APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1997, p. 785–789 Vol. 63, No. 2
0099-2240/97/$04.00+0

In Vitro Stabilization and In Vivo Solubilization of Foreign Proteins by the β Subunit of a Chaperonin from the Hyperthermophilic Archaeon *Pyrococcus* sp. Strain KOD1

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The gene encoding the β subunit of a molecular chaperonin from the hyperthermophilic archaeon *Pyrococcus* sp. strain KOD1 (*cpkB*) was cloned, sequenced, and expressed in *Escherichia coli*. The *cpkB* gene is composed of 1,641 nucleotides, encoding a protein (546 amino acids) with a molecular mass of 59,140 Da. The enhancing effect of CpkB on enzyme stability was examined by using *Saccharomyces cerevisiae* alcohol dehydrogenase (ADH). Purified recombinant CpkB prevents thermal denaturation and enhances thermostability of ADH. CpkB requires ATP for its chaperonin function at a low CpkB concentration; however, CpkB functions without ATP when present in excess. In vivo chaperonin function for the solubilization of insoluble proteins was also studied by coexpressing CpkB and CobQ (cobryic acid synthase), indicating that CpkB is useful for solubilizing the insoluble proteins in vivo. These results suggest that the β subunit plays a major role in chaperonin activity and is functional without the α subunit.

Chaperonins are a group of molecular chaperones and are a subset of the heat shock proteins (HSPs), whose members are widely distributed from prokaryotes to eukaryotes and first came to light because of their specific induction during the cellular response of all organisms to heat shock. It is now clear that the majority of these proteins are expressed constitutively and abundantly in the absence of any stress, and genetic studies show that many of them are essential for cell viability under normal growth conditions (3, 4, 7). Many HSP family members, including those that do not respond significantly to heat shock, are induced under a variety of other stress conditions whose common denominator may be the accumulation of unfolded or malfolded proteins in cells (4, 8, 19, 21). They also mediate the correct assembly of polypeptides and the translocation of proteins across membranes but are not themselves components of the final structures (15).

According to structure, molecular mass, and function, HSPs have previously been divided into several families (2, 7, 10, 11, 35): the stress-70 protein family, the stress-90 protein family, and the GroEL/chaperonin family. In hyperthermophilic archaea which can grow above 80°C, chaperonin plays an essential role in hindering protein denaturation (13, 14, 24). New HSPs identified from mammals and archaea have been classified into a new chaperonin group (17, 36). Even though these two proteins' primary structures are different from that of GroEL, they have similar quaternary structures, and they may be involved in de novo protein folding and assembling. The mammalian chaperonin is a t-complex polypeptide-1 (TCP-1), a ubiquitous eukaryotic protein, organized as a multisubunit toroid; it requires ATP for activity and has the ability to catalyze the refolding of denatured proteins (6, 17). The other protein is thermophilic factor 55 (TF55), isolated from the thermophilic archaeon *Sulfolobus shibatae* (30, 31). TF55 is a hetero-oligomeric complex (α and β) having two stacked nine-membered rings, and it binds unfolded polypeptides in vitro and has ATPase activity (14, 23, 24). Some TF55 homologs from various hyperthermophilic archaea have also been reported (16, 22, 23). Recently, it was reported that TF55 exists in two distinct conformational states termed open and closed complexes, both of which are involved in the refolding cycle. The closed complex binds ATP and shifts itself to the open complex. Upon ATP hydrolysis, the open complex dissociates into subunits (24). It is suggested that the binding and hydrolysis of ATP act as a switch between the two conformational forms of the archaeon chaperonin. Both α and β subunits can form a complex without their counterpart. Moreover, both subunits can bind proteins, but the binding activity of the β subunit is 5 to 10 times higher than that of the α subunit (24). These results suggest that the β subunit plays an important role in protein refolding.

