A Nonconserved Carboxy-Terminal Segment of GroEL Contributes to Reaction Temperature

Takamichi Nakamura,1 Mitsuko Tanaka,1 Akihiko Maruyama,2 Yowsuke Higashi,2 and Yasurou Kurusu1,4

1Laboratory of Molecular Microbiology, College of Agriculture, Ibaraki University, Ami 3-21-1, Inashiki, Ibaraki 300-0393, Japan
2National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8566, Japan

Received June 17, 2004; Accepted September 21, 2004

The role of the C-terminal segment of the GroEL equatorial domain was analyzed. To understand the molecular basis for the different active temperatures of GroEL from three bacteria, we constructed a series of chimeric GroELs combining the C-terminal segment of the equatorial domain from one species with the remainder of GroEL from another. In each case, the foreign C-terminal segment substantially altered the active temperature range of the chimera. Substitution of L524 of Escherichia coli GroEL with the corresponding residue (isoleucine) from psychrophilic GroEL resulted in a GroEL with approximately wild-type activity at 25°C, but also at 10°C, a temperature at which wild-type E. coli GroEL is inactive. In a detailed look at the temperature dependence of the GroELs, normal E. coli GroEL and the L524I mutant became highly active above 14°C and 12°C respectively. Similar temperature dependences were observed in a surface plasmon resonance assay of GroES binding. These results suggested that the C-terminal segment of the GroEL equatorial domain has an important role in the temperature dependence of GroEL. Moreover, E. coli acquired the ability to grow at low temperature through the introduction of cold-adapted chimeric or L524I mutant groEL genes.

Key words: GroEL; psychrophile; mesophile; thermo-philie; temperature-adaptation

The GroE chaperone system from Escherichia coli prevents the aggregation and supports the folding of polypeptides.1) It consists of GroEL, a homotetradecameric double-ring cylinder composed of ~57 kDa subunits2,3) and its co-chaperone GroES, a homohexameric dome-shaped ring composed of ~10 kDa subunits.4) A GroEL subunit consists of an apical, an intermediate, and an equatorial domain.2,3) The apical domains form the entrance to the GroEL cavity and include the residues involved in binding to GroES and unfolded proteins.5) The small intermediate domain has potential hinge regions at its connection to the equatorial and apical domains.6) The equatorial domain, which includes both the N- and C-termini of GroEL, contains the ATP binding site and most of the residues that make inter-subunit contacts.2) The equatorial domain does not contact the intermediate or apical domain of the neighboring subunits. An anti-parallel β-loop (residues 36–50 in GroEL) projects from the body of the domain towards the inner surface of its right-hand neighbor, where it forms a parallel β-structure near its neighbor’s C-terminal segment (residues 519–522).2) Deletion analysis of the C-terminal segment shows the important role of intersubunit interactions in GroEL assembly,7) but the amino acid sequences of GroEL from many bacteria show that most of the N-terminal segment is highly conserved while the C-terminal segment is not.

Here we investigate the role of the sequence diversity of the C-terminal segment of the GroEL equatorial domain using GroEL from psychrophilic, mesophilic, and thermophilic bacteria (Pseudoalteromonas sp. PS1M3, E. coli, and Thermus sp. TB1 respectively). By construction of chimeric GroELs, we attempt to show that the C-terminal segment governs the different active temperatures of GroE from these bacteria. Specifically, the amino acid residues of E. coli GroEL are identified by site-directed mutagenesis and surface plasmon resonance (SPR). Furthermore, we show the cold-adaptation of E. coli harboring a psychrophilic groEL gene.

Materials and Methods

Bacterial strains, plasmids, and growth conditions. E. coli strains were grown in LB medium supplemented with ampicillin at a final concentration of 50 μg/ml or kanamycin at a final concentration of 50 μg/ml, if required. E. coli DH5αΔlacZΔM15 endA1 recA1 hsdR17 (r−m−) supE44 thi-1 λ− gyrA relA1 F− Δ

1 To whom correspondence should be addressed. Fax: +81-29-888-8525; E-mail: krsy@mx.ibaraki.ac.jp
H60PS-F, 5′ AB057417) were amplified using primers as follows: *Pseudoalteromonas* groEL control were amplified by PCR from genomic DNA of three bacteria used as a host for routine cloning. Plasmids pQE30 (containing the groEL gene with an N-terminal hexa-histidine tag and ampicillin resistance gene) and pQE60 (containing the groES gene with a C-terminal hexa-histidine tag and ampicillin resistance gene) were used for the expression of the recombinant groE gene in *E. coli* SG13009 (Qiagen).

