Comparative Biochemical Characterization of Two GroEL Homologs from the Cyanobacterium Synechococcus elongatus PCC 7942

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Unlike Escherichia coli, cyanobacteria generally contain two GroEL homologs. The chaperone function of cyanobacterial GroELs was examined in vitro for the first time with GroEL1 and GroEL2 of Synechococcus elongatus PCC 7942. Both GroELs prevented aggregation of heat-denatured proteins. The ATPase activity of GroEL1 was approximately one-sixth that of Escherichia coli GroEL, while that of GroEL2 was insignificant. The activities of both GroELs were enhanced by GroES, while that of Escherichia coli GroEL was suppressed. The ATPase activity of GroEL1 was greatly enhanced in the presence of GroEL2, but the folding activities of GroEL1 and GroEL2 were much lower than that of Escherichia coli GroEL, regardless of the co-presence of the counterpart or GroES. Both native and recombinant GroEL1 forms a tetradecamer like Escherichia coli GroEL, while GroEL2 forms a heptamer or dimer, but the GroEL1 and GroEL2 oligomers were extremely unstable. In sum, we concluded that the cyanobacterial GroELs are mutually distinct and different from Escherichia coli GroEL.

Key words: ATPase activity; chaperonin; cyanobacterium; GroEL; GroES

Among heat shock proteins, Escherichia coli GroEL, a member of the Hsp60 or chaperonin (cpn) family has been intensively studied for its ability to facilitate protein folding in vitro. GroEL forms a large oligomer with two rings of seven subunits.3) Inside the tetradecameric cylinder there is a cavity. The co-cpn GroES heptameric ring closes the cavity, generating a folding chamber in which an unfolded/folding polypeptide undergoes productive folding.

The majority of bacterial species including E. coli have only one groESL operon, where it is essential for bacterial growth at all temperatures,1,2) but close to one-third of completely sequenced bacterial genomes contain more than one copy of the groEL operon or additional groEL genes.5) For example, the root nodulator Rhizobium leguminosarum has three groEL operons,4) while the Mycobacterium sp. contains two groEL genes, one of which is arranged in an operon with groES.5) The presence of multiple copies of groEL is not limited to bacterial species, for chloroplasts of higher plants also possess two kinds of cpns (ch-cpn60).6) The functions of multiple GroELs are not fully understood, except for the few examples explored.7,8) The R. leguminosarum three GroEL homologs show distinct in vitro properties, and only one of the three groEL genes is required for the growth of this organism.9,10)

Photosynthetic prokaryotic cyanobacteria generally have two copies of the groEL gene.10,11) The groEL1 gene forms an operon with groES, whereas groEL2 is not accompanied by groES. A phylogenetic tree constructed with GroELs from various cyanobacteria showed higher conservation in the GroEL1 amino acid sequences than in GroEL2s.12) GroELs confer thermo-tolerance when GroEL1 and GroEL2 are overexpressed in Synechocystis sp. PCC 6803, while a groEL2 mutant of Thermosynechococcus elongatus is high-temperature sensitive.14,15) The mutant is also cold sensitive, which is consistent with the fact that the groEL2 gene is uniquely cold-induced.15) GroEL1 can replace GroEL functionally in E. coli, while GroEL2 cannot.11,16,17)

In this study, we characterized the chaperone functions of GroEL1 and GroEL2 from the mesophilic cyanobacterium Synechococcus elongatus PCC 7942. The two homologs shared 62% identity in primary structure. The biochemical functions of the two homologs were found to be distinct. This is the first report regarding in vitro functional characterization of any cyanobacterial GroEL.

Materials and Methods

Purification of recombinant proteins expressed in E. coli. GroEL and GroES proteins were overexpressed in and purified from E. coli. The coding regions of groEL1 (NCBI-GI: 81301122), groEL2 (NCBI-GI: 81299496), and groES (NCBI-GI: 81301123) were PCR-amplified with genomic DNA from S. elongatus PCC 7942 and primers 5′-AAATATGTGGCAACGATCATG-3′ (Ndel) and 5′-AATCTGAGGTAGTGCAAGTGGCCCTAT-3′ (Xhol), 5′-AACAATATGTGGCAACGATCATG-3′ (Ndel) and 5′-AAATATGTGGCAACGATCATG-3′ (Xhol), respectively. The primers contained an Ndel, Xhol, or HinIII site at