Pyrococcus sp. strain KOD1 is a hyperthermophilic archaeon newly isolated from a solfatara at a wharf on Kodakara Island, Kagoshima, Japan (20), and possesses a single circular genome of 2,036 kb (5). Interestingly, some enzymes produced by strain KOD1 show eukaryotic features (13, 20, 25). In order to further investigate archaeon chaperonin, especially the role of the β subunit, cloning of the β-subunit gene from KOD1 was attempted. PCR was performed, using primers designed based on conserved regions of known HSPs and the β subunit, with the nucleotide sequences 5′-GGGNGTACCACNAT(T/A/C)ACNAA(T/C)GA(T/C)GGNGC-3′ and 5′-GGCATNCC(G/A)AA(G/A)AGGAT(A/T/C)GA(G/A)AA(T/C)GC-3′. Amplified DNA was used as a probe for Southern hybridization, and a 4.5-kb *Eco*RI-*Hin*dIII fragment which possesses the entire *cpkB* (chaperonin-like protein β from KOD1) gene was cloned in the pUC18 plasmid. The nucleotide sequence was deter-

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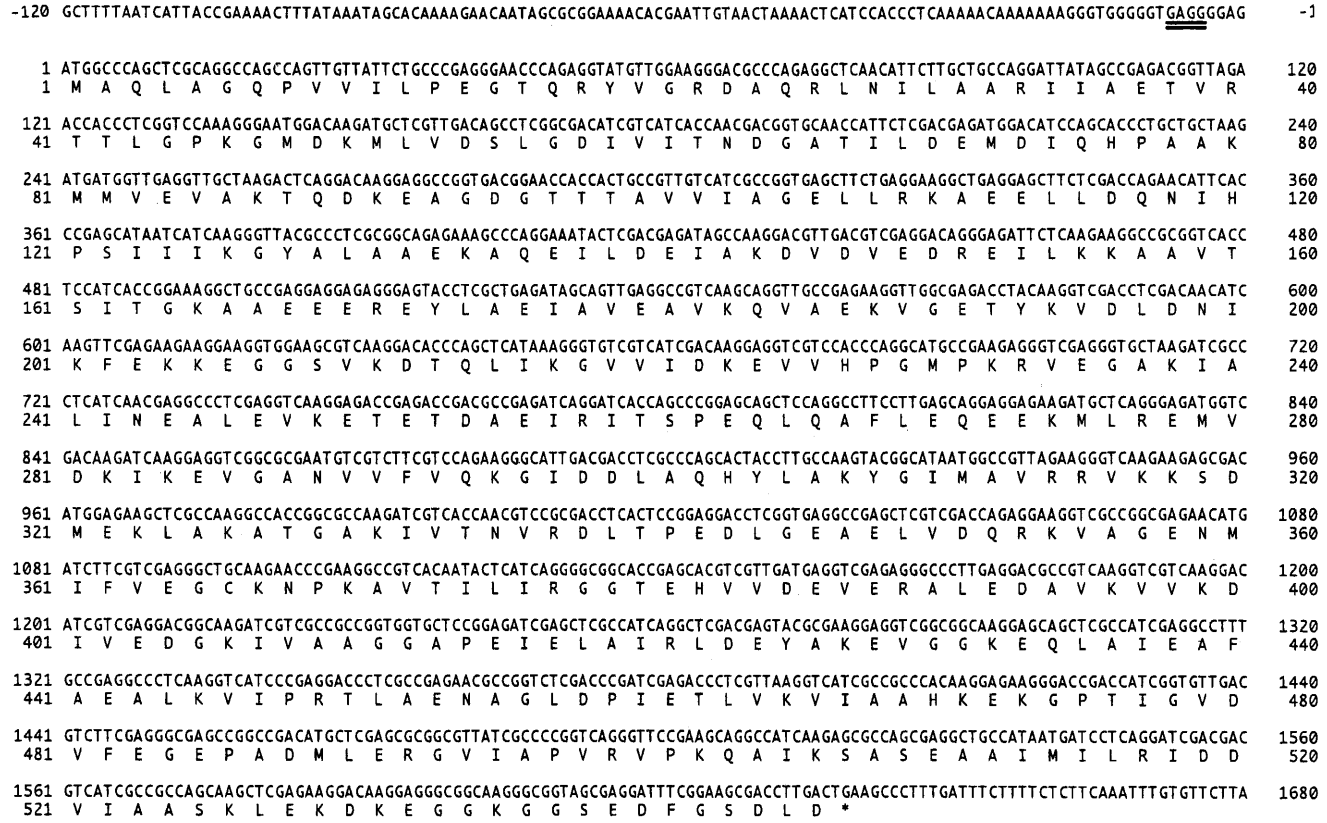