Construction of recombinant groE genes. Wild-type groEL and groES genes of three bacteria were used as a control were amplified by PCR from genomic DNA as follows. groEL and groES genes of the psychrophilic *Pseudoalteromonas* sp. PS1M3 (accession no. AB057417) were amplified using primers as follows: H60PS-F, 5′ TTCCATGGCCGACATTCGTCCTTTAACA 3′ (NcoI site underlined) and H60PS-R, 5′ TTAGATCTGCTCAATGCTAAATGT 3′ (BglII site underlined) for the groE gene; and H30PL-F, 5′ TTGGATCCGACATGACGCCCATG 3′ (BamHI site underlined) and H30PL-R, 5′ GGAAAGCTTATCATCATGACGCCCATG 3′ (HindIII site underlined) for the groE gene.

The groEL and groES genes of *E. coli* (accession no. X07850) were amplified using primers as follows: H60ES-F, 5′ TTCCATGGCCGACATTCGTCCTTTAACA 3′ (NcoI site underlined) and H60ES-R, 5′ TTAGATCTGCTCAATGCTAAATGT 3′ (BglII site underlined) for the groE gene; and H30EL-F, 5′ CCGGCTCTGACATGACGCCCATG 3′ (BamHI site underlined) and H30EL-R, 5′ GGAAAGCTTATCATCATGACGCCCATG 3′ (HindIII site underlined) for the groE gene. The amplified groEL and groES genes of *E. coli* were separately amplified using primers as follows: I525L-F, 5′ TTCCATGGCCGACATTCGTCCTTTAACA 3′ (NcoI site underlined) and I525L-R, 5′TTGGATCCGACATGACGCCCATG 3′ (BamHI site underlined) for the *E. coli* groEL segment (residues 1–502). The amplified DNAs were digested with appropriate restriction enzymes and cloned into pQE30 and vector pQE60. The chimeric FPEL gene was constructed by PCR with *Pseudoalteromonas* sp. PS1M3 and *E. coli* genomic DNA as follows. Psychrophilic groEL (residues 1–502) and the C-terminal segment (residues 502–549) of *E. coli* groEL were separately amplified using primers as follows: H30PL-F (described above) and FPL-R, 5′ TTAGATCTGCTTCATTTAGTTGGATC 3′ (BglII site underlined); FEL-F, 5′ TTAGATCTGCTGAGTCGACAGCTC 3′ (BglII site underlined) and H30EL-R (described above). The fused FTEL gene was constructed by PCR with *Thermus* sp. TB1 and *E. coli* genomic DNA as follows. Thermophilic groEL (residues 1–502) and the C-terminal segment (residues 502–549) of *E. coli* groEL were separately amplified using primers as follows: H30TL-F (described above) and FTL-R, 5′ TTAGATCTGCTTACCTGGCAGGGT 3′ (BglII site underlined); FEL-F and H30EL-R (described above). Both amplified DNAs were mixed and digested with BglII, and re-ligated. The ligated DNA were digested with appropriate restriction enzymes and cloned into the pQE30.

Various groEL mutants of *E. coli* were constructed by two-step PCR as described previously.8) Primers H30EL-F and H30EL-R (described above) were used in the usual ways and other primers were used as follows: Y506F-F, 5′ TGCTCTGAGTTCGACAGCTC 3′ (mutation site underlined) and Y506F-R, 5′ CGACGCTGACGAGCTC 3′ (mutation site underlined) for Y506F; V510I-F, 5′ CGACGCTGACGAGCTC 3′ (mutation site underlined) and V510I-R, 5′ CCATGACGAGCTC 3′ (mutation site underlined) for V510I; T522A-F, 5′ ATGACGACGAGCTC 3′ (mutation site underlined) and T522A-R, 5′ CTGGGCGACGAGCTC 3′ (mutation site underlined) for T522A. The amplified DNAs were digested with appropriate restriction enzymes and cloned into pQE30.

