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Isolation and Characterization of a Second Subunit of Molecular Chaperonin from *Pyrococcus kodakaraensis* KOD1: Analysis of an ATPase-Deficient Mutant Enzyme

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The *cpkA* gene encoding a second (α) subunit of archaeal chaperonin from *Pyrococcus kodakaraensis* KOD1 was cloned, sequenced, and expressed in *Escherichia coli*. Recombinant CpkA was studied for chaperonin functions in comparison with CpkB (β subunit). The effect on decreasing the insoluble form of proteins was examined by coexpressing CpkA or CpkB with CobQ (cobyric acid synthase from *P. kodakaraensis*) in *E. coli*. The results indicate that both CpkA and CpkB effectively decrease the amount of the insoluble form of CobQ. Both CpkA and CpkB possessed the same ATPase activity as other bacterial and eukaryal chaperonins. The ATPase-deficient mutant proteins CpkA-D95K and CpkB-D95K were constructed by changing conserved Asp^{95} to Lys. Effect of the mutation on the ATPase activity and CobQ solubilization was examined. Neither mutant exhibited ATPase activity in vitro. Nevertheless, they decreased the amount of the insoluble form of CobQ by coexpression as did wild-type CpkA and CpkB. These results implied that both CpkA and CpkB could assist protein folding for nascent protein in *E. coli* without requiring energy from ATP hydrolysis.

Chaperonins have been classified into two distinct groups, I and II (12). Group I chaperonins (the GroEL family) were found in bacteria, chloroplasts, and mitochondria of eukaryotic cells. They are composed of two kinds of subunits, with molecular masses of about 60 and 10 kDa, and form the sevenfold rotational symmetric double-ring structures. Members of group II chaperonins (TCP-1 [t-complex polypeptide-1]; the thermosome family) occur in the cytosol of eukaryotes and archaea. They also form toroidal structures with variations in the numbers of subunits. The eukaryotic cytosolic chaperonin complex (CCT [chaperonin-containing TCP-1]) consists of up to eight or nine kinds of TCP-1 units (15). It has been shown that CCT is involved in the folding of actin, tubulin, and firefly luciferase in an ATPase-dependent manner in vitro (4, 6, 30) and that newly synthesized actin and tubulin monomers are bound by CCT in vivo (25). Archaeal chaperonins, the thermophilic factor 55 (TF55) of *Sulfolobus shibatae* (27), thermosome of *Pyrodictium occultum* (20), and *Thermococcus* strain KS-1 (33), were found to be members of a related family of high-molecular-mass ATPase complexes. They are able to bind several denatured polypeptides in vitro (8, 27, 28) in an ATPase-dependent manner and are ubiquitous in the archaea. Most archaeal chaperonins, except those of methanogens, consist of two kinds of subunits with diverse stoichiometry and rotational symmetry (10). The subunit stoichiometry of the ninefold symmetric chaperonin complex from *Sulfolobus solfataricus* is reported to be 2:1 (14). Chaperonins from *P. occultum* and *Thermoplasma acidophilum* appear to contain two subunits in 1:1 stoichiometry which form two stacked rings with eightfold symmetry. On the other hand, the structures of chaperonins from methanogens seem to be different from those of other archaeal chaperonins. The chaperonin from *Methanopyrus kandleri* was found to be a homo-oligomer with eightfold symmetry (1). In the case of *Methanococcus jannaschii*, only one gene was found in the genome (2).

Although the functional consequences of ATP binding and hydrolysis for folding of polypeptide substrate seem to have been conserved between the catalytic cycles of group I and group II chaperonins, the effects of nucleotides on the overall structure of the two chaperonin groups apparently differ. ATP binding drives group II chaperonins from the open, substrate binding conformation into the closed conformation where substrate folds in the central cavity (13). ATP hydrolysis would allow the chaperonin to return to the open conformation with subsequent release of folded substrate. In contrast, GroEL, group I chaperonins, binds its substrates in a compact conformation, and ATP causes its apical domains to move outward to allow binding of GroES and thus closure of the cavity. ATP binding and hydrolysis seem to be essential for chaperonin functions. On the other hand, ATP-independent activity of molecular chaperonin has been reported. Upon incubation with the nonhydrolysis ATP analog AMP-PNP, α-tubulin previously bound to TRiC-CCT undergoes at least partial folding without releasing from the chaperonin (3). In addition, the ATPase activity of chaperonin from *M. kandleri* was not detected (1). Our previous studies also showed that recombinant β subunit of chaperonin-like protein from *Pyrococcus kodakaraensis* KOD1 (CpkB) prevents thermal denaturation and enhances thermostability of yeast (*Saccharomyces cerevisiae*) alcohol dehydrogenase in the absence of ATP, when CpkB is present in excess (31). In the present study, the gene encoding the chaperonin α subunit was cloned from *P. kodakaraensis* KOD1, expressed in *E. coli*, and examined for biochemical properties as a molecular chaperone. ATPase-deficient mutant proteins were constructed, and their characteristics were compared with those of wild-type chaperonins.