Abbreviations: cpn, chaperonin; ch-cpn60, chloroplast chaperonin; LDH, lactate dehydrogenase; MDH, malate dehydrogenase
their 5’ ends (underlined in the above primer sequences). The PCR fragments were cloned into pT7Blue T-Vector (Novagen, Madison, WI), digested with the respective enzymes and subcloned into the same sites in pET21a vector (Novagen), and introduced into E. coli BL21 (DE3). DNA sequencing confirmed insertion of the expected DNA fragments. Overexpressed C-terminal His-tagged proteins were purified by nickel affinity column (Ni Sepharose 6 Fast Flow, GE Healthcare, Piscataway, NJ). Protein concentrations were measured using a Protein Assay Kit (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard. E. coli GroEL and GroES were purchased from Takara (Kyoto, Japan).

Organism and culture conditions. S. elongatus PCC 7942 cells were grown photoautotrophically in BG-11 inorganic liquid medium in glass tubes or on BG-11 plates containing 1.5% w/v agar and 0.3% w/v sodium thiosulfate at 30°C. The liquid cultures were continuously aerated and illuminated with a light intensity of 30 μE/m²/s. Construction of a ΔgroEL2 mutant strain, in which the groEL2 gene was deleted from the genome of the S. elongatus PCC 7942 strain and replaced with a kanamycin resistant gene, will be described elsewhere. Correct insertion and complete segregation of the mutation in all the copies of the genome was confirmed by PCR.

Protein preparation from S. elongatus PCC 7942 cells for native PAGE and Western blot analysis. S. elongatus PCC 7942 wild-type and ΔgroEL2 cultures growing exponentially at 30°C were shifted to 42°C for 4 h to induce GroEL expression. The E. coli cells were shifted to 42°C for 3 h from 37°C. Cells were harvested by centrifugation. The cell pellet was collected and suspended in a buffer containing 50 mM Hepes-KOH (pH 8.0), 10 mM MgCl₂, 5 mM ATP, and a protease inhibitor cocktail (Sigma, St. Louis, MO). The harvested cells were disrupted with an approximately equal volume of glass beads (Sigma) by vigorous vortexing for 30 min at 4°C. The resulting suspension was centrifuged at 16,000 g for 1 h. The supernatant (soluble whole cell extract) was analyzed by native PAGE (4% Tris-glycine gel containing 2% w/v 2-mercaptoethanol). The protein concentration was determined by absorbance at 280 nm. A small amount of protein was loaded on a 10 or 15% SDS–PAGE gel. The column was equilibrated with at least 3 bed volumes of running buffer containing 50 mM Hepes-KOH (pH 8.0), 300 mM NaCl, and 10 mM MgCl₂. The activity was monitored using the Smart System (Pharmacia Biotech, Uppsala, Sweden) equipped with a Superose 12 PC 3.2/30 column. The column was equilibrated with at least 3 bed volumes of running buffer containing 50 mM Hepes-KOH (pH 8.0), 300 mM NaCl, and 10 mM MgCl₂ prior to each run. A flow rate of 0.5 ml/min was maintained. Absorbance at 280 nm was monitored to determine elution of proteins.

Results

Purification and anti-aggregation activity of cyanobacterial GroEL1 and GroEL2

We cloned genes encoding groES, groEL1, and groEL2 from S. elongatus PCC 7942 and overexpressed them individually in E. coli. SDS–PAGE (15%) showed that these recombinant proteins were highly enough purified to study their biochemical properties (Fig. 1A). Recombinant GroELs containing a His-tag at the C-terminus were expressed in a soluble form that was purified by immobilized metal affinity chromatography. This His-tagging does not affect the assembly or activity of the E. coli GroEL.23)

All major molecular chaperones, including GroEL, have anti-aggregation activity to bind and suppress denatured proteins. The binding of a molecular chaperone to a substrate protein is the first step in the chaperone cycle. Hence the purified cyanobacterial GroELs were first examined for their ability to prevent aggregation of heat (45°C) denatured MDH as a model substrate. Both GroEL1 and GroEL2 prevented aggregation of the MDH at a molar ratio of 1:7 in the absence of ATP (Fig. 1B), like E. coli GroEL (data not shown).