Protein expression and purification. Plasmids pQE harboring various groEL and groES genes were transformed into *E. coli* SG13009 host cells (Qiagen). Large-scale expression and non-denaturing purification by nickel/nitriloacetic (Ni-NTA; Qiagen) metal-affinity chromatography were performed according to the manufacturer’s instructions. This chromatography was applied at least twice, with buffer containing 5 mM ATP. The N-terminal amino acid sequence of the purified protein was determined by Edman degradation using an ABI 473A amino acid analyzer. The amount of protein was determined by Bio-Rad protein determination kit with BSA as the standard. The purified proteins were dialyzed to buffer the subsequent experiments properly.
Refolding assay. Refolding of denatured Rhodanese (Sigma) was measured as follows. 0.5 μM of Rhodanese was denatured in 6 M guanidine–HCl (GuHCl) and diluted 100-fold into buffer A (20 mM MOPS/KOH [pH 7.5], 100 mM KCl, and 5 mM Mg(CH$_3$COO)$_2$) in the absence or presence of GroEL at various temperatures, as specified in the figure legends. A 2-fold molar excess of GroES over GroEL was added and refolding was initiated with the addition of 5 mM ATP. GroEL action was stopped with the addition of 50 mM CDTA. Refolding of rhodanese was analyzed according to methods described previously. For a high temperature analysis at 37°C, Achromobacter protease I (API: Wako) (E.C. 3.4.21.50), a relatively thermo-stable enzyme, was used as substrate. API was denatured in 20 mM Tris–HCl (pH 8.0) and 8 M guanidine HCl at 30°C for 30 min. API refolding was assayed by cleavage of API substrate Bz-lys-p-nitroanilide. Refolding was started by the addition of 20 μl of denatured API (0.03 nM) into 1,180 μl of reaction buffer containing 50 mM Tris–HCl (pH 8.0), 2 mM ATP, 10 mM Mg(CH$_3$COO)$_2$, and 0.2 mM Bz-lys-p-nitroanilide, and both GroEL and GroES were added in the reaction buffer at 0.6 nM. Refolding of API at different temperatures was continuously monitored at 405 nm.

Wild-type GroE with the histidine-tag of E. coli prepared as described above had fully refolding activity (95%) compared to GroE with no histidine-tag of E. coli (Takara) (data not shown), these results indicated that plasmids pQE30 (for the groEL gene with an N-terminal hexa-histidine tag) and pQE60 (for the groES gene with a C-terminal hexa-histidine tag) were suitable for the expression of various recombinant groE genes in E. coli.

Binding assay by Surface Plasmon Resonance. The experimental procedure was as previously described. E. coli GroES was purified as described above under “Protein expression and purification” and immobilized (300 RU) via a thioether linkage on a CM5 biosensor chip (Biacore 2000 SPR instrument, Biacore X, Uppsala, Sweden). GroEL binding was followed using buffer A containing 0.2 mM ADP at a flow rate of 10 μl per minute at various temperatures. The concentration of GroEL was 100 nM.