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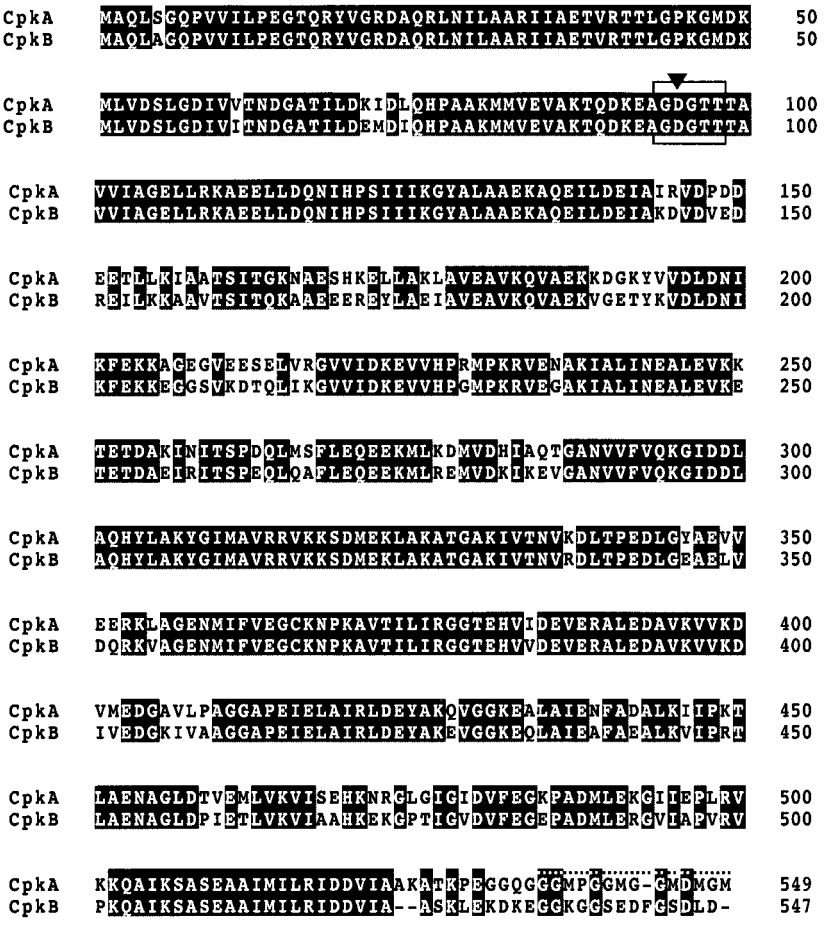

FIG. 1. Comparison of deduced amino acid sequences of CpkA and CpkB chaperonin subunits from *P. kodakaraensis* KOD1. White letters on a black background are amino acids identical between CpkA and CpkB. The G-M motif region is indicated by a dotted line above the sequence. The conserved region for nucleotide binding is boxed. The arrowhead indicates Asp^{95} for site-directed mutagenesis.