ATPase activity of cyanobacterial GroELs

ATP hydrolysis plays an essential role in GroEL-mediated protein folding that has been well characterized for E. coli GroEL.23) A bound denatured substrate must be released from the chaperone when it has refolded. ATP-binding and hydrolysis by GroEL is required at this important step. Hence we measured the

ATPase assay. Steady-state ATPase activity of GroEL was measured at 37°C with an ATP regeneration system.21) The assay mixture (1 ml) consisted of 100 mM Hepes-KOH (pH 8.0), 6 mM MgCl₂, 20 mM KCl, 0.05 mg of LDH, 2 mM phosphoenolpyruvate, 0.2 mM NADH, 0.03 mg of pyruvate kinase, and 1 mM ATP. The reaction mixture was pre-incubated at 37°C for 3 min. ATP hydrolysis was initiated by the addition of 0.4 μM GroEL to the reaction mixture with and without 0.8 μM GroES. The decrease in absorbance at 340 nm was monitored continuously with a Shimadzu UV-1600PC spectrophotometer (Shimadzu, Kyoto, Japan). To analyze the effect of GroEL2 on the ATPase activity of GroEL1, increasing concentrations of GroEL2 from 0.4 μM to 3 μM were added in the presence of 0.4 μM GroEL1.

Gel filtration chromatography. Gel filtration chromatography was done at room temperature using the Smart system (Pharmacia Biotech, Uppsala, Sweden) equipped with a Superose 12 PC 3.2/30 column. The column was equilibrated with at least 3 bed volumes of running buffer containing 50 mM Hepes-KOH (pH 8.0), 300 mM NaCl, and 10 mM MgCl₂ to 3 M NaCl. Each sample contained 2 μM or 0.5 μM GroEL and 20 μM bovine serum albumin (BSA) as the standard, respectively.

4,4′-Dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS) fluorescence spectroscopy. A bis-ANS fluorescence experiment was performed following the protocol described in reference 7, with an excitation wavelength of 395 nm and an emission wavelength of 400-600 nm with a Hitachi 850 spectrophotometer (Hitachi, Tokyo, Japan). Each sample contained 2 μM or 0.5 μM GroEL and 20 μM bis-ANS in 20 mM Hepes-KOH (pH 8.0).

References
ATPase activity of the GroELs. The $k_{cat}$ values for *E. coli* GroEL and *S. elongatus* PCC 7942 GroEL1 were $6.91 \pm 0.28 \text{ min}^{-1}$ and $1.15 \pm 0.05 \text{ min}^{-1}$ respectively (Fig. 2). The activity of GroEL2 was very low. Its $k_{cat}$ was only $0.28 \pm 0.07 \text{ min}^{-1}$. ATPase activity in the presence of GroES was also measured. Consistently with a previous report, ATP hydrolysis was inhibited by about 50% by the addition of GroES in the case of *E. coli* GroEL. In contrast, the addition of 2-fold GroES to GroEL (0.4 $\mu M$) stimulated ATP hydrolysis by GroEL1 and by GroEL2 (Fig. 2). The ATPase activity of GroEL2 increased about 6-fold in the presence of GroES.

Refolding of heat-denatured MDH and LDH by cyanobacterial GroELs

GroEL mediates the folding of a non-native protein. A classical way to monitor the folding of a protein to its native form is to measure the recovery of activity of a denatured enzyme. In order to assess the ability of cyanobacterial GroELs to facilitate folding of substrate proteins, we analyzed the *in vitro* refolding of heat-denatured MDH by the GroELs. We denatured MDH in the presence of GroEL, and then initiated its refolding by the addition of GroES and ATP. The MDH refolding yield was 5% of activity prior to denaturation after 4 h of incubation at 25°C in the absence of any GroEL and the presence of BSA, a control protein. Thus spontaneous refolding was very low (Fig. 3A). *E. coli* GroEL with its GroES showed high refolding activity in the presence of ATP, at a final refolding yield of 99%. On the other hand, GroEL1 and GroEL2 increased the refolding yield to 35% and 25% after 4 h, respectively. We evaluated the dependence of refolding on GroES. *E. coli* GroEL-mediated refolding dropped from 99% to 40% in the absence of GroES (Fig. 3A). In contrast, no significant effect of cyanobacterial GroES was observed on the refolding activity of GroEL1 or GroEL2. In the same way, the dependence of refolding on ATP was assessed. The final yield of *E. coli* GroEL-mediated refolding was severely diminished in the absence of ATP, at a final refolding yield of 14% (Fig. 3B). In the absence of ATP, the polypeptide may remain tightly bound to *E. coli* GroEL. Again, the absence of ATP did not cause any effect on the refolding activity of cyanobacterial GroEL1 or GroEL2. GroEL2-mediated refolding was very similar to *E. coli* GroEL-mediated refolding in the absence of ATP. GroEL1 showed refolding activity higher than GroEL2 (Fig. 3A and B).

