Lane M; molecular mass markers (kDa).

lcross-inking between adjacent toroids. Thus, GroELx appeared to be in two stacks of homo-7-mer subunits.

However, GroESx and GroELx at various molar ratios in a solution could not be cross-linked even after 3 h incubation in 0.5% GA. They also did not form a detectable GroESLx complex in the analysis of sucrose density gradient centrifugation. This could be due to transient interaction between GroESx and GroELx similar to those of *Clostridium thermocellum* (Cross et al., 1996) and *Thermoanaerobacter brockii* (Truscott et al., 1994). The absence of GroESLx complex in ultracentrifugation or in chemical cross-linking is a clear deviation from the GroESL complex of *E. coli*. The GroESL complexes of *E. coli* can be visualized as a football shape or a bullet shape in electron microscopic images. The football shape consists of GroEL and two bound GroES rings and the bullet shape consists of GroEL and one bound GroES ring (Beissinger *et al.*, 1999; Grallert and Buchner, 2001).

In the analysis of oligomeric stability, GroELx was stable for 1 h in 50 mM Tris-HCl at 4 or 37℃. GroELx underwent partial dissociation into monomer subunits by incubation in 50 mM Tris-HCl at 37℃ containing 2 mM ATP (Fig. 6). The dissociation could be blocked by addition of GroESx at 37℃. However, the blocking was not effective at 24℃ or 4℃. GroEL of *E. coli*, *Buchnera* sp. and symbionin also underwent partial dissociation upon incubation with Mg-ATP (Kakeda

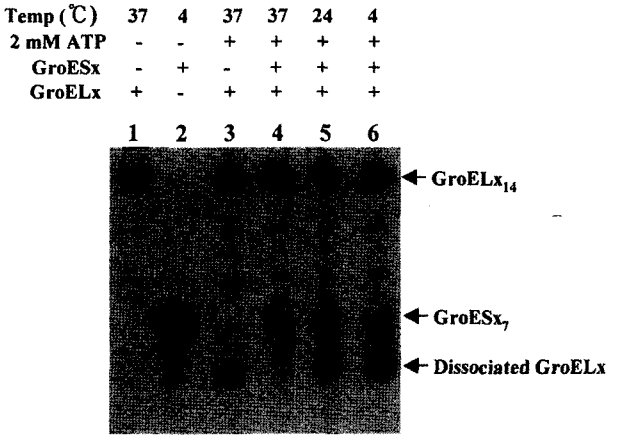

Fig. 6. Changes in oligomeric stability of GroELx by GroESx, ATP and temperature. An aliquot of purified GroELx (10 μg protein) in 50 mM Tris-HCl were incubated alone or in mixture with ATP or GroESx at various temperatures for 1 h and analyzed by non-denaturing polyacrylamide gel (5%) electrophoresis.

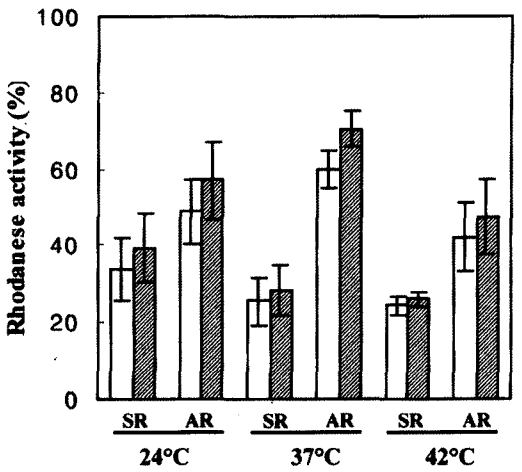

Fig. 7. Comparisons of spontaneous (SR) and GroESLx-assisted refolding (AR) of urea-denatured rhodanese at different temperatures. Open bars; refolded for 80 min, filled bars; for 150 min.

and Ishikawa, 1991; Lissin, 1995; van den Heuvel et al., 1997). This observation suggests that the structure of GroELx could be modified by ATP and stabilized optimally by GroESx at 37℃. GroESx inhibits ATPase activity of GroELx to 60% level of control activity at a 1:1 stoichiometry of $GroESx_7$ to $GroELx_{14}$ (Jung and Ahn 2001).

Lowering the incubation temperature from 37℃ to 24 ℃ reduced the ATPase activity of GroELx to 30% of that at 37℃ (data not shown). The chaperonin activity of GroESLx at 24℃ in the recovery of urea-denatured rhodanese was about a half of that at 37℃ (Fig. 7). Thus, the unstability of GroELx caused by ATP and temperature apparently affected the ATPase activity and chaperonin function of GroESLx. The optimal temperature for the growth of xD strain of *A. proteus* harboring the symbiotic X-bacteria is 24℃. The overproduction of GroELx in the symbiotic X-bacteria could be due to reduced stability of GroELx at 24℃.

In terms of the stability change of GroELx in the presence of ATP our results support the chaperonin model for GroESL of *E. coli* (Sigler et al., 1998). In the model GroES was known to stabilize large conformational changes in GroEL promoted by nucleotide binding and to regulate polypeptide binding and dissociation. It was reported that nucleotide binding, even in the absence of GroES, is known to induce considerable conformational changes that extend beyond the apical domain into the opposite GroEL-ring (Ranson et al., 1998).

A fully functional GroE chaperone system cycles through different conformational stages, which allows binding, folding, and release of substrate proteins (Grallert and Buchner, 2001). In the absence of ATP or presence of ADP GroEL exists in a "tight" conformational state that binds partially folded or misfolded proteins. Binding of ATP shifts GroEL to a more open, "relaxed" state, which releases the folded protein (Roseman et al., 1996). In the ATP-dependent step, GroEL expands and the protein exits GroEL by a process assisted by GroES which caps the ends of GroEL (Gottesman and Hendrickson, 2000).

The present study confirms the oligomeric structure of GroESx and GroELx that are homologous to GroES and GroEL of *E. coli*, respectively. However, they are different in the formation and the stability of GroESLx complex that could not be detected either in chemical cross-linking or in ultracentrifugation. The stability change of GroELx in the presence of ATP may require large accumulation of GroESLx in the adaptation of X-bacteria in symbiosis at 24℃. This can be achieved by the presence of strong promoters and the potent transcription terminator in the *groEx* operon so that the gene could be expressed even in the absence of heat shock (Ahn et al., 1994; Lee et al., 2002). Thus, X-bacteria may survive within the potentially hostile intracellular environment by employing GroESLx chaperonin (Ahn et al., 1994).

Acknowledgements

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