Cloning and sequencing of the chaperonin gene. Chaperonins of most microorganisms are known to contain two kinds of subunits. Archaeal chaperonins are likely to form hetero-oligomeric double-ring structures. We previously reported cloning and sequencing analysis of the *cpkB* gene encoding the β subunit of KOD1 chaperonin (31). In order to obtain the α subunit gene from KOD1, PCR using primers based on the conserved regions of group II chaperonins [primer 1, 5′-GGG NGTACCACNAT(T/A/C)ACNAA(T/C)GA(T/C)GGNGC-3′; primer 2, 5′-GGCATNCC(G/A)AA(G/A)AGGAT(A/T/C) GA(G/A)AA(T/C)GC-3′] was performed. Chromosomal DNA (1 μg) and 200 pmol of each primer in 100 μl of reaction buffer were used for PCR. Southern hybridization was performed using the PCR product as a probe. This probe strongly hybridized with two distinct *Hin*dIII fragments whose sizes are 4.2 and 1.8 kb. These fragments were cloned separately into pUC19 and sequenced. Sequence analysis revealed that the 4.2-kb fragment contained the entire *cpkB* gene. The 1.8-kb fragment possessed an open reading frame encoding a protein with 549 amino acid residues, giving a predicted molecular weight of 59,166.4. The obtained gene was designated *cpkA*, which stands for α subunit of chaperonin-like protein from *P. kodakaraensis* KOD1. The constructed plasmid harboring the 1.8-kb fragment was termed pCPA.

The deduced amino acid sequence from the newly identified open reading frame (*cpkA*) showed a high degree of similarity (77.1% identity) to that of CpkB (Fig. 1). The most conspicuous difference between the two chaperonins was found at the carboxyl termini. CpkA has a glycine-methionine (G-M) motif, which is typically found in bacterial GroEL chaperonin (18), while the G-M motif was not found in CpkB or other eukaryal chaperonin TCP-1. This region of *cpkA* might have been transferred from the bacterial counterpart in the course of evolution. Another difference between the two genes was observed in codon usage. When codon usage was examined using 20 cloned genes, some codons were not frequently utilized in KOD1. The typical rare codons for strain KOD1, such as CTT for Leu, AGT for Ser, and GCA for Ala, were found in the 5′ region of the *cpkA* gene (data not shown). This fact implied that the translational efficiency of *cpkA* is lower than that of *cpkB*.

In order to determine whether these genes were located close together in chromosomal DNA of strain KOD1, Southern hybridization was performed. A physical map of the KOD1 chromosome was previously constructed for *Asc*I, *Pme*I, and *Pac*I (5). In order to determine the location of *cpkA*, cloned *Hin*dIII fragment (1,804 bp) was used as a probe for Southern hybridization. Major signals were detected at the *Asc*I-D, *Pme*I-D, and *Pac*I-D fragments, and minor signals were detected at the *Asc*I-E, *Pme*I-A, and *Pac*I-A fragments. When the DNA fragment which carries *cpkB* was used as a probe for Southern hybridization, the *Asc*I-E, *Pme*I-A, and *Pac*I-A frag-

ments were highlighted. As mentioned above, *cpkA* shows high sequence identity to *cpkB*. The weak signals of *Asc*I-E, *Pme*I-A, and *Pac*I-A detected by the *cpkA* probe were considered to be due to high sequence identity between *cpkA* and *cpkB*. The locus of the *cpkA* gene was defined at the overlapped region of *Asc*I-D, *Pme*I-D, and *Pac*I-D. Two genes were located at distinct loci on the physical map.

