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ORIGINAL PAPER

Distinct features of protein folding by the GroEL system from a psychrophilic bacterium, *Colwellia psychrerythraea* 34H

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Abstract We investigated the protein folding mechanism of the GroEL system of a psychrophilic bacterium, *Colwellia psychrerythraea* 34H. The amount of mRNA of the *groESL* operon of *C. psychrerythraea* was increased about 6-fold after a temperature upshift from 8 to 18 °C for 30 min, suggesting that this temperature causes heat stress in this bacterium. A σ^{32}-type promoter was found upstream of the *groESL*, suggesting that the *C. psychrerythraea groESL* is regulated by the σ^{32} system, like the *groESL* in *E. coli*. The maximum ATPase and CTPase activities of *Cp*GroEL were observed at 45 and 35 °C, respectively, which are much higher than the growth temperatures of *C. psychrerythraea*. We found that the refolding activity of the *Cp*GroEL system in the presence of ATP is lower than that in the presence of CTP. This suggests that ATP is not the optimum energy source of the *Cp*GroEL system. Analyses for the interaction of *Cp*GroEL–*Cp*GroES revealed that CTP could weaken this interaction, resulting in effective refolding function of the *Cp*GroEL system. From these findings, we consider that the *Cp*GroEL system possesses an energy-saving mechanism for avoiding excess consumption of ATP to ensure growth in a low-temperature environment.

Keywords GroEL · Psychrophilic bacterium · *Colwellia psychrerythraea* · Protein folding · Nucleotide selectivity

Abbreviations

Cp	*Colwellia psychrerythraea*
Ec	*Escherichia coli*
HSP	Heat shock protein
MDH	Malate dehydrogenase
SR1	Single-ring mutant of *E. coli* GroEL
Oa	*Oleispira antarctica*
(His_6)	Hexahistidine

Introduction

All organisms synthesize a set of highly conserved proteins called heat shock proteins (HSPs) in response to changes in environmental conditions, such as heat stress (Richter et al. 2010). These proteins include the molecular chaperones that help proper folding of cellular proteins (Walter and Buchner 2002) and proteases that degrade denatured proteins (Hengge and Bukau 2003). The chaperonin GroEL is one of the best-characterized molecular chaperones. GroEL forms an ~800 kDa cylindrical complex consisting of two

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heptameric rings of 57 kDa subunits. Each subunit of GroEL is composed of three domains, namely, an apical domain containing the binding sites for substrate proteins and for co-chaperonin GroES, an equatorial domain containing the ATP binding site, and an intermediate domain, which connects the apical and the equatorial domains (Xu et al. 1997). In cooperation with GroES, GroEL assists the proper folding of various cellular proteins including essential ones through an ATP-dependent process (Kerner et al. 2005).

The GroEL-assisted folding pathway established so far is as follows. Substrate proteins bind to the apical domain of GroEL. Binding of ATP to the equatorial domain of GroEL leads to the interaction with GroES, and a ternary complex containing GroEL, GroES, and the substrate protein is obtained. In this state, the substrate protein is encapsulated into the central cavity of GroEL, where proper folding is performed. After ATP hydrolysis, both GroES and the substrate protein are released, and the next round of folding reaction proceeds in the opposite ring of GroEL (Horwich et al. 2006). Furthermore, other folding pathways, such as GroES- and/or ATP-independent pathways, have been reported (Hansen and Gafni 1993; Kawata et al. 1994). Recently, it has also been described that substrate proteins that are too large to be encapsulated can utilize GroEL/GroES for proper folding (Chaudhuri et al. 2001; Paul et al. 2007). In addition to these well-known pathways in *E. coli*, there are many varieties in the functional pathways of GroEL. For example, GroEL1 from *Chlamydophila pneumoniae* has a broader selectivity for nucleotides in the refolding reaction, and the intermediate domain is the major contributor to this selectivity (Okuda et al. 2008). In GroEL1 from *Mycobacterium tuberculosis*, the transition between the single- and double-ring states is mediated by phosphorylation (Kumar et al. 2009). Thus, GroEL has evolved a wide range of characteristics depending on the conditions and bacterial species, among other factors.

Colwellia psychrerythraea 34H is a psychrophilic bacterium that was isolated from Arctic marine sediments (Huston et al. 2000). Optimum and lethal temperatures for this bacterium are 8 and 19 °C, respectively. Thus, a relatively low temperature, such as 16 °C, may cause heat stress in this bacterium, whereas this temperature induces the cold shock response in most mesophilic organisms (Sakamoto et al. 1997; Yamanaka 1999). We previously described that, in a psychrophilic bacterium, *Colwellia maris* ABE-1, a temperature of 20 °C induced the *groESL* and *dnaK* genes, which is a much lower temperature than for mesophilic bacteria (Yamauchi et al. 2003, 2004). These results suggest that molecular chaperones in psychrophilic bacteria are essential for their lives at a low temperature.

Although many reports have described GroEL from mesophilic and thermophilic bacteria, little is known about the biochemical properties of GroEL from psychrophilic bacteria. Recently, GroEL characterization has been reported for a few psychrophilic bacteria. Tosco et al. (2003) have described that no obvious difference was shown between GroEL from *Pseudoalteromonas haloplanktis* TAC 125 and a mesophilic one. In contrast, GroEL from *Oleispira antarctica* RB-8 is a cold-adapted chaperonin that transits between single- and double-heptameric-ring states in a temperature-dependent manner (Ferrer et al. 2004). These results suggest that various types of GroEL exist in psychrophilic bacteria. Thus, the study of GroEL from psychrophilic bacteria will yield more information about not only specific features involved in temperature dependence but also a novel mechanism for protein folding.

In this paper, we describe novel features of GroEL derived from a psychrophilic bacterium, *C. psychrerythraea* 34H (*Cp*GroEL). Our biochemical analysis demonstrated that the nucleotide selectivity of *Cp*GroEL was different from those of *E. coli* GroEL (*Ec*GroEL). In addition, we also investigated the transcriptional regulation of the *C. psychrerythraea groESL* under heat stress conditions.

Materials and methods

Bacteria, culture conditions, and DNA preparation

The psychrophilic bacterium *Colwellia psychrerythraea* 34H was grown to stationary phase in Marine Broth (Difco, Lawrence, KS, USA) at 8 °C. *E. coli* JM109 and BL21(λDE3) were used for the propagation of plasmids and the expression of recombinant proteins, respectively. Unless otherwise stated, these *E. coli* strains were grown at 37 °C with vigorous shaking in LB medium supplemented with 50 μg ml^{-1} ampicillin. Genomic DNAs of *C. psychrerythraea* and *E. coli* were prepared by the method described previously (Yamauchi et al. 2003).

RNA preparation

Total RNA was extracted from cells that had been incubated at the indicated temperatures for 60 min or at 18 °C for the indicated times. After incubation, 3 ml culture was mixed with an equal volume of ethanol containing 10 % (w/v) phenol. Cells were collected by centrifugation, and were resuspended in TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) with lysozyme (0.4 mg/ml). After incubation at room temperature for 5 min, total RNA was isolated using the SV total RNA isolation kit according to the manufacturers' instructions (Promega, Madison, WI, USA).

