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Overexpression, Purification, and Characterization of β-Subunit of Group II Chaperonin from Hyperthermophilic *Aeropyrum pernix* K1

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In the present study, overexpression, purification, and characterization of *Aeropyrum pernix* K1 chaperonin B in *E. coli* were investigated. The chaperonin β-subunit gene (*ApCpnB*, 1,665 bp ORF) from the hyperthermophilic archaeon *A. pernix* K1 was amplified by PCR and subcloned into vector pET21a. The constructed pET21a-ApCpnB (6.9 kb) was transformed into *E. coli* BL21 Codonplus (DE3). The transformant cell successfully expressed ApCpnB, and the expression of ApCpnB (61.2 kDa) was identified through analysis of the fractions by SDS–PAGE (14% gel). The recombinant ApCpnB was purified to higher than 94% by using heat-shock treatment at 90°C for 20 min and fast protein liquid chromatography on a HiTrap Q column step. The purified ApCpnB showed ATPase activity and its activity was dependent on temperature. In the presence of ATP, ApCpnB effectively protected citrate synthase (CS) and alcohol dehydrogenase (ADH) from thermal aggregation and inactivation at 43°C and 50°C, respectively. Specifically, the activity of malate dehydrogenase (MDH) at 85°C was greatly stabilized by the addition of ApCpnB and ATP. Coexpression of pro-carboxypeptidase B (pro-CPB) and ApCpnB in *E. coli* BL21 Codonplus (DE3) had a marked effect on the yield of pro-CPB as a soluble and active form, speculating that ApCpnB facilitates the correct folding of pro-CPB. These results suggest that ApCpnB has both foldase and holdase activities and can be used as a powerful molecular machinery for the production of recombinant proteins as soluble and active forms in *E. coli*.

Keywords: Chaperonin, *Aeropyrum pernix*, ATPase activity, citrate synthase, alcohol dehydrogenase, malate dehydrogenase, pro-carboxypeptidase B

The high-level expression of recombinant gene in *E. coli* often results in three-dimensional folding errors of the proteins of interest and their subsequent degradation by proteases or their accumulation into the biologically inert inclusion bodies. The protein aggregation problem in *E. coli* limited significantly the total downstream processing yield of aggregated protein in the refolding steps [10, 16, 20]. It is widely recognized that coexpression of molecular chaperones or foldases can assist protein folding, and this leads to an increase in the production of active protein [23, 24]. Moreover, heat-shock proteins such as Hsp60 and Hsp70 are molecular chaperones that not only regulate the heat-shock response, but are also required for folding a newly synthesized polypeptide under normal growth conditions. The chaperone system is composed of two major functions (called a foldase and holdase). The foldase activity is that where a molecular chaperone binds to nascent polypeptides, and subsequently actively assists the folding of partially denatured substrates to their native state [32]. The holdase activity is that where a molecular chaperone tightly binds to nonnative folding intermediates or aggregation-liable proteins, efficiently prevents their aggregation, and maintains them in a folding competent conformation. Thus, some chaperone systems such as the GroEL/ES or the DnaK/DnaJ/GrpE work as "foldase," and the other chaperones such as DnaJ or small heat-shock proteins work as "holdase" [13]. Most of the conducted study has been focused on the interaction between GroEL and substrate proteins [22].

Recently, the chaperonins are well known as essential cellular proteins that form barrel-shaped high molecular weight complexes. They are thought to assist the folding of newly synthesized polypeptides and promote the refolding of denatured ones [12]. The chaperonins are classified into two distinct groups, I and II [17]. The group I chaperonins (the GroEL family) were found in bacteria, chloroplasts, and eukaryote organelles. They form a tetradecameric double-toroid oligomer of an about 60 kDa subunit and require a heptameric ring of a 10 kDa subunit [26]. Members of group II chaperonins are known as CCT [cytosolic chaperonin-containing T complex polypeptide-1 (TCP-1)], which occur in the cytosol of eukaryotes and archaea

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[28]. The folding of substrate proteins by groups I and II chaperonins occurs within the central cavity formed by each ring [25, 35]. This cavity can be sealed off from the external environment by a lid, allowing the thus-isolated substrate protein to fold more efficiently. The lid function for group I chaperonins is provided by a separate co-chaperonin protein (GroES in bacteria). In contrast, group II chaperonins exist as an eight- or nine-membered rotationally symmetrical double-ring in a toridal structure composed of homologous subunits of about 60 kDa. A unique structural feature, termed the helical protrusion, might act as a built-in lid to seal off the central cavity of group II chaperonins during folding [3, 8, 11, 21]. The group II chaperonins have been reported from thermophilic archaea, such as *Sulfolobus shibatae* TF55 [29], *Pyrodictium occultum* [27], *Sulfolobus solfataricus* [18], *Thermoplasma acidophilum* [30], *Methanopyrus kandleri* [1], *Methanococcus jannaschii* [5], *Pyrococcus* sp. KOD1 [14], and *Thermococcus* strain KS-1 [34]. However, to date, in archaeal group II chaperonins, there is not much information regarding the protein-folding mechanism. Lately, the complete genome sequence of a hyperthermophilic archaeum *Aeropyrum pernix* K1 revealed that this strain has two kinds of thermosome subunit genes (α-subunit, *ApcpnA*; β-subunit, *ApCpnB*) [15]. ApcpnA and ApCpnB have 58.56% identity of amino acid sequence. Our previous study also showed that a recombinant α-subunit of chaperonin-like protein from the hyperthermophilic archaeon *A. pernix* K1 prevents thermal denaturation of bovine liver rhodanese and enhances the thermostability of yeast (*Saccharomyces cerevisiae*) alcohol dehydrogenase in the absence of ATP, when *ApCpnA* is present in excess [28].