FIG. 1. Nucleotide sequence of the KOD1 *cpkB* gene. The deduced CpkB amino acid sequence is shown below the nucleotide sequence. Nucleotides and amino acids are numbered on the top and bottom, respectively. The stop codon (asterisk) and a putative ribosomal binding site (double underlined) are indicated. DNA sequences were determined in both directions of the strands by the dideoxy chain termination method using a DNA sequencer (ALF Express; Pharmacia). DNA sequence data was analyzed with a DNASIS system (Hitachi Software Co., Yokohama, Japan).

mined by the dideoxy chain termination method using an automatic sequencer (ALF Express; Pharmacia, Uppsala, Sweden) and is shown in Fig. 1. Its primary structure, deduced from the cloned genomic DNA sequence, predicts a polypeptide of 546 amino acids and was highly similar to the TF55 β subunit and mouse TCP-1, with homology between them of 56.3 and 42.8%, respectively. However, it is less homologous to GroEL (20 to 24%) and shows no homology with DnaK in prokaryotes. These results support the idea that archaea are evolutionarily more closely related to eukaryotes than to eubacteria (9, 12).

The KOD1 *cpkB* gene was cloned into the *Nco*I and *Bam*HI sites of the pET-8c expression vector (Novagen, Madison, Wis.). The protein was produced by *Escherichia coli* BL21 (DE3) via induction with isopropyl-β-D-thiogalactopyranoside (IPTG) addition at 37°C and purified by heat treatment and ion-exchange chromatography using a fast protein liquid chromatography system (Pharmacia, Uppsala, Sweden). The purity of the recombinant protein was demonstrated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, which showed a single polypeptide of 60 kDa (Fig. 2), consistent with the calculated molecular mass.

The function of chaperonin in stabilizing foreign proteins has been well studied with bacterial GroES/GroEL systems (8, 28, 34). To confirm the possible chaperonin function of CpkB, we examined its ability to stabilize *Saccharomyces cerevisiae* alcohol dehydrogenase (ADH) under heat stress in vitro. The yeast ADH activity was assayed by monitoring ethanol-dependent NAD reduction at 340 nm (27). Yeast ADH has its highest activity at 30°C, and the activity decreased sharply with increasing temperatures above 50°C. No activity was observed at 70°C. In contrast, when CpkB was incubated with native ADH, the thermal inactivation slowed down (Fig. 3a), indicating that the β subunit of archaeon chaperonin is functional as a molecular chaperonin. CpkB might have bound to the thermally unfolded or partially unfolded ADH, prevented its denaturation, and aided its refolding.

We also reproduced in vitro chaperonin function for maintaining ADH in an enzymatically active state at 50°C. After a 20-min heat treatment, the remaining activity of native ADH was about 11%, but it approached 100% in the presence of CpkB. The enhancement effect was more significant with an increase in the CpkB concentration (Fig. 3B). The result also revealed that ATP could enhance CpkB's thermostability effect at a low concentration of CpkB. In contrast, at higher concentrations, CpkB seems to be able to function without ATP. The experiments were repeated several times, and the patterns of each experiment were reproducible. ATP-independent chaperonin activity of CpkB is not attributable to prebound ATP, because purified CpkB degraded ATP with its ATPase activity, as described below.