Primer extension and Northern blotting

Restriction endonucleases and DNA-modifying enzymes for plasmid construction were obtained from New England Biolabs (Beverly, MA, USA) and Takara Shuzo (Kyoto, Japan), respectively. For performing a cycle sequence in the primer extension analysis, a 681-bp DNA fragment that contains the *C. psychrerythraea groES* and its upstream region was amplified by PCR using the primers *groES*681-FW, 5′-TGGCAATGTCTATCAGCATG-3′, and *groES*681-RV, 5′-GAATCTAAACACTCAAGCAGC-3′. The amplified fragment was cloned into the pGEM-T vector (Promega), yielding pT-G681. To determine the site of transcriptional initiation, a 5′-DIG-labeled oligonucleotide, 5′-ATGTAGTGGACGAATGCTCAT-3′, which is complementary to nucleotides +1 to +21 relative to the *groES* initiation codon, was used. Total RNA (20 μg) and the DIG-labeled primer (2 pmol) were combined, and primer extension analysis was performed as described previously (Yamauchi et al. 2006).

To prepare the probe for Northern blotting, pT-G681 was digested with *Spe*I and the resultant fragment containing the *groES* was cloned into the corresponding site of the pBluescript (pBS) II KS (+) vector (Stratagene, La Jolla, CA, USA). The resultant plasmid was designated as pBS-GroES. The DIG-labeled probe was prepared from pBS-GroES with the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Basel, Switzerland). Northern blotting was performed as described previously (Yamauchi et al. 2003). Hybridization with the probe containing a part of the gene encoding 16S rRNA was used as a loading control (Yamauchi et al. 2003). Hybridized signals were quantified by ImageJ software (Abràmoff et al. 2004).

Expression and purification of recombinant proteins in *E. coli*

The coding regions of the *groES* and *groEL* from *C. psychrerythraea* (*CpgroES* and *CpgroEL*) and *E. coli* (*EcgroES* and *EcgroEL*) were amplified from genomic DNA by PCR with KOD plus DNA polymerase (Toyobo, Osaka, Japan) and the primers *CpgroES*-FW, 5′-CATATGAGCA TTCGTCCACTACA-3′, and *CpgroES*-RV, 5′-CTCGA GCTACTCAACGATCGCTAAAAT-3′, for *CpgroES*, and *CpgroEL*-FW, 5′-CATATGGCTGCAAAAGACGTATT A-3′, and *CpgroEL*-RV, 5′-CTCGAGTTTAGACAAGCAT AAATTACATC-3′, for *CpgroEL*, and *EcgroES*-FW, 5′-CATATGAATATTCGTCCATTGCAT-3′, and *EcgroES*-RV, 5′-CTCGAGTTACGCTTCAACAATTGCCA-3′, for *EcgroES*, and *EcgroEL*-FW, 5′-CATATGGCAGCTA AAGACGTAAAAT-3′, and *EcgroEL*-RV, 5′-CTCGAGA GGTGCAGGGCAATTACATC-3′, for *EcgroEL*. The PCR products were digested with *Nde*I and *Xho*I. The resultant DNA fragments for *groES* were cloned into the same sites of the pET-15b vector (Novagen, Darmstadt, Germany) to produce hexahistidine (His_6)-tagged proteins at the N-terminus. The resultant DNA fragments for *groEL* were cloned into the pET-21b vector (Novagen). These plasmids were used to transform *E. coli* BL21(λDE3), and the expression of recombinant proteins was induced by the addition of 0.1 mM IPTG at 30 °C.

For purification of recombinant GroELs, cells from 300 ml culture were harvested, and suspended in 7 ml of a solution containing 25 mM Tris–HCl (pH 7.5), 1 mM EDTA, and 1 mM DTT (buffer A). After sonication, ammonium sulfate was added to the cell extracts at a final concentration of 20 %. Supernatant fractions were applied to a Butyl-Toyopearl column (Tosoh, Tokyo, Japan) that had been equilibrated with buffer A containing 20 % ammonium sulfate, at a flow rate of 1 ml min^{-1}. The column was washed with buffer A containing 20 % ammonium sulfate and 20 % methanol, and recombinant GroELs were eluted with a linear gradient (20-0 % ammonium sulfate). GroEL fractions were pooled and loaded onto a Hiprep Sephacryl S-300 HR 16/60 gel-filtration column (GE Healthcare, Little Chalfont, UK), which had been equilibrated with buffer B (25 mM Tris–HCl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$) at a flow rate of 0.4 ml min^{-1}. The purified GroELs were concentrated using a Centricon YM-100 centrifugal filter device (Millipore, Billerica, MA, USA), and then stored at −80 °C until use.

For purification of recombinant (His_6)-tagged GroESs, cells from 200 ml culture were harvested and suspended in 5 ml of a solution containing 50 mM Tris–HCl (pH 7.5) and 500 mM NaCl (buffer C). After sonication, supernatant fractions were applied onto a 1-ml HiTrap chelating column (GE Healthcare) that had been equilibrated with buffer C containing 20 mM imidazole. The column was washed with buffer D (50 mM Tris–HCl, pH 7.5, 50 mM NaCl, and 40 mM imidazole), and (His_6)-tagged GroESs were then eluted with buffer D containing 400 mM instead of 40 mM imidazole. Eluted fractions were applied onto a 5-ml HiTrap desalting column (GE Healthcare) that had been equilibrated with buffer B containing 10 % glycerol, and purified (His_6)-tagged GroESs were stored at −80 °C until use. All purified proteins were checked by SDS-PAGE analysis, and protein concentration was determined using the Protein Assay Kit (Bio-Rad, Hercules, CA, USA) with BSA as the standard.

Gel filtration chromatography and native PAGE

Purified GroELs (1.8 μM oligomer) were prepared in 200 μl of the refolding buffer (50 mM Hepes–KOH, pH 7.4, 100 mM KCl, and 10 mM $MgCl_2$) with or without

2 mM ATP. Gel filtration chromatography was performed using the FPLC system (GE Healthcare) equipped with a Superose 6 10/300 GL column (GE Healthcare) pre-equilibrated with the refolding buffer supplemented or not with 100 μM ATP. Separation was performed at 4 °C and at a flow rate of 0.3 ml min^{-1}. The following proteins were used to calibrate the gel filtration column: thyroglobulin (669 kDa), ferritin (440 kDa), and ovalbumin (44 kDa).

For native PAGE analysis, purified GroELs (3 μg) were resolved by a 6 % polyacrylamide gel.

Refolding assay

Unless otherwise stated, all refolding reactions were performed at 20 °C. Malate dehydrogenase (MDH) from pig heart (Oriental Yeast, Tokyo, Japan) was denatured in 6 M guanidine hydrochloride and 1 mM DTT at 25 °C for 1 h. The denatured MDH (15 μM) was diluted 100-fold in the refolding buffer containing 0.15 μM GroEL (oligomer), 0.3 μM GroES (oligomer), 5 mM DTT, and 2 mM nucleotide. At the indicated times, 30 μl aliquot was added into 800 μl of a solution containing 100 mM Tris–HCl, pH 7.8, 1 mM DTT, 0.2 mM NADH (Nacalai Tesque, Kyoto, Japan), and 1 mM oxaloacetic acid (Sigma-Aldrich, St. Louis, MO, USA). The rate of conversion from NADH to NAD^+ was measured at 340 nm with a U-1800 spectrophotometer (Hitachi, Tokyo, Japan).