Results

Replacement of the C-terminal segment in GroEL

The amino acid sequences of the N-terminal equatorial domain (residues 22–42) of psychrophilic and thermophilic GroEL are highly homologous to that of E. coli GroEL (91%, and 85% respectively) (Fig. 1A). Although the C-terminal domains (residues 507–536) of both psychrophilic and thermophilic GroEL have amino acids residues contributing to intersubunit contacts, the homology between these segments and that of E. coli is relatively low (62%, and 33% respectively). Therefore, we focused on the C-terminal segment of GroEL and constructed three chimeric GroELs to evaluate the role of this region in protein folding at different temperatures. These chimeric GroELs are indicated in Fig. 1B. The FEPL was constructed by combining E. coli GroEL (residues 1–501) with psychrophilic GroEL (residues 503–549), and the FPEL vice versa. The FTEL was constructed by combining thermophilic GroEL (residues 1–502) with E. coli GroEL (residues 502–548). The native GroELs were used as controls and showed different patterns of activity at 10°C (Fig. 2A), 25°C (Fig. 2B), and 37°C (Fig. 2C). Although E. coli GroEL was not active at 10°C, the FEPL with an E. coli GroES was nearly as active as the psychrophilic GroE (Fig. 2A). Moreover, the activity of FTEL with thermophilic GroES effectively increased at 37°C compared with the thermophilic GroE (Fig. 2C). On the other hand, the activity of the FPEL with psychrophilic GroES decreased at 10°C but slightly increased at 25°C compared to the psychrophilic GroE (Fig. 2A and 2B). These results suggest that the C-terminal segment of a GroEL equatorial domain governs the temperature dependence of GroE activity.
Site-directed mutagenesis of the C-terminal segment in *E. coli* GroEL

To determine the amino acid residues in the C-terminal segment that contribute to temperature dependence, several mutated GroELs were constructed by site-directed mutagenesis. Four amino acids in the C-terminal segment (residues 506–535) of *E. coli* GroEL were substituted for with the corresponding residues in the psychrophilic type (Fig. 1A). A L524I GroEL was as active as wild-type GroEL at 25°C (Fig. 3B), but, unlike the wild type, they were active at 10°C (Fig. 3A). Y506F, V510I, and T522A were not active at 10°C (data not shown). On the other hand, I525L of psychrophilic GroEL with the corresponding residue in the mesophilic type was constructed and showed that the activity of I525L decreased at 10°C (Fig. 3A), but still remained at 25°C (Fig. 3B). These results suggested that L524, which is close to the residues that make inter-subunit contacts (residues 516–522), is critical for temperature-adaptation of *E. coli* GroE activity.

**Fig. 2.** Refolding Activity of GroE at Different Temperatures.

The assay method is described in “Materials and Methods”. The experiment was performed three times and the average of these is indicated. The time-course of GroE from three bacteria exhibited at 10°C (A), 25°C (B), and 37°C (C). Rhodanese as substrate for GroE were used in Fig. 2A and Fig. 2B and API was used in Fig. 2C. Each enzyme activity was calculated from the activities of native (non-denatured) enzyme as a control. The thermophilic GroE was not active at 10 and 25°C (data not shown). Closed circles, psychrophilic GroE; closed squares, *E. coli* GroE; open squares, the FEPL with *E. coli* GroES; open circles, the FPEL with psychrophilic GroES; closed diamonds, thermophilic GroE; open diamonds, FTEL with thermophilic GroES; X, no GroE added.

**Fig. 3.** Refolding Activity of Mutated GroEL at 10 and 25°C.

The assay method using rhodanese as substrate is described in “Materials and Methods”. The experiment was performed three times and the average of these is indicated. The time-course of the GroE reaction is exhibited at 10°C (3A) and 25°C (3B). Enzyme activity was calculated from the activity of native (non-denatured) rhodanese as a control. Closed triangles, L524I with *E. coli* GroES; open triangles, I525L with psychrophilic GroES; X, no GroE added.
Refolding and SPR analysis of GroE at different temperatures

To estimate the critical temperature of GroE function, we analyzed GroE refolding activity and GroES–GroEL binding at temperatures from 10 to 20 °C (Fig. 4). Although the FEPL and psychrophilic GroE had high refolding activities across the whole temperature range, the significant refolding activity of wild-type E. coli GroEL and the L524I were elevated above 14 °C and 12 °C respectively (Fig. 4A). Furthermore, SPR analysis indicated that the binding ability of wild-type E. coli GroEL and the L524I with GroES increased starting at 14 °C and 12 °C respectively (Fig. 4B), suggesting that GroEL assembly and binding with GroES depends on temperature. Taken together, the above results suggested that the C-terminal segment of the GroEL equatorial domain has an important role in the temperature dependence of GroEL assembly into mature protein in order to bind with GroES.