In the GroEL/GroES system, the binding and release of proteins are associated with ATP hydrolysis (4, 7, 26). The chaperonins recognize and stabilize partially folded intermediates during polypeptide folding, assembly, and disassembly. ATP hydrolysis is considered to be important for the release of proteins from chaperonin. In the case of archaeon chaperonin, ATP plays an important role in the formation of an active open

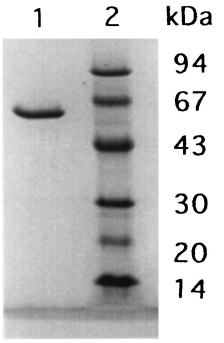

FIG. 2. SDS-polyacrylamide gel electrophoresis of purified recombinant CpkB. Lanes: 1, purified CpkB (3 μg); 2, protein standard markers (molecular masses are shown on the right). The DNA fragment carrying the *cpkB* gene was amplified by PCR using two primers which have *Nco*I and *Bam*HI recognition sequences (5′-AGGGGCCATGGCCCAGCTCGCAGGCCAGC-3′ and 5′-AAAAGGGATCCAAGGTCATCAGTCAAGG-3′). The amplified DNA was cloned between *Nco*I and *Bam*HI sites of plasmid pET-8c (Novagen), and the constructed plasmid was designated pECPK. CpkB was overproduced in *E. coli* BL21(DE3), and purification was carried out. *E. coli* cells harboring pECPK were induced by 0.1 mM IPTG at mid-exponential phase and incubated for 6 h at 37°C. The cells (from a 3-liter culture) were centrifuged, and the pellet was washed with 50 mM phosphate buffer (pH 7.5). The cells were disrupted by sonication, and the supernatant fraction was recovered by centrifugation at 27,000 × *g* for 20 min at 4°C. The supernatant was heat treated at 90°C for 20 min and centrifuged again at 27,000 × *g* for 15 min at 4°C. The supernatant obtained was brought to 75% ammonium sulfate saturation and kept at 4°C overnight. The precipitate was collected by centrifugation at 27,000 × *g* for 30 min, dissolved in 20 ml of 50 mM phosphate buffer (pH 7.5), and dialyzed overnight against the same buffer. The dialysate was applied to an anion-exchange column (HiTrap Q; Pharmacia) and equilibrated in 50 mM phosphate buffer (pH 7.5), and CpKB was eluted with a linear gradient of NaCl by using a fast protein liquid chromatography system (Pharmacia). CpkB was further purified by repeating anion-exchange chromatography with a Resource Q column (Pharmacia).

complex and in the dissociation of the subunits (24). Therefore, purified recombinant CpkB was tested for ATPase activity. An ATPase assay was performed by monitoring ADP formation using [α-^{32}P]ATP (400 Ci/mmol; Amersham) on polyethyleneimine-cellulose thin-layer sheets (Macherey-Nagel, Düren, Germany) based on a previously reported procedure (26, 27). The substrate and products of the reaction were separated by one-dimensional chromatography using 1 M LiCl (29, 32). ATP and ADP spots were cut out, and the radioactivity was determined by liquid scintillation counting. It was shown that CpkB possesses a heat-stable ATPase activity at 95°C (Fig. 4a), which is consistent with the optimal growth temperature of KOD1 (20). The K_m of the ATPase hydrolysis reaction was estimated to be 6.6 μM by Lineweaver-Burk plotting as shown in Fig. 4b. The value (6.6 μM) was approximately the same as those of known HSP molecules which require ATP hydrolysis (1, 18).

At present, the reason CpkB at a high concentration doesn't require ATP for protein refolding is unclear. The recombinant β subunit may make two forms which are dependent on concentration. As a hypothesis, one form, at a low CpkB concentration, might refold the unfolded proteins by using ATP. In contrast, at a high CpkB concentration, the β subunit might possess another form and function as a chaperonin without ATP. Under stress conditions which require a large quantity of chaperonin molecules, the β subunit may possess this second form and refold the unfolded proteins without ATP.