Alpha-glucosidase from *Saccharomyces cerevisiae* (Sigma-Aldrich) was denatured at 46 °C for 1 h in the refolding buffer containing 0.375 μM GroEL (oligomer). To initiate the α-glucosidase refolding, (His_6)-tagged GroES and nucleotide were added to final concentrations of 0.75 μM (oligomer) and 2 mM, respectively. At the indicated times, 20 μl aliquot was added into 410 μl of a solution containing 50 mM sodium phosphate buffer (pH 6.8) and 2 mM ρ-nitrophenyl-α-D-glucopyranoside (Sigma-Aldrich), and then incubated at 37 °C for 5 min. To terminate the α-glucosidase reaction, 430 μl of 0.2 M Na_2CO_3 was added, and the absorbance at 405 nm, resulting from the release of ρ-nitrophenol, was measured.

ATPase assay

GroELs (0.05 μM oligomer) was incubated in 200 μl of solution (50 mM Tris–HCl pH 7.4, 100 mM KCl, 10 mM $MgCl_2$) at 20 °C for 10 min. When needed, twofold molar of (His_6)-tagged GroES (oligomer) was added to the reaction solution. The reaction was initiated by the addition of 1 mM nucleotide, and then stopped by the addition of 0.3 M perchloric acid. Quantification of liberated inorganic phosphate was performed by malachite green assay (Lanzetta et al. 1979; Kodama et al. 1986). Standards were prepared using KH_2PO_4 solution as inorganic phosphate.

*Cp*GroEL digestion with proteinase K

*Cp*GroEL (0.3 mg ml^{-1}) was incubated with (His_6)-tagged *Cp*GroES (0.1 mg ml^{-1}) and 2 mM nucleotide in the refolding buffer at 20 °C for 15 min, and then treated for various times at 20 °C with 7.5 mg ml^{-1} proteinase K. The proteolysis was stopped by the addition of 1 mM phenylmethanesulfonyl fluoride, and the residual amount of *Cp*GroEL was analyzed by 12.5 % SDS-PAGE. Protein bands on the CBBR-stained gel were quantified by ImageJ software (Abràmoff et al. 2004).

Pull-down assay

*Cp*GroEL (1.5 μM oligomer) was incubated with (His_6)-tagged *Cp*GroES (3 μM oligomer) and 4 mM nucleotides in the refolding buffer at 20 °C for 30 min. The samples were then loaded onto a 1 ml HiTrap chelating column (GE Healthcare) that had been equilibrated with the refolding buffer. The column was washed with 5 ml of the refolding buffer containing 20 mM imidazole, and then incubated at 20 °C for 15 min. After washing with 8 ml of the same buffer again, the proteins were eluted with the refolding buffer containing 400 mM imidazole. Aliquots of each fraction were analyzed by 12.5 % SDS-PAGE.

Results

Transcriptional regulation of the *C. psychrerythraea groESL*

To examine the temperature dependence of the *C. psychrerythraea groEL* gene, we first analyzed levels of *groEL* mRNA under heat stress conditions by Northern blot analysis. Total RNAs were extracted from *C. psychrerythraea* cells incubated at various temperatures for 60 min, and hybridized with a 472-bp DIG-labeled probe specific to the *groES*. The DIG-labeled probe was hybridized to a 2.1-kb transcript (Fig. 1a). Judging from the size, this transcript could be assigned as a composite of the *groES* (288 bp) and *groEL* (1647 bp). Although a low level of *groESL* transcript was found in cells kept at growth temperature (8 °C), obvious increase of the transcript was observed when the cells were treated at 14–18 °C (Fig. 1a). The increase of *groESL* mRNA reached a maximum of about 6-fold around 30 min after treatment at 18 °C (Fig. 1b).

To identify the promoter region of *groESL*, the initiation site of its transcription was determined by primer extension analysis with 5′ DIG-labeled primer complementary to the 5′ end of the *groES*, and total RNAs from cells under heat shock (18 °C) and normal growth (8 °C) conditions. We

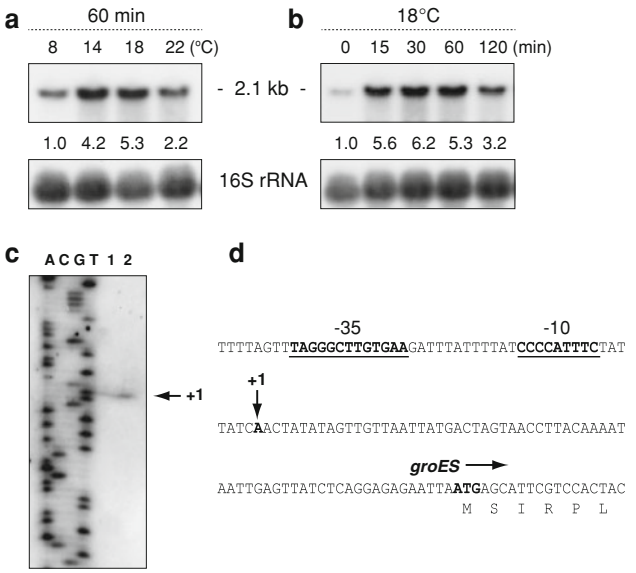

Fig. 1 Transcriptional analysis of the *C. psychrerythraea groESL*. **a**, **b** Northern blotting of the *C. psychrerythraea groESL*. Thirteen micrograms of total RNA per lane was analyzed. **a** Total RNA was extracted from cells exposed to various temperatures for 60 min. **b** Total RNA was extracted from cells that had been exposed to 18 °C for various periods of time. The transcript size is marked between the panels. Expression of *groESL* mRNA was normalized to that of 16SrRNA of *C. maris* (*lower panels*). **c** Primer extension analysis of the *C. psychrerythraea groESL*. The product of the primer extension reaction is indicated by an *arrow* (+1). *Lane 1* cells grown at 8 °C, *lane 2* cells exposed to 18 °C for 60 min. Sequence of the upstream region of the *C. psychrerythraea groESL* is shown on the *right-hand side*. The *vertical arrow* indicates the transcriptional start site. Potential −10 and −35 sequences of the promoter are *underlined*

found one extension product, the level of which was increased after a temperature upshift from 8 to 18 °C (Fig. 1c). The 5′ end nucleotide was A, which was located at 67 nucleotides upstream of the initiation codon (Fig. 1d). The nucleotide sequences of the −35 and −10 regions of the putative promoter for the *groESL* were TAGGGCT TGTGAA and CCCCATTTC, respectively, which are similar to the consensus sequence of the σ^{32}-specific promoter in *E. coli* (Fig. 1d).

Oligomeric state of *C. psychrerythraea* GroEL

*Cp*GroEL and *Cp*GroES consisted of 548 and 96 amino acid residues, and their molecular masses were 57418 and 10301 Da, respectively. The deduced amino acid sequences of *Cp*GroEL and *Cp*GroES were highly homologous to those of other bacteria. The overall identities with GroEL of psychrophilic bacteria, such as *C. maris* (Yamauchi et al. 2003), *Oleispira antarctica* (Ferrer et al. 2004), and *Pseudoalteromonas haloplanktis* (Tosco et al. 2003), a mesophilic bacterium, such as *E. coli* (accession no. X07850), a thermophilic bacterium, such as *Thermus thermophilus* (Amada et al. 1995), were 94, 74, 80, 82, and 60 %, respectively. The overall identities with GroES were 94, 57, 71, 87, and 52 % in the same order.