The low refolding activity of GroEL1 and GroEL2 might have been due to their substrate specificity. Hence we did refolding assays with a different substrate, LDH (Fig. 3C). In contrast to MDH refolding, *E. coli* GroEL can mediate LDH refolding in a GroES-independent manner. Thus the refolding assay allowed us to compare cyanobacterial GroELs with *E. coli* GroEL without taking account of any effects in refolding that may result from interaction between GroEL and GroES.

**Fig. 1.** Purification of *S. elongatus* PCC 7942 GroEL1, GroEL2, and GroES and Analysis of Their Anti-Aggregation Activity. A, SDS–PAGE of purified GroEL1, GroEL2, and GroES. Lane 1, markers; lane 2, GroEL1 (17.6 $\mu g$); lane 3, GroEL2 (17.7 $\mu g$); lane 4, GroES (3.6 $\mu g$). B, Aggregation of 0.2 $\mu M$ MDH at 45°C in the absence (no addition) and the presence of 1.4 $\mu M$ GroEL1 or GroEL2.

**Fig. 2.** ATPase Activities of *S. elongatus* PCC 7942 GroEL1, GroEL2, and *E. coli* GroEL. ATPase activity of 0.4 $\mu M$ GroEL1, GroEL2, and *E. coli* GroEL with and without 0.8 $\mu M$ GroES. Activity was measured in the presence of 1 $\text{mM}$ ATP at 37°C. The data shown are averages for four independent experiments $\pm$ SD.
After heat denaturation at 45 °C for 2 h, the remaining activity of LDH was 16%. The presence of BSA did not increase the activity during a 4-h incubation at 25 °C, indicating that no spontaneous refolding took place. Ninety-one percent of LDH activity was recovered after 4 h of incubation in the presence of 2.8 mM *E. coli* GroEL (Fig. 3C). In contrast, no refolding was observed in the presence of 2.8 mM GroEL1 or GroEL2, the same GroEL concentration used in the MDH refolding assays. We repeated the same experiment in the presence of 25 mM GroEL1 and GroEL2, since higher concentrations favored tetradecamer formation by GroEL1 as explained below, but the increase in GroEL concentration did not affect the refolding activities of GroEL1 or GroEL2.

**Oligomeric states of GroELs**

Functional differences in ATP hydrolysis and refolding activities between the GroEL homologs led us to examine their structural differences. It is well-established that *E. coli* GroEL function requires assembly into a double ring of tetradecamer, though mutants of *E. coli* GroEL which form a single ring also retain a chaperone function. Hence GroELs were analyzed by gel filtration column chromatography to determine their oligomeric state. At a 2-μM loading concentration of GroEL1 or GroEL2, no absorbance peaks were detected in the chromatography fractions, while *E. coli* GroEL formed a tetradecamer at this concentration (data not shown). Reconstitution of ch-cpn60 showed an increased percentage of tetradecamerization, with a gradual increase in protomer concentration. Hence GroEL oligomerization was assessed at higher loading concentrations. When 50 μM GroEL1 was applied, peaks ranging from tetradecamer to tetramer were detected (Fig. 4A). One of the two major peaks eluted immediately after the void volume, like that of *E. coli* GroEL. Thus, it must have been a tetradecamer. The other eluted at a size corresponding to larger than a heptamer, based on molecular size markers. At the same concentration, GroEL2 eluted as a dimer peak (78% of the total mass) which is likely to have been a monomer (data not shown).
Bis-ANS fluorescence measurement of GroELs

Loss of oligomerization can result in the exposure of hydrophobic residues, which causes increases in the fluorescence of bis-ANS, a dye generally used to detect conformational changes in proteins hydrophobic interaction sites. As shown in Fig. 4B, bis-ANS fluorescence greatly increased upon binding to GroEL2. GroEL1 binding also increased the fluorescence, but the increase was lower than in the presence of GroEL2. The lowest increase was observed in the presence of E. coli GroEL. These results indicate that cyanobacterial GroELs, especially GroEL2, have a greater tendency to expose hydrophobic regions due to dissociation of higher oligomers than does E. coli GroEL.