Since CpkB shows in vitro chaperonin activity, we expected protein solubilization activity in vivo. When recombinant proteins are overexpressed in *E. coli*, inclusion bodies are often formed. It has been observed that molecular chaperonin is very effective for solubilizing these proteins when it is coexpressed (37). In order to determine the possible function of CpkB in vivo, the effect of CpkB on foreign protein solubilization was examined. The *cobQ* gene of KOD1, which encodes cobyric acid synthase, makes an insoluble inclusion complex when it is overexpressed in *E. coli*, as shown in Fig. 5. However, when both CpkB and CobQ were expressed together, a significant amount of protein was found in a soluble fraction. This result suggests that the β subunit is functional as a molecular chaperonin without the α subunit in vivo. Coexpression of recombinant protein and CpkB also provides a

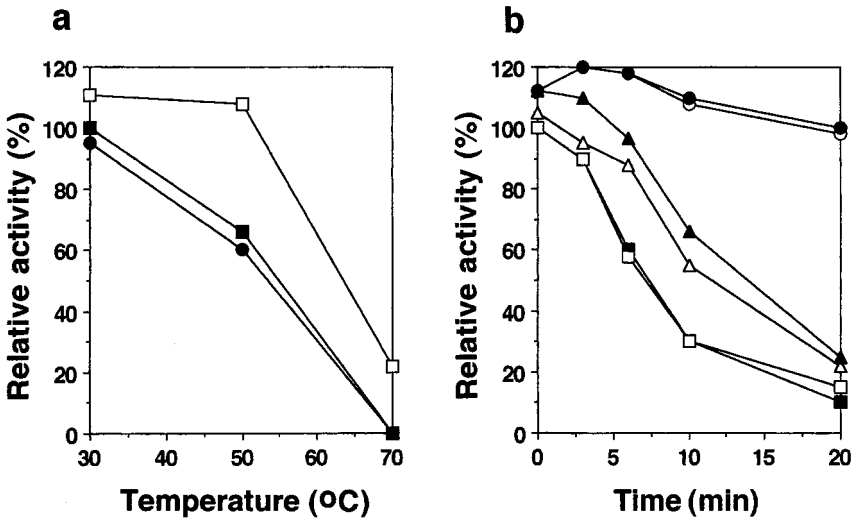

FIG. 3. Effect of KOD1 CpkB on heat stabilization of ADH. (a) Thermal inactivation of ADH and KOD1 CpkB-mediated reactivation in vitro. Yeast ADH (0.025 μM) was incubated for 6 min at temperatures between 30 and 70°C in the absence (■) or presence (□) of purified recombinant KOD1 CpkB (0.25 μM), and remaining ADH activity (●) was assayed in the presence of bovine serum albumin (0.25 μM). (b) CpkB-dependent stabilization of ADH. The ADH (0.025 μM) was incubated at 50°C either without CpkB (■) or with 0.25 μM CpkB (●), 0.25 μM CpkB and 10 mM ATP (○), 0.025 μM CpkB (△), 0.025 μM CpkB and 10 mM ATP (▲), or 0.25 μM CpkB and 0.25 μM bovine serum albumin (□). ADH activity was measured at the times indicated.