Most bacterial GroELs form a double ring-shaped tetradecameric structure, which is considered to be essential for their protein folding function (Xu et al. 1997). We first analyzed the oligomeric state of *Cp*GroEL by gel filtration chromatography and native PAGE. The proteins used for these experiments were purified from overexpressed *E. coli* by a column chromatography, and purity of the proteins was greater than 95 % as judged by SDS-PAGE analysis (Fig. 2a).

The purified recombinant *Cp*GroEL was eluted at a position corresponding to *Ec*GroEL by gel filtration chromatography, and its elution pattern did not change in the presence of ATP (Fig. 2b). However, in native-PAGE analysis, *Cp*GroEL was mainly detected at a position corresponding to a molecular mass of 400 kDa, which corresponds to the size in the heptameric state (Fig. 2c). Thus, ring-to-ring interaction within the double ring of *Cp*GroEL appears to be disrupted under electrophoretic conditions. Taken together, these results indicate that *Cp*GroEL forms the double-ring tetradecamer, although its oligomeric state is less stable than that of *Ec*GroEL.

Refolding of chemically denatured MDH by the *Cp*GroEL system

To discriminate chaperone function of *Cp*GroEL from that of *Ec*GroEL, we performed refolding assays in vitro. We first used malate dehydrogenase (MDH) from pig heart (molecular mass 35 kDa) as a substrate protein, whose refolding is absolutely dependent on the GroEL system (Miller et al. 1993; Hayer-Hartl 2000). MDH denatured with 6 M guanidine-HCl was diluted in a refolding buffer containing *Cp*GroEL, (His_6)-tagged *Cp*GroES, and nucleotide, and samples were then incubated at 20 °C. Remaining MDH activity was not found after denaturation, and the spontaneous refolding was approximately 30 % after incubation for 120 min. In both *Cp*GroEL and *Ec*GroEL systems, we observed essential recovery of chemically denatured MDH in the presence of both ATP and CTP (Fig. 3a, b), but recovery by the *Cp*GroEL system seemed to be more efficient in the presence of CTP than ATP (Fig. 3a). For example, after incubation for 40 min, the activity of MDH was recovered to 25 % in the presence of ATP and 54 % in the presence of CTP (Fig. 3c). On the other hand, by the *Ec*GroEL system, the activity of MDH was recovered to 56 % in the presence of ATP and 47 % in the presence of CTP (Fig. 3d). In both GroEL systems, no significant refolding of MDH was found with ADP, and without (His_6)-tagged GroES and/or nucleotide (Fig. 3c, d). Regarding GTP and UTP, the same selectivity was observed between *Cp*GroEL/*Cp*GroES and

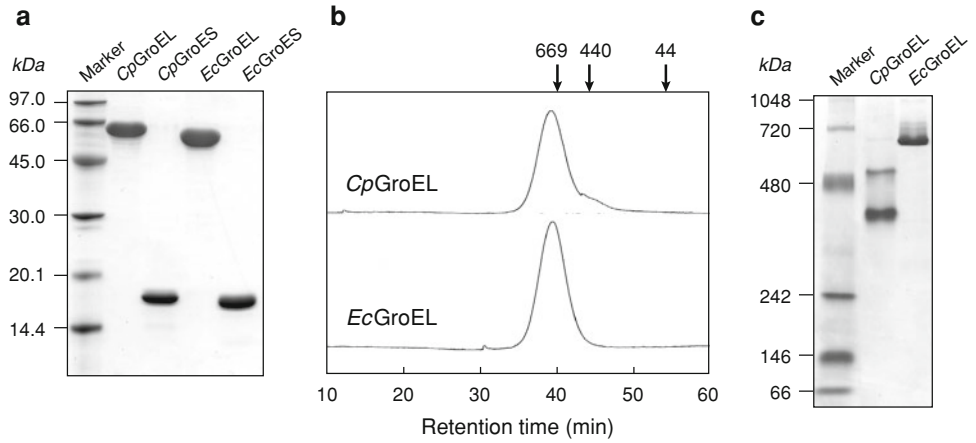

Fig. 2 Purified proteins and oligomeric state of *Cp*GroEL. **a** Purified GroELs and GroESs. Each protein (2 μg) was resolved by 15 % SDS-PAGE and visualized by CBBR staining. **b** A gel filtration chromatograph of *Cp*GroEL and *Ec*GroEL on a Superose 6 10/300 GL column. Elution positions of molecular mass standards are shown at the *top* of the figure. **c** Native-PAGE analysis of the purified GroELs. Purified *Cp*GroEL and *Ec*GroEL (3 μg) were resolved on a 6 % polyacrylamide gel and visualized by CBBR staining

*Ec*GroEL/*Ec*GroES, namely, both GroEL systems could use UTP but not GTP for MDH refolding (Fig. 3c, d). We also analyzed MDH refolding activity of the *Cp*GroEL system in the presence of ATP or CTP at temperatures between 8 and 20 °C. Although the *Cp*GroEL system did not show a significant refolding activity at 8 °C, the recovery rates of MDH activity increased as the incubation temperature is raised. Furthermore, the same ATP and CTP selectivity was found at 12, 16, and 20 °C (Fig. S1). Taken together, these results indicate that the nucleotide selectivity, especially in ATP, is different between *Cp*GroEL/*Cp*GroES and *Ec*GroEL/*Ec*GroES. Thus, we extensively studied the effect of ATP and CTP on refolding function of the *Cp*GroEL system at 20 °C.

Refolding of thermally denatured α-glucosidase by the *Cp*GroEL system

We next performed the refolding assay using α-glucosidase as a substrate protein. The α-glucosidase from *Saccharomyces cerevisiae* (molecular mass 68 kDa) is too large to be encapsulated within the cavity of GroEL, and its refolding is mediated by both GroES-dependent and -independent pathways (Höll-Neugebauer et al. 1991). When we denatured α-glucosidase by incubation at 46 °C for 1 h in the absence of *Cp*GroEL, remaining activity of α-glucosidase was approximately 5 %, and no spontaneous refolding was observed after subsequent incubation at 20 °C for 180 min (Fig. 4a, b). If no nucleotide was added, recovery of the α-glucosidase activity by *Cp*GroEL was quite small (up to 14 %), in both the presence and the absence of (His_6)-tagged *Cp*GroES (Fig. 4a, b). Particularly, in the presence of CTP, the α-glucosidase activity was efficiently recovered by *Cp*GroEL/*Cp*GroES as is the case with MDH. In the presence of ATP or CTP, the activity of α-glucosidase was recovered to 22 or 42 % of the original level, respectively, after incubation for 180 min (Fig. 4a, c). In contrast, without (His_6)-tagged *Cp*GroES, the activity of α-glucosidase was recovered more efficiently with ATP (43 %) than with CTP (36 %) (Fig. 4b, c). These results indicate that the utilization efficiency of ATP in α-glucosidase refolding by the *Cp*GroEL system is different between the *Cp*GroES-dependent and -independent pathways. As shown in Fig. 4d, both ATP and CTP permitted α-glucosidase refolding in *Ec*GroEL/*Ec*GroES, whereas only ATP permitted it in the absence of (His_6)-tagged *Ec*GroES. Taking these findings together, we reconfirmed the difference of nucleotide selectivity between *Cp*GroEL and *Ec*GroEL systems.

In this study, we used (His_6)-tagged GroESs in all investigations. We also performed the refolding assays with no tagged *Cp*GroES purified using a Butyl-Toyopearl column, and the same nucleotide selectivity as for (His_6)-tagged *Cp*GroES was observed. This result indicates that (His_6)-tag of *Cp*GroES does not affect the nucleotide selectivity of the *Cp*GroEL system.