Oligomeric states of cyanobacterial GroELs in cell extracts

The S. elongatus PCC 7942 recombinant GroEL oligomers were very unstable to dissociate. This may be an artifact due to His-tagging to the recombinant proteins, but this kind of unusual instability has also been observed in native GroELs from Synechocystis sp. PCC 6803. A GroEL tetradecamer from this cyanobacterium was detected only on native PAGE with a gel containing 17% glycerol, while without glycerol, 7-mer and monomer were detected. To evaluate the stability of S. elongatus PCC 7942 native GroELs, we analyzed oligomeric forms of GroEL in crude soluble protein extracts from the S. elongatus PCC 7942 wild type and the ΔgroEL2 mutant by native PAGE, followed by Western blot analysis. We used cell extracts of both the wild type and the ΔgroEL2 mutant in order to discriminate GroEL1 from GroEL2 on the gel. Similarly to Synechocystis sp. PCC 6803 GroELs, native GroELs from S. elongatus PCC 7942 did not form a 14-mer in the absence of glycerol in the gel (data not shown), but the presence of glycerol stabilized the tetradecamer, which was detected (Fig. 4C). GroEL in E. coli cell extract was used as positive control to identify the position of the GroEL 14-mer. Multiple GroEL oligomers were observed in wild-type S. elongatus PCC 7942 cell extracts, including a tetradecamer. A tetradecamer band was also detected in the ΔgroEL2 cell extracts (Fig. 4C). Thus the band must correspond to the GroEL1 tetradecamer. Two lower oligomeric species of GroELs were detected between 67 kDa and 140 kDa in the wild-type cell extracts, while these bands were absent in the ΔgroEL2 cell extracts (Fig. 4C). Thus they were probably GroEL2 oligomers. This also confirms that the C-terminal His-tagging did not cause the lower oligomerization of the GroELs.

Effect of GroEL2 on ATPase and refolding activities of GroEL1

As shown in Fig. 2, GroEL1 alone showed low ATPase activity. In a cell, GroEL1 and GroEL2 are located together. Consequently the effects of varying concentrations of GroEL2 on the GroEL1 ATPase activity were analyzed. GroEL2 stimulated the ATPase activity of GroEL1 greatly (Fig. 5A).
GroEL1 increased about 5-fold in a 1:7.5 molar ratio of GroEL1:GroEL2.

We analyzed MDH refolding in the presence of GroEL1 and GroEL2, since GroEL2 dramatically increased the ATPase activity of GroEL1. The coexistence of GroEL1 and GroEL2 did not result in refolding of MDH (Fig. 5B). The refolding yield in the presence of E. coli GroEL was slightly higher than 100%. This may have been due to an experimental deviation or to refolding of denatured MDH that was present before heat denaturation.

Discussion

As explained in the introduction, rather many bacterial genomes contain multiple copies of groEL genes. These bacteria might regulate genes differentially and/or confer distinct, non-overlapping functions on gene products. Cyanobacteria usually contain two GroELs, GroEL1 and GroEL2. There is evidence of some specificity of in vivo function in these two GroELs. GroEL1 appears to be essential, since complete segregation of a disruptant of groEL1 of S. elongatus PCC 7942 was not observed,28) while the groEL2 gene of Thermosynechococcus elongatus is dispensable under normal growth conditions.13) The E. coli groEL mutant returns almost to the wild type on complementation test with the S. vulcanus and with the Synechocystis sp. PCC 6803 groEL1 gene, but not with groEL2 of these thermophilic and mesophilic cyanobacteria.11,16,17) Although GroEL1 is present, GroEL2, which is dispensable under normal conditions, becomes essential under heat and cold stress in Thermosynechococcus elongatus.15) Thus GroEL1 and GroEL2 may be non-replaceable with each other. We postulate that GroEL1 plays an essential housekeeping role, like E. coli GroEL, whereas GroEL2 has evolved to play a role under stress conditions. These in vivo studies suggest that GroEL1 and GroEL2 have distinct biochemical properties, but the in vitro function of cyanobacterial GroEL homologs remains unexplored. In the present study, E. coli GroEL was used as positive control, since it is well characterized.