Expression and purification. The region for *cpkA* was amplified by PCR with the following set of forward and reverse primers to introduce restriction enzyme recognition sites: CPAU (5′-TT*CCATGG*CACAGCTTAGTGGACAGCCG GT-3′) and CPAR′ (5′-AT*GGATCC*TGCTGGAAGGAAAA GAGAAGTG-3′). The forward and reverse primers possess additional *Nco*I and *Bam*HI sites at the 5′-terminal regions, respectively, as shown in italic letters in the sequences. The amplified DNA fragment was inserted into the *Nco*I and *Bam*HI sites of pET-8c. *E. coli* BL21(DE3) cells were transformed by the recombinant plasmid, and overexpression was performed. However, expressed CpkA was not detected in the cell extract. Some codons of the 5′ terminus are occupied with rare codons which are not efficiently utilized in *E. coli*. It has been reported that rare codons located near the 5′ end of the gene are negatively effective for an efficient translation (7, 16). In order to achieve the efficient expression, several rare codons for N-terminal amino acids were changed to codons which are frequently used in *E. coli*. CTT for Leu[4], AGT for Ser[5], and GGA for Gly[6] were replaced by CTG, AGC, and GGC, respectively. Primer CPKU2 (5′-TT*CCATGG*CACAGCTGAGCGG CACAGCCGGT-3′) was used for PCR instead of CPAU2. The constructed plasmid was designated pCPAE. *E. coli* BL21(DE3) cells harboring pCPAE efficiently expressed *cpkA*. The expression plasmid for CpkB carrying *cpkB* gene was constructed as described by Yan et al. (31). The constructed plasmid was designated pECPK. *E. coli* BL21(DE3) cells carrying each plasmid were grown at 37°C in NZCYM medium (1% NZ amine, 0.5% NaCl, 0.5% yeast extract, 0.1% Casamino Acids, 0.2% $MgSO_4 \cdot 7H_2O$ adjusted to pH 7.0 with NaOH) containing ampicillin (50 μg/ml) until the optical density at 660 nm reached 0.4. Chaperonin expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h. After harvesting the cells by centrifugation (6,000 × *g*, 20 min), the pellet was frozen, thawed, and resuspended in buffer A (50 mM Tris-HCl [pH 7.5], 30 mM NaCl, 1 mM dithiothreitol [DTT], 20% [vol/vol] glycerol). The cells were disrupted by sonication in buffer A and then centrifuged at 24,000 × *g* for 1 h at 4°C. CpkA or CpkB was recovered in a soluble fraction, and the crude extract was treated at 80°C for 20 min followed by centrifugation (24,000 × *g*, 1 h). Most proteins derived from the host *E. coli* cell were precipitated and removed as an insoluble inclusion complex.

Relationship between ATP hydrolysis and chaperonin function. ATP hydrolysis is considered important for the release of folded proteins from chaperonin. Based on the comparative sequence analysis, the *cpkA* and *cpkB* genes possess the conserved region which is related to putative nucleotide binding (the GDGTT motif at amino acids 94 to 98; Fig. 1). Recombinant CpkA and CpkB were examined for ATPase activity. An ATPase assay was performed by monitoring ADP formation on polyethylenimine-cellulose thin-layer sheets in accordance with a previously reported procedure (22, 23). As expected, both CpkA and CpkB exhibit ATPase activity at 80°C (Fig. 2). These ATPase activities were maintained even at 90°C (data not shown).

In order to confirm the possible chaperonin function of CpkA, the effect of CpkA on decreasing insoluble foreign protein was examined. When some foreign proteins are overexpressed in *E. coli*, insoluble inclusion bodies are often formed. It has been observed that molecular chaperone is effective for decreasing insoluble proteins when it is coexpressed (32). The *cobQ* gene of KOD1, which encodes cobyric acid synthase, forms an insoluble inclusion complex when it is overexpressed in *E. coli* (31). It is reported that CpkB is functional in vivo and is effective to decrease the amount of the insoluble form of CobQ in *E. coli* by coexpression. The *cobQ* gene was previously cloned in pET-8c, and the derivative plasmid was designated pCOB (31). The *Nru*I-*Cla*I fragments of plasmid pCPAE and pECPK were recloned into the respective sites of plasmid pACYC184, which is compatible with pET-8c, and the constructed plasmids were designated pCPAE2 and pCPK, respectively. Both *cobQ* and the chaperonin genes are inducible with IPTG by the use of a T7 promoter system. *E. coli* BL21(DE3) cells were transformed by plasmids pCOB and pCPAE2 or by pCOB and pCPK and were grown in NZCYM medium containing ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml). Overexpression of proteins was induced by the addition of IPTG (1 mM) for 4 h. Cells were then harvested by centrifugation. The pellet was suspended in 1 ml of buffer A. After the cells were disrupted by sonication and centrifuged, the supernatant was rescued as the soluble fraction. The pellet was washed with 1 ml of buffer A and suspended in 1 ml of sample buffer (50 mM Tris-HCl [pH 6.8], 100 mM DTT, 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, 10% glycerol), boiled for 5 min, and centrifuged. The supernatant was recovered in an insoluble fraction. Each fraction (20 μl) was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie brilliant blue R-250 staining. When CpkA was coexpressed with CobQ, a significant amount of insoluble CobQ was kept soluble, indicating that CpkA also functions to decrease the insoluble form (Fig. 3A, lanes 3 and 4). CpkA is thought to trap unfolded forms of polypeptides and to correct them in accordance with properly folded ones in *E. coli*. CpkA and CpkB seem to function to prevent CobQ insolubilization, thus keeping its form soluble.