ATPase and CTPase activities of *Cp*GroEL

We demonstrated that ATP is less effective for refolding function of the *Cp*GroEL system (Figs. 3, 4). Since nucleotide hydrolysis plays an important role in the turnover of GroEL-assisted folding reaction, we examined the ATPase and CTPase activities of *Cp*GroEL. For temperature profile, the ATPase activity of *Cp*GroEL was higher than the CTPase activity at all temperatures ranged from 8 to 60 °C. The maximum ATPase and CTPase activities

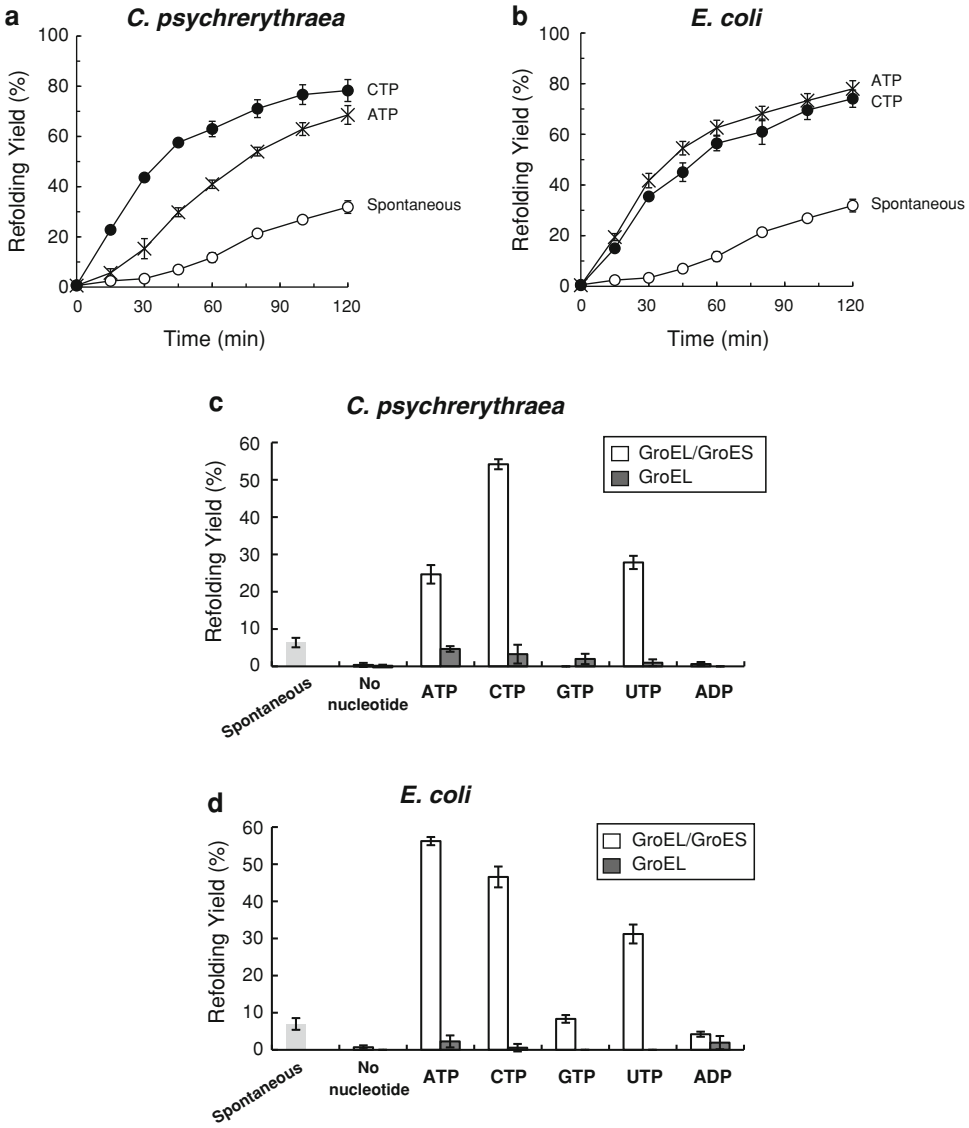

Fig. 3 Refolding assay of MDH with the *Cp*GroEL system. Time course of MDH refolding with the *Cp*GroEL system (**a**) and *Ec*GroEL system (**b**). Chemically denatured MDH (15 μM) was diluted into a refolding buffer containing 0.15 μM GroEL (oligomer), 0.3 μM GroES (oligomer) in the presence of 2 mM ATP (*cross*), and CTP (*closed circle*). *Open circle* shows the spontaneous refolding of MDH. Refolding activities are indicated as a percentage of the enzymatic activity of the same amount of native MDH. Effects of nucleotides on the MDH refolding by the *Cp*GroEL system (**b**) and *Ec*GroEL system (**c**). Refolding of the denatured MDH was performed in the refolding buffer containing GroEL and the indicated nucleotide in the presence (*white bar*) or absence (*black bar*) of GroES at 20 °C for 40 min. Refolding activities are indicated as a percentage of the enzymatic activity of the same amount of native MDH

were obtained at 45 and 35 °C, respectively, which are much higher than the growth temperatures of *C. psychrerythraea* (Fig. S2).

Next, we compared the ATPase and CTPase activities of *Cp*GroEL at 20 °C with those of *Ec*GroEL in the presence or absence of (His_6)-tagged GroES. As shown in Fig. 5a, the ATPase and CTPase activities of *Cp*GroEL were lower as a whole than those of *Ec*GroEL. However, in both cases, the CTPase activity was about 60 % of the ATPase activity. Both ATPase and CTPase of *Ec*GroEL were inhibited approximately 60 % by (His_6)-tagged *Ec*GroES, consistent with previous reports (Fig. 5b) (Todd et al. 1993; Tang et al. 2008; Machida et al. 2009). However, in the case of the GroEL system of *C. psychrerythraea*, the ATPase activity of *Cp*GroEL was inhibited approximately 80 %, and the CTPase activity was inhibited 60 % by (His_6)-tagged *Cp*GroES (Fig. 5b). These results demonstrate that the inhibitory effects of GroES to ATPase and CTPase activities are different between the *Cp*GroEL and *Ec*GroEL system.

Effects of nucleotides on *Cp*GroEL–*Cp*GroES interaction

To understand nucleotide specificity of the *Cp*GroEL system, we compared the effects of nucleotides on *Cp*GroEL–*Cp*GroES interaction. In the absence of nucleotide, *Cp*GroEL was digested by proteinase K (Fig. 6a, e), differing from *Ec*GroEL, which is known to be resistant to proteinase K digestion. When *Cp*GroEL was incubated with (His_6)-tagged *Cp*GroES and ADP, *Cp*GroEL exhibited resistance to digestion with proteinase K (Fig. 6b, e). This result is attributed to the interaction of *Cp*GroEL with (His_6)-tagged *Cp*GroES mediated by ADP binding