We prepared recombinant GroEL1, GroEL2, and GroES that were pure enough to study their biochemical properties (Fig. 1A). It is necessary to have a highly purified GroEL to measure its extremely low ATPase activity without interference from other ATP-hydrolyzing enzymes. Both GroEL1 and GroEL2 prevented the aggregation of heat-denatured MDH (Fig. 1B). Thus they have the capability to interact with a non-native protein. The association of ATP and GroES to a denatured protein-bound GroEL ring, one of the double rings, encapsulates the protein substrate in the central cavity of the ring, causing it to fold in an environment where interaction with other non-native proteins is blocked. ATP hydrolysis of that ring initiates a series of reactions that leads to release of the protein. In order to determine whether the paradigm well-established for E. coli GroEL is applicable to cyanobacterial GroELs, we measured their ATPase activities and analyzed the effect of GroES on the activity. We detected ATPase activity for both cyanobacterial GroELs, indicating that they can bind and hydrolyze ATP. GroEL1, especially GroEL2, showed lower ATPase activity than E. coli GroEL (Fig. 2). The addition of GroES stimulated ATP hydrolysis by both GroEL1 and GroEL2, whereas it inhibited the ATPase activity of E. coli GroEL. This stimulating effect of GroES as well as low ATPase activity in the absence of GroES has been observed in a single-ring mutant (SR-A92T) of E. coli GroEL.29) The single-ring mutant of E. coli GroEL has reduced affinity for GroES.29)

Both GroEL1 and GroEL2 were inefficient in the refolding of heat-denatured substrate proteins, even though they possessed some ATPase activity, at least in the presence of GroES. MDH refolding in the presence of GroEL1 or GroEL2 was dependent neither on GroES nor on ATP, although the refolding yield was higher than that of spontaneous refolding (Fig. 3A and B). Hence the increase in the refolding yield was not due to the active refolding well-established for E. coli
The lack of active refolding might have been caused by reduced affinity for GroES, as discussed above. In order to evaluate this possibility, we resorted to LDH refolding assay. In contrast to MDH, heat-denatured LDH can be refolded fully in the absence of GroES by *E. coli* GroEL. Hence one can ignore effect of GroES on GroEL refolding activity. Neither GroEL1 nor GroEL2 refolded LDH (Fig. 3C). Hence it is likely that both GroEL1 and GroEL2 lacked the capability to refold a protein substrate, consistently with the insensitivity of their MDH refolding activity with respect to GroES and ATP. The refolding yield of MDH in the presence of GroEL1 and of GroEL2 was significantly higher than the yield in the absence of the GroELs (Fig. 3A and B), while that of LDH was almost the same regardless of the presence or absence of the GroELs (Fig. 3C). The higher refolding of MDH may have resulted from suppression of the irreversible aggregation of the denatured substrates. We do not know why the presence of GroEL1 or GroEL2 did not increase the refolding yield of LDH. For some reason, LDH might have remained tightly bound to GroEL1 or GroEL2. As shown in Fig. 4B, GroEL1 and GroEL2 expose more hydrophobic regions than *E. coli* GroEL, which may lead to tighter binding to a denatured substrate.

The tetradecameric organization of the GroEL assembly is crucial to the folding and ATPase activities of GroEL. GroEL1 formed a tetradecamer at high concentration, while GroEL2 did not (Fig. 4A). The oligomers of both GroELs were very unstable, and dissociated when they were diluted. This unusual instability of the GroEL1 and GroEL2 oligomers is not due to His-tagging to the recombinant proteins, since oligomers smaller than tetradecamer were also detected with native GroEL1 and GroEL2 of cell extracts (Fig. 4C). We examined to determine whether the low refolding activity of the GroELs was due to the low concentrations employed in the experiments (and thus due to dissociation of the oligomers). However, great increases from 2.8 to 25 μM in the GroEL1 and of the GroEL2 concentration in a refolding solution did not result in LDH refolding (Fig. 3C).

GroEL2 enhanced the ATPase activity of GroEL1 greatly, although it did not enhance the refolding activity of GroEL1 (Fig. 5). Hence GroEL1 and GroEL2 can interact with each other. There are two types of GroEL homologs, ch-cpn60, in chloroplasts. They form hetero-oligomers. Further studies are underway to explain the presence of such a hetero-oligomer of GroEL1 and GroEL2 in cyanobacteria.