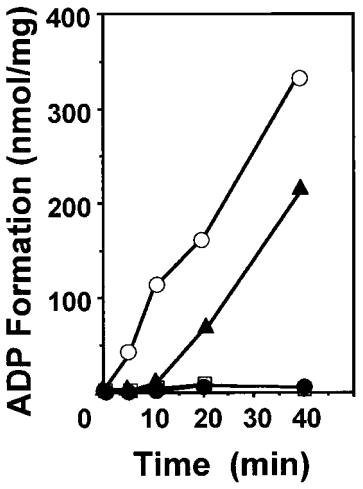

FIG. 2. ATPase activities of the wild-type and mutant chaperonins at 80°C. ATPase activity was determined in reaction mixtures containing 40 mM HEPES [pH 7.2], 75 mM KCl, 4.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 1 mM ATP in a total volume of 20 μl including [α-^{32}P]ATP (400 Ci/mmol). The reaction was started by the addition of the extracts and terminated by rapid cooling to 0°C. ATP hydrolysis was examined by spotting an aliquot (2 μl) on polyethylenimine-cellulose thin-layer sheets (Macherey-Nagel, Duren, Germany). The substrate and products of the reaction were separated by one-dimensional chromatography using 1 M LiCl. The spots were cut out, and the radioactivity was determined by liquid scintillation counting. Symbols: ▲, CpkA; ○, CpkB; ●, CpkA-D95K; □, CpkB-D95K.

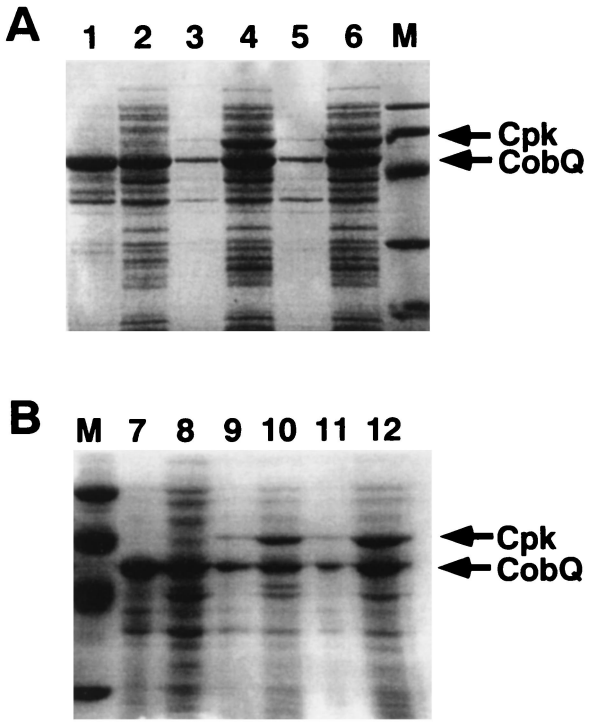

FIG. 3. Increased solubility of CobQ protein by coexpression with wild-type (A) and mutant (B) chaperonins in *E. coli*. Soluble and insoluble forms of CobQ (cobyric acid synthase from *P. kodakaraensis*) were monitored by SDS-PAGE. Lanes: 1, insoluble fraction from *E. coli* (pCOBQ); 2, soluble fraction from *E. coli* (pCOB); 3, insoluble fraction from *E. coli* (pCPAE2/pCOB): 4, soluble fraction from *E. coli* (pCPAE2/pCOB); 5, insoluble fraction from *E. coli* (pCPK/pCOB); 6, soluble fraction from *E. coli* (pCPK/pCOB); 7, insoluble fraction from *E. coli* (pCOB); 8, soluble fraction from *E. coli* (pCOB); 9, insoluble fraction from *E. coli* (pCPAE2-D95K/pCOB); 10, soluble fraction from *E. coli* (pCPAE2-D95K/pCOB); 11, insoluble fraction from *E. coli* (pCPK-D95K/pCOB); 12, soluble fraction from *E. coli* (pCPK-D95K/pCOB); M, molecular mass markers (94 kDa, rabbit muscle phosphorylase; 67 kDa, bovine serum albumin; 43 kDa, egg white ovalbumin; 30 kDa, bovine erythrocyte carbonic anhydrase; 20.1 kDa, soybean trypsin inhibitor).