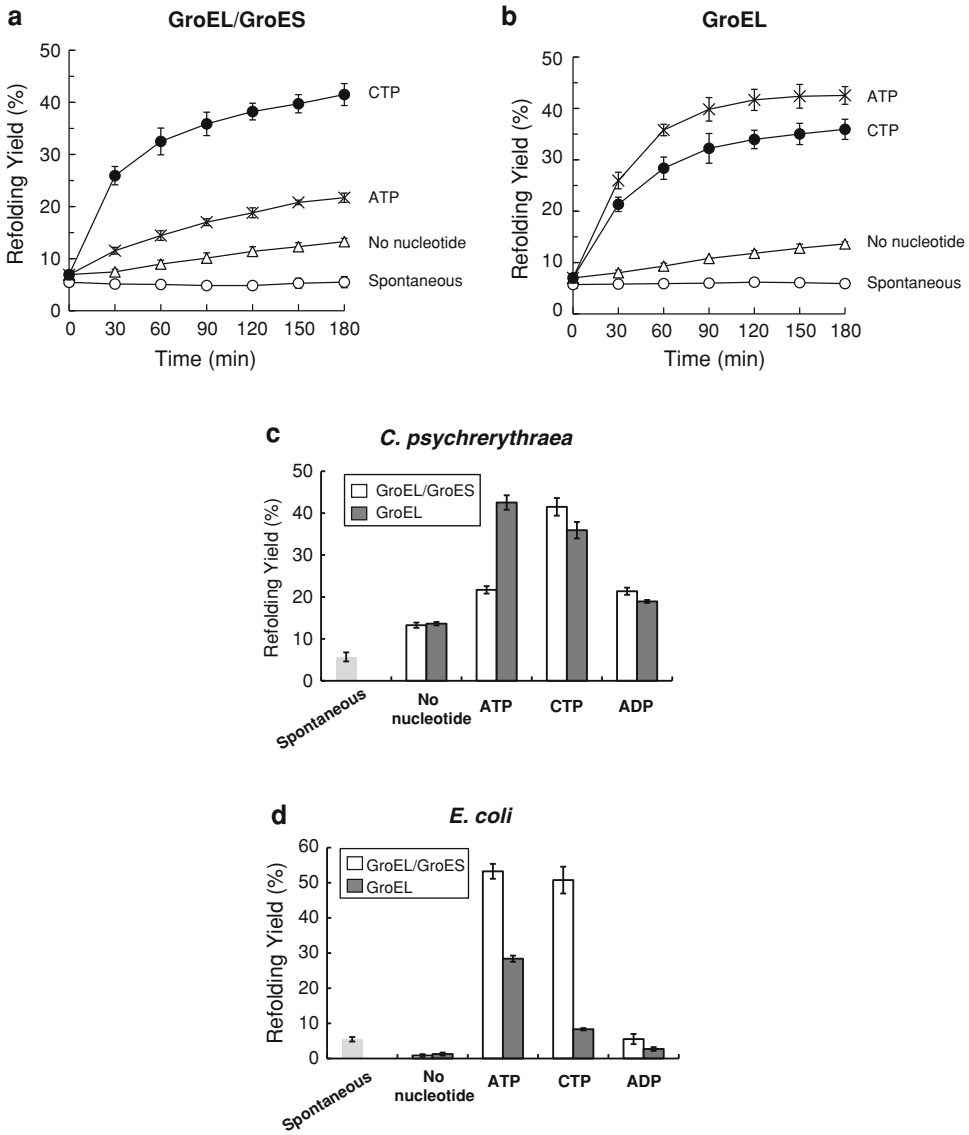

Fig. 4 Refolding assay of α-glucosidase by the *Cp*GroEL system. **a**, **b** Time course of α-glucosidase refolding with the *Cp*GroEL system. Alpha-glucosidase (0.075 μM) was thermally denatured in a refolding buffer containing 0.375 μM *Cp*GroEL (oligomer) at 46 °C for 1 h. Refolding reactions were initiated by the addition of 2 mM ATP (*cross*) and CTP (*closed circle*) in the presence (**a**) or absence (**b**) of 0.75 μM *Cp*GroES (oligomer). Refolding activities in a refolding buffer containing only *Cp*GroEL (*open triangle*) and no chaperones (*open circle*) are also shown. Refolding activities are indicated as a percentage of the enzymatic activity of the same amount of native α-glucosidase. Effects of nucleotides on α-glucosidase refolding by the *Cp*GroEL system (**c**) and *Ec*GroEL system (**d**). Refolding of the denatured α-glucosidase was performed in a refolding buffer containing GroEL and the indicated nucleotide in the presence (*white bar*) or absence (*black bar*) of GroES at 20 °C for 180 min (**c**) or for 60 min (**d**). Refolding activities are indicated as a percentage of the enzymatic activity of the same amount of native α-glucosidase

followed by structural change of *Cp*GroEL. When incubated with (His_6)-tagged *Cp*GroES and CTP, *Cp*GroEL was rapidly digested with proteinase K similar to the case of no nucleotide (Fig. 6d, e). In contrast, *Cp*GroEL exhibited resistance to digestion in the presence of (His_6)-tagged *Cp*GroES and ATP (Fig. 6c, e). These results suggest that *Cp*GroEL–*Cp*GroES interaction mediated by ATP binding is stronger than that mediated by CTP binding.

To investigate *Cp*GroEL–*Cp*GroES interaction directly, we performed in vitro pull-down assay. *Cp*GroES with (His_6)-tag was incubated with *Cp*GroEL and nucleotides, followed by trapping using a Ni-affinity column. The trapped proteins were eluted with a buffer containing 400 mM imidazole and analyzed by SDS-PAGE. There was almost no interaction between *Cp*GroEL and (His_6)-tagged *Cp*GroES in the absence of nucleotide (Fig. 7a). However, significant levels of *Cp*GroEL were co-eluted with (His_6)-tagged *Cp*GroES in the presence of ADP (Fig. 7b), confirming that nucleotide is essential for interaction of *Cp*GroES with *Cp*GroEL, as reported previously for *Ec*GroEL (Roseman et al. 1996). When *Cp*GroEL was incubated with (His_6)-tagged *Cp*GroES and CTP, weak interaction of *Cp*GroEL with (His_6)-tagged *Cp*GroES was found (Fig. 7d). In contrast, in the presence of ATP, *Cp*GroEL co-eluted with (His_6)-tagged *Cp*GroES was markedly increased, similarly to the case of ADP (Fig. 7c). These results clearly demonstrated that *Cp*GroEL–*Cp*GroES interaction mediated by ATP binding is stronger than that mediated by CTP binding.

Discussion

GroEL assists in the proper folding of newly translated and denatured proteins in cells, and thus plays important roles