Cold-adapted growth of E. coli harboring various groEL genes

The psychrophilic and engineered GroELs are cold-adapted proteins that showed higher activity at low temperature than the native E. coli GroEL. Cold-adapted GroEL may be important for the growth of psychrophilic bacteria at low temperatures. To evaluate the physiological role of GroEL, we examined the growth of E. coli harboring a plasmid containing various groEL inserts. The results in Fig. 5 indicate that the cold-adapted groEL genes conferred increased growth at 10 °C. The growth rates were correlated with the different levels of activity of the various GroELs at 10 °C (Fig. 2). It was found that bacterial growth was adaptable to the active temperature of GroE and confirmed that the groEL and groES genes from psychrophilic bacterium Oleispira antarctica RB8 show a positive effect on E. coli growth at low temperatures.

Discussion

Temperature is undoubtedly one of the major factors influencing bacterial growth and survival. Consequently, it is of considerable significance how extremophiles survive in their natural environments and how they adapt to temperatures exceeding their normal growth range. In this respect, it noteworthy that all organisms studied to date acquire thermo-tolerance to normally lethal temperatures when preexposed to milder, non-lethal heat shock. This phenomenon depends on heat shock proteins.

This report examined how the activity of heat shock protein GroEL is affected by the residues of the C-terminal segment of its equatorial domain. First, from
replacement analysis of the C-terminal segment (residues 503–548) of GroEL from psychrophilic, mesophilic, and thermophilic bacteria, we found that this segment governs the temperature of GroE activity. Furthermore, by site-directed mutagenesis analysis, Leu524 in this segment, close to the residues 516–522 contributing to intersubunit contacts, was to be critical for the temperature-adaptation of E. coli GroE activity. Moreover, protein refolding and SPR assays indicated that the both assembly of GroES–GroEL and ATPase activity occurred at 10 to 37°C. The L524I mutation of the C-terminal segment, close to the residues 516–522 surrounding residues 516–522 is responsible for the temperature-adaptation of GroEL monomers into a single ring (the GroEL heptamer). Also, these events are correlated with the active temperature of GroEL, and residue 524 is important for both phenomena. The hydrophobicity of the C-terminal region is 8.2 for psychrophilic (residues 517–524), 6.4 (residues 517–524) for E. coli, and 5.4 (residues 518–525) for thermophilic GroEL respectively. Thus it seems that the interaction between the conserved N-terminal equatorial domain and the C-terminal equatorial domain depends on the hydrophobicity of the C-terminal segment. The L524I mutation increased the hydrophobicity of the E. coli segment to 7.6, supporting this hypothesis.

On the other hand, ATPase activity of GroEL from psychrophilic Pseudoalteromonas haloplanktis has been reported for comparison with that of mesophilic E. coli. It was found that no significant difference in ATPase activity occurred at 10 to 37°C and that the only difference was that maximum activity occurred at 47°C, some 8°C lower than for mesophilic activity. The amino acid sequence of GroEL from P. haloplanktis is highly homologous to that of PS1M3 (95.4%), used in this study. We also measured the ATPase activities of various GroELs according to the previous method and found that significant differences, as well as the results of refolding activity, described above, did not occur in the 10 to 37°C temperature range (data not shown). Taken together, the above results suggest that the temperature dependence of GroEL activity is due to the C-terminal segment of the GroEL equatorial domain rather than to ATPase activity.

The second aspect of this work is to clarify the mechanisms underlying bacterial adaptation to temperature. Despite many biochemical reports on psychrophilic and thermophilic bacteria, the importance of GroE for growth at various temperatures has yet to be demonstrated directly. It is interesting that the introduction of a cold-adapted GroEL allowed E. coli, a mesophilic bacterium, to shorten its doubling time at low temperatures. Recently, it was reported that the groEL and groES genes from psychrophilic bacterium Oleispira antarctica RB8 showed a positive effect on E. coli growth at low temperatures. It appears that cold-adapted GroE speeds the folding and maturation of many cellular proteins at low temperatures, facilitating bacterial adaptation to the low temperatures.

Acknowledgments

This research was funded by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan through the Special Coordination Fund “Archaeon Park” project. We would like to thank T. Urabe (University of Tokyo) and K. Marumo (AIST) for their support during isolation of a thermophilic bacterium, Thermus sp. TB1, from the Toyoha Mines in Japan.

References