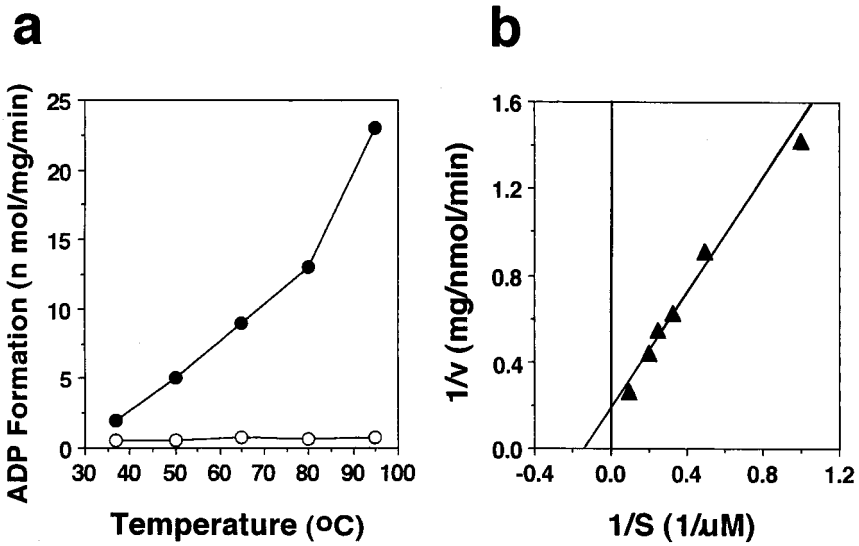

FIG. 4. Influence of temperature on ATPase activity. (a) ATPase activity of KOD1 HSP at the indicated temperatures. ADP formation using [α-^{32}P]ATP (400 Ci/mmol; Amersham) on polyethyleneimine-cellulose thin-layer sheets (Macherey-Nagel) was monitored with (●) or without (○) CpkB. (b) Lineweaver-Burk plot of the rate versus ATP concentration.

novel simple approach for efficient recovery of foreign proteins.

Nucleotide sequence accession number. The DNA sequence of the KOD1 *cpkB* gene has been submitted to GenBank/EMBL/DDBJ, and the accession number is D29672.

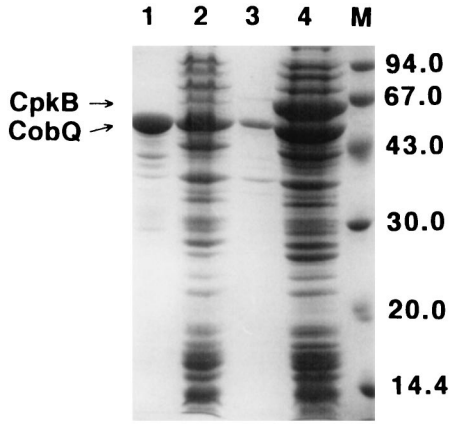

FIG. 5. Solubilization of CobQ protein by coexpression with CpkB in *E. coli*. The *cobQ* gene was cloned in expression plasmid pET-8c, and the derivative plasmid was designated pCOB. *cpkB* was cloned in plasmid pACYC184, which is compatible with pET-8c, and the constructed plasmid was named pCPK. Both *cobQ* and *cpkB* are inducible by use of a T7 promoter system with IPTG addition. *E. coli* BL21(DE3) was transformed by plasmid pCOB and/or pCPK, and overexpression of proteins was induced by IPTG addition (0.1 mM). After 3 h of induction, cells were harvested by centrifugation. The pellet was suspended in 1 ml of 30 mM Tris-HCl (pH 8.0)–30 mM NaCl buffer. 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 30 mM Tris-HCl (pH 8.0)–30 mM NaCl and suspended in 1 ml of sample buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), boiled for 3 min, and centrifuged. The supernatant was rescued as the insoluble fraction. Each fraction (20 μl) was loaded on a 0.1% SDS–10% polyacrylamide gel and subjected to electrophoresis followed by Coomassie brilliant blue R-250 staining. Lanes: 1, insoluble fraction from *E. coli*(pCOB); 2, soluble fraction from *E. coli*(pCOB); 3, insoluble fraction from *E. coli*(pCOB and pCPK); 4, soluble fraction from *E. coli*(pCOB1 and pCPK); M, molecular weight marker (weights shown in thousands on the right).

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