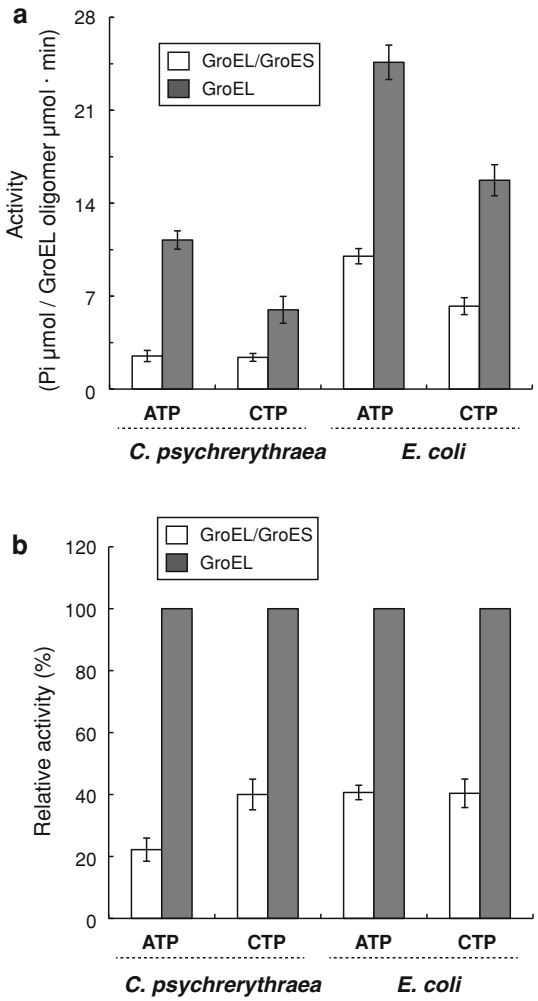

Fig. 5 ATPase and CTPase activities of *Cp*GroEL. **a** Reactions were initiated in a solution containing GroEL (0.05 μM oligomer) by the addition of 1 mM nucleotides with (*white bar*) or without (*black bar*) GroES (double molar to GroEL) at 20 °C. The released inorganic phosphate was quantified by malachite green assay. **b** Inhibitory effects of *Cp*GroES on the ATPase and CTPase activities of *Cp*GroEL. ATPase and CTPase activities of GroEL in the presence of GroES were expressed as a percentage relative to the activities in the absence of GroES

under non-stress conditions as well as stress conditions (Fayet et al. 1989). *C. psychrerythraea* 34H is a psychrophilic bacterium that grows optimally at 8 °C, and it has been reported that this bacterium produces cold-adapted enzymes (Huston et al. 2004, 2008). Even though they function optimally at a low temperature and exhibit heat instability, they should retain their correct folding under a certain range of temperature fluctuation. Thus, like other bacteria, molecular chaperones should play the important roles for the maintenance of proper folding of cellular proteins in *C. psychrerythraea*.

In this report, we described the transcriptional regulation and biochemical characterization of the GroEL system from *C. psychrerythraea*. The transcription of the *C. psychrerythraea groESL* was induced at 14–18 °C (Fig. 1a). This temperature is much lower than that for the transcription of the *groESL* of mesophilic bacteria. Several reports have described the HSP expression in psychrophilic bacteria due to heat stress. In *C. maris* that grows optimally at 10–15 °C, *groESL* and *dnaK* were induced at 20 °C (Yamauchi et al. 2003, 2004). Furthermore, the *groESL* of *P. haloplanktis* TAC125, whose optimum temperature is 15 °C, was induced at 27 °C (Tosco et al. 2003). These results suggest that psychrophilic bacteria, like other bacteria, respond to heat stress and produce HSPs.

The primer extension analysis showed that the *C. psychrerythraea groESL* had a σ^{32}-type promoter, called heat shock promoter (Fig. 1c, d). This result indicates that the *C. psychrerythraea groESL* is regulated by the σ^{32} system, like *groESL* in several mesophilic bacteria. Our previous study revealed that, in *C. maris*, the specific features of *rpoH* encoding σ^{32}, such as the stability and secondary structure of *rpoH* mRNA, were optimized to ensure the HSP expression at a relatively low temperature (Yamauchi et al. 2006). Further study is needed to reveal whether these features are also essential for the *C. psychrerythraea rpoH*.

With respect to the functional characteristics, in contrast to the *Ec*GroEL system, a marked difference in nucleotide selectivity was observed in the *Cp*GroEL system. It has been reported that several GroELs can utilize ATP as well as other nucleotides for efficient chaperone function. For example, the *Ec*GroEL system exhibits effective refolding activity with ATP, CTP, and UTP but not GTP. In addition, *Ec*GroEL utilizes ATP more effectively than CTP and UTP (Fig. 3d) (Kubo et al. 1993). On the other hand, archaeal type II chaperonins from *Thermococcus* strain KS-1 and *Thermoplasma acidophilum* can utilize ATP, CTP, UTP, and GTP for effective chaperone function (Yoshida et al. 2002; Hirai et al. 2008). Broader nucleotide selectivity has also been reported GroEL1 from *Chlamydophila pneumoniae* (Okuda et al. 2008). These results suggest that GroEL has individual selectivity for nucleotides depending on the conditions and bacterial species, among other factors. In the case of *Cp*GroEL, ATP did not enhance the recovery rates of MDH and α-glucosidase efficiently in the presence of *Cp*GroES (Figs. 3, 4). However, in the absence of *Cp*GroES, *Cp*GroEL could use ATP for α-glucosidase refolding (Fig. 4b, c). These results indicate that ATP is not the optimum energy source of *Cp*GroEL at least in the *Cp*GroES-dependent process.

We assumed three possibilities concerning the reason why ATP is not optimum for the *Cp*GroEL system: (1) lower affinity of *Cp*GroEL for ATP; (2) lower activity of the ATPase of *Cp*GroEL; and (3) stronger interaction of *Cp*GroEL–*Cp*GroES mediated by ATP binding. Regarding

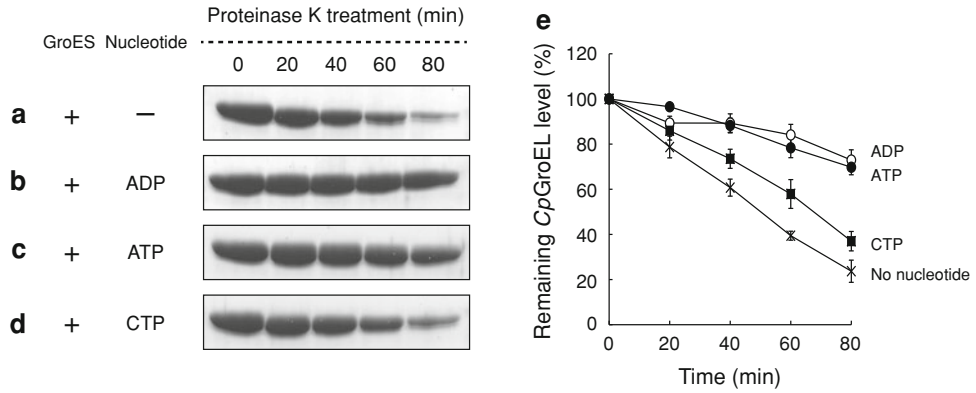

Fig. 6 *Cp*GroEL digestion by proteinase K. *Cp*GroEL was incubated in the buffer with *Cp*GroES without nucleotide (**a**), and with the indicated nucleotide, ADP (**b**), ATP (**c**), and CTP (**d**), at 20 °C for 15 min, followed by treatment with 7.5 μg/ml proteinase K. The samples were withdrawn every 20 min, and residual amount of *Cp*GroEL was analyzed by 12.5 % SDS-PAGE. **e** Relative amount of *Cp*GroEL level. The level of *Cp*GroEL was shown as a percentage relative to the amount before addition of proteinase K. *Open circle* ADP, *closed circle* ATP, *closed square* CTP, *cross* no nucleotide