In the GroEL-GroES chaperonin system, the binding and releasing of target proteins are associated with ATP hydrolysis. Chaperonin TCP-1 and thermophilic factor TF55 from the thermophilic archaeon *S. shibatae* also require ATP for refolding of denatured proteins. Several protein folding cycles have been proposed for bacterial chaperonin (17, 19, 24, 26, 29). Quaite-Randall et al. (21) have suggested the conformational cycle of the archaeosome, which includes a change in conformation and in the oligomerization state. According to their model, the binding or hydrolysis of ATP acts as a switch between two conformational forms of chaperonin, open and closed complexes. They suggested that as previously reported for GroEL (26), the thermodynamic barriers separating protein-bound and -free archaeosome states are overcome by ATP hydrolysis. Our previous studies, however, revealed that CpkB functions as a chaperonin in the absence of ATP when it is present in an excess amount. In order to examine whether ATPase activity is necessary for chaperonin functions, ATPase-deficient mutant proteins CpkA-D95K (in which Asp^{95} was replaced by Lys) and CpkB-D95K (in which Asp^{95} was replaced by Lys) were constructed by site-directed mutagenesis. For CpkA-D95K construction, primers CPKU2 and DAK2′ (5′-AGACTCAGGACAAGGAGGCCGGTAAA GGTACTACC-3′) were annealed with plasmid pCPA, and an intermediate DNA (about 300 bp) was produced by PCR. Primers CPAR′ and DAK1′ (5′-ATGACGACGGCAGTGG TAGTACCTTTACCGGCCTC-3′) were also annealed with pCPA, and another intermediate DNA (1.5 kbp) was produced by PCR. Those intermediates were joined by PCR with the two outer primers CPKU2 and CPAR′. The synthesized DNA was then digested with *Nco*I and *Bam*HI and introduced between the *Nco*I and *Bam*HI sites of pET-8c. The resulting plasmid was named pCPAE-D95K. For CpkB-D95K construction, primers KODHSPF, KODHSPB, DBK1′ (5′-AGACTCAGGACA AGGAGGCCGGTAAAGGAACCACC-3′), and DBK2′ (5′-ATGACAACGGCAGTGGTGGTTCCTTTACCGGCCTC-3′) were replaced with CPKU2, CPAR′, DAK1′, and DAK2′, respectively, using plasmid pECPK (31) as a template for PCR. The construct was designated pECPK-D95K. As shown in Fig. 2, neither mutant did exhibited ATPase activity. In order to confirm their chaperonin function, the coexpression effect on preventing insoluble CobQ formation was examined. The *Nru*I-*Cla*I fragments of plasmid pCPAE-D95K and pECPK-D95K were recloned into the respective sites of plasmid pACYC184, which is compatible with pET-8c, and the constructed plasmids were designated pCPAE2-D95K and pCPK-D95K, respectively. Although CpkA-D95K and CpkB-D95K exhibited no ATPase activity, both mutants are functional for decreasing insoluble CobQ, as shown in Fig. 3B. This result suggested that CpkA and CpkB are functional in the absence of ATP. The chaperonin function of CpkA-D95K or CpkB-D95K might be cooperative action with a host-derivative chaperonin, such as GroEL.

At present, the exact reason why CpkA and CpkB do not require ATPase activity for their chaperonin functions is unclear. ATP-independent action was also observed for molecular chaperonin from *Thermococcus* sp. strain KS-1 (32a). ATP is easily degraded at high temperatures (the half-lives for ATP and ADP at 90°C were 115 and 750 min, respectively [11]). At the extremely high temperatures at which hyperthermophiles grow, available ATP would be scarce. In the present study, the functions of CpkA and CpkB were examined only for their solubilization activities for the recombinant protein expressed in *E. coli*. Further biochemical analysis in vitro is in progress.

Nucleotide sequence accession number. The *cpkA* gene produced in this study has been assigned GenBank /EMBL/DDBJ accession no. AB018432.

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