The in vitro biochemical properties of GroEL1 and GroEL2 are summarized in Table 1. They were compared with *E. coli* GroEL and GroELs (Cpn60s) from other bacterial species that contain multiple GroELs. Somewhat similar results to ours have been obtained with *Mycobacterium tuberculosis* GroELs. The genome of this bacterium also carries a duplicate set of groEL genes, one of which forms the groESL operon, while the other is separately arranged on the chromosome. The recombinant GroELs of this bacterium also exist as lower oligomers but not as tetradecamers. However, the tetradecamer of one of the GroELs, Cpn60.1, has been detected in cell extracts. The tetradecameric form is phosphorylated while the heptameric form is not.

### Table 1. Comparison of the Biochemical Functions of *E. coli* GroEL with Some Multiple GroEL Possessing Bacteria

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>GroEL homologs</th>
<th>Anti-aggregation activity</th>
<th>ATPase activity ($k_{cat}$ (% relative value))</th>
<th>Folding activity (% relative value)</th>
<th>Oligomeric state</th>
<th>Notes</th>
<th>Dispensable under normal growth conditions</th>
<th>References</th>
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<td><em>E. coli</em></td>
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NA, Information not available.

*S* Spontaneous refolding was 5%.

**Spontaneous refolding was 58%.

***Spontaneous refolding was 25%. 

GroEL, The lack of active refolding might have been caused by reduced affinity for GroES, as discussed above. In order to evaluate this possibility, we resorted to LDH refolding assay. In contrast to MDH, heat-denatured LDH can be refolded fully in the absence of GroES by *E. coli* GroEL. Hence one can ignore effect of GroES on GroEL refolding activity. Neither GroEL1 nor GroEL2 refolded LDH (Fig. 3C). It is likely that both GroEL1 and GroEL2 lacked the capability to refold a protein substrate, consistently with the insensitivity of their MDH refolding activity with respect to GroES and ATP. The refolding yield of MDH in the presence of GroEL1 and of GroEL2 was significantly higher than the yield in the absence of the GroELs (Fig. 3A and B), while that of LDH was almost the same regardless of the presence or absence of the GroELs (Fig. 3C). The higher refolding of MDH may have resulted from suppression of the irreversible aggregation of the denatured substrates. We do not know why the presence of GroEL1 or GroEL2 did not increase the refolding yield of LDH. For some reason, LDH might have remained tightly bound to GroEL1 or GroEL2. As shown in Fig. 4B, GroEL1 and GroEL2 expose more hydrophobic regions than *E. coli* GroEL, which may lead to tighter binding to a denatured substrate.

The tetradecameric organization of the GroEL assembly is crucial to the folding and ATPase activities of GroEL. GroEL1 formed a tetradecamer at high concentration, while GroEL2 did not (Fig. 4A). The oligomers of both GroELs were very unstable, and dissociated when they were diluted. This unusual instability of the GroEL1 and GroEL2 oligomers is not...
indicating phosphorylation-mediated regulation of the GroEL oligomeric states. The GroELs possess very weak ATPase activity. They show low or no active refolding, because the apparent refolding in their presence is lower than spontaneous refolding (Table 1). The diversity in ATPase and refolding activities among the three GroELs of *R. leguminosarum* suggests that they have evolved to acquire functional diversity, although this diversity is not related to their oligomeric states.

This study clearly indicates that the biochemical properties of cyanobacterial GroELs are different from those of *E. coli* GroEL. The functional differences, i.e., lower ATPase activity, stimulation of ATPase activity by GroES, and a lack of active refolding activity, may be related to the oligomeric states of GroEL1 and GroEL2. Assuming that the *E. coli* GroEL chaperone mechanism is highly conserved in other bacterial GroELs, we propose that GroEL1 forms a homo- or hetero-tetradecamer (with GroEL2) in *vivo*, acts as a foldase to fold and refold non-native proteins, and plays an essential housekeeping role, like *E. coli* GroEL. The tetracamer might be stabilized by an unknown mechanism. GroEL2 does not form a tetracamer, since we have not seen any evidence in *vivo* or in *vitro* that indicates its presence. In that it is a lower oligomer with a larger hydrophobic surface (Fig. 4), GroEL2 acts as a holdase to suppress aggregation of denatured proteins (Fig. 1). It is also possible that it controls the function of GroEL1, as shown in Fig. 5.

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