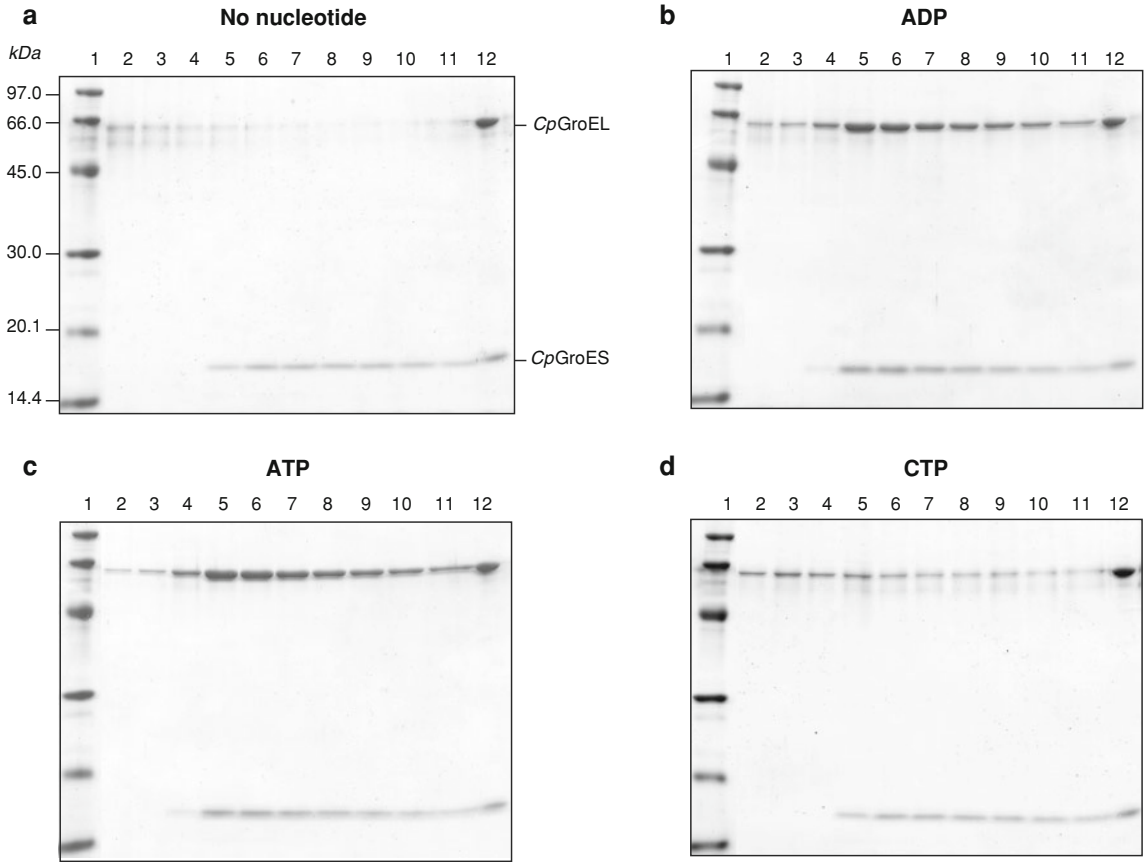

Fig. 7 Effects of nucleotides on *Cp*GroEL–*Cp*GroES interaction. *Cp*GroEL was incubated with *Cp*GroES and the indicated nucleotide at 20 °C for 30 min, followed by loading onto a Hitrap chelating column. Eluted proteins were analyzed by 12.5 % SDS-PAGE. Co-eluted *Cp*GroEL in the absence of nucleotide (**a**), or in the presence of ADP (**b**), ATP (**c**), and CTP (**d**), is shown. *Lane 1* protein marker, *lane 2–11* eluted proteins, *lane 12* an aliquot of the samples before loading onto a Hitrap chelating column

the first and second possibilities, we found that the ATPase activity of *Cp*GroEL was higher than the CTPase activity, similar to the *Ec*GroEL (Fig. 5a). In addition, the amino acid residues concerning nucleotide binding and hydrolysis were perfectly conserved between *Cp*GroEL and *Ec*GroEL (Brocchieri and Karlin 2000). These results suggest that the molecular mechanism of nucleotide hydrolysis of *Cp*GroEL is almost the same as that of *Ec*GroEL.

Recently, Ferrer et al. (2004) described the characterization of GroEL from a psychrophilic bacterium, *O. antarctica* (*Oa*GroEL). *Oa*GroEL existed as a single ring at low temperatures, and its ATPase activity was strongly inhibited by interacting with *Oa*GroES. From this result, they proposed the ATP-saving mechanism of *Oa*GroEL to overcome severe growth conditions, such as low temperatures and lack of nutrients. Thus, we focused our analysis on the third possibility, namely, differences of the strength of *Cp*GroEL–*Cp*GroES interaction mediated by nucleotide binding.

Usability of ATP and CTP could be evaluated by analysis of the GroES–GroEL interaction in the presence of ATP and CTP. Previous studies showed that *Ec*GroES inhibits the ATPase activity of *Ec*GroEL by approximately 50 % (Todd et al. 1993), and we also obtained the same result in this study. This is due to the binding of *Ec*GroES to one ring of *Ec*GroEL. On the other hand, *Cp*GroES markedly inhibited the ATPase activity of *Cp*GroEL by approximately 80 % (Fig. 5b). Similar results have been reported in a single-ring mutant of *E. coli* GroEL, named SR1, whose ATPase activity was inhibited by up to 90 % in the presence of GroES (Weissman et al. 1995). Since SR1 lacks any ring-to-ring interaction, GroES and substrate proteins cannot be dissociated from the SR1 ring, resulting in functional impairment (Weissman et al. 1995, 1996). By means of gel filtration chromatography, we revealed that *Cp*GroEL exists as the double-ring tetradecameric state irrespective of the presence or absence of ATP (Fig. 2b). However, our results of ATPase activity indicate that *Cp*GroES tightly binds and is difficult to dissociate from *Cp*GroEL in the presence of ATP. In contrast, the inhibitory effect of *Cp*GroES on the CTPase activity was weaker than that on the ATPase activity, suggesting that *Cp*GroES can dissociate from *Cp*GroEL in the presence of CTP.

In this study, we assessed *Cp*GroEL–*Cp*GroES interaction in the presence of nucleotides by two approaches. First, by taking advantage of the fact that *Cp*GroEL is sensitive to proteinase K, we examined digestion of *Cp*GroEL in the presence of *Cp*GroES and nucleotides. Although the addition of ATP did not allow proteinase K to digest *Cp*GroEL, the addition of CTP easily caused the digestion of *Cp*GroEL (Fig. 6). This result suggests that the interaction of *Cp*GroEL–*Cp*GroES mediated by CTP binding is weaker than that mediated by ATP binding. Second, the results of in vitro pull-down assay demonstrated smaller contents of *Cp*GroEL in the coeluted fractions in the presence of CTP (Fig. 7), suggesting that the interaction of *Cp*GroEL–*Cp*GroES mediated by CTP binding is weaker than that mediated by ATP binding. Taking all these findings together, we concluded that CTP could weaken the interaction of *Cp*GroEL–*Cp*GroES, resulting in effective refolding function of the *Cp*GroEL system.

In summary, we demonstrated the difference in nucleotide selectivity of the *Cp*GroEL system, and that ATP was not the optimum energy source for its refolding function. Our findings raise the question of why the *Cp*GroEL system cannot use ATP efficiently. We consider that the *Cp*GroEL system possesses the mechanisms for avoiding excess consumption of ATP similar to the *Oa*GroEL system (Ferrer et al. 2004), and saved ATP is supplied to other crucial processes of the cell. Further characterization will be needed to answer the question of whether the energy-saving mechanism is a common feature of molecular chaperones in psychrophilic bacteria. In addition, identification of the *Cp*GroEL regions involved in the nucleotide selectivity will provide important information to understand molecular mechanisms of the folding process in the *Cp*GroEL system.

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