In this study, the chaperonin β-subunit gene (*ApCpnB*, 1,665 bp ORF) from the hyperthermophilic archaeon *A. pernix* K1 was overexpressed in *E. coli*, purified, and characterized. The foldase and holdase activities of ApCpnB as a molecular chaperone were estimated using citrate synthase (CS), alcohol dehydrogenase (ADH), and malate dehydrogenase (MDH) as substrates. To produce porcine pancreas pro-carboxypeptidase B (pro-CPB) as soluble and active forms in *E. coli*, the coexpression of pro-CPB and ApCpnB was also performed.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Reagents

In this study, *E. coli* DH5α was used for the preparation and amplication of plasmids, and BL21 Codonplus (*E. coli* B F- *ompT hsdS*(rB- mB-) *dcm*+ Tet^r *gal* 1 (DE3) *endA* Hte [*argU ileY leuW* Cam^r]) was used for the expression of *ApCpnB* (APE2072) and porcine pancreas pro-carboxypeptidase B (pro-CPB). The shotgun clone of *A. pernix* K1 containing a chaperonin ORF, APE2072 (*ApCpnB*), was purchased from NITE Biological Resource Center (NBRC, Chiba, Japan). The pET21a plasmid for expressing *ApCpnB* was purchased from Novagen Inc. (San Diego, CA, U.S.A.) and the pET24a-Mpcb plasmid containing the pro-CPB gene was obtained from Dr. J. H. Seo (Seoul National University, Seoul, Korea). Restriction enzymes, Ex*Taq* DNA polymerase, and other reagents for gene manipulation were purchased from TaKaRa Shuzo (Kyoto, Japan). Citrate synthase (CS, E.C. 4.1.3.7) from porcine heart was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Alcohol dehydrogenase (ADH, E.C. 1.1.1.1), malate dehydrogenase (MDH, E.C. 1.1.1.37), ATP, and hippuryl-L-arg were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Construction and Transformation of Expression Plasmids for ApCpnB and pro-CPB

A shotgun clone of *A. pernix* K1 containing a chaperonin ORF, APE2072, was used as a template for PCR amplification of the chaperonin gene. The *ApCpnB* gene was amplified by PCR using two primers (5'-CCGGAACATATGGTTGACCGTGTGATCGA-3' and 5'-GATTGGATCCTTAGAACTCGAACTTGCTGC-3'; the underlined sequences denote the *Nde*I and *Bam*HI restriction enzyme sites, respectively) and ligated into *Nde*I/*Bam*HI-digested pET21a, resulting in the chaperonin B gene expression plasmid, pET21a-ApCpnB (6.9 kb). The constructed pET21a-ApCpnB (6.9 kb) was transformed into *E. coli* BL21 Codonplus (DE3).

For coexpression of pro-CPB with the molecular chaperone ApCpnB, BL21 Codonplus (DE3) cells were cotransformed with plasmids pET24a-Mpcb (kanamycin-resistant plasmid) and pET21a-ApCpnB (ampicillin-resistant plasmid). Transformant *E. coli* CodonPlus/[pET24a-Mpcb+pET21a-ApCpnB] cells were selected on LB agar plates containing 50 μg/ml kanamycin and 50 μg/ml ampicillin.

Expression and Purification of ApCpnB

The *E. coli* BL21 Codonplus cells were transformed with pET21a-ApCpnB plasmid and selected on LB agar plates containing 50 μg/ml ampicillin for the selection of pET21a-ApCpnB. Then, the transformants were cultivated in LB medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl) at 37°C until the optical density at 600 nm reached 0.8. Overexpression of ApCpnB was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 8 h. After harvesting the cells by centrifugation at 9,800 ×*g* for 10 min at 4°C, the collected cells were resuspended in buffer A (50 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol, 20 mM NaCl, and 1 mM DTT), and disrupted by sonication (Sonoplus HD2070; Bandelin, Germany) for 1 min on ice, and then centrifuged at 9,800 ×*g* for 10 min to separate the soluble and insoluble fractions.

In order to purify the expressed ApCpnB, the soluble fraction was treated at 90°C for 20 min and the resulting denatured proteins were removed by centrifugation. The supernatant fractions were loaded by fast protein liquid chromatography (FPLC) on a HiTrap Q column (Amersham Biosciences, Piscataway, NJ, U.S.A.) equilibrated with the buffer A and the bound proteins were eluted with a linear gradient of NaCl (20 mM~1 M in the same buffer).

Measurement of ATPase Activity

The ATPase activity of recombinant ApCpnB was measured by colorimetrical quantitation of released phosphate (Pi) with a malachite green assay as previously described [22, 26]. The standard assay was performed with 150 μl of buffer B (50 mM BisTris-HCl buffer, pH 5.0, containing 2 mM ATP, 100 mM KCl, 5 mM $MnCl_2$, and 8 μg/ml ApCpnB). The reaction of purified ApCpnB was initiated

by adding ATP to 2 mM and allowed to be proceeded at 37~85°C for 10 min with 8 μg/ml ApCpnB concentration. After the reaction, the samples were placed on ice and briefly centrifuged. With the addition of 800 μl of malachite green reagent and 100 μl of 34% (v/w) trisodium citrate, the samples were incubated at room temperature for 5 min. The absorbance at 660 nm of the reaction mixture was measured with a UV–visible spectrophotometer (Shimadzu Corp., Tokyo, Japan). To calculate the amount of released Pi, a calibration curve in the range of 0~100 μM Pi was determined in parallel at every assay.

Measurement of Thermal Aggregation of CS and ADH

The thermal aggregation of citrate synthase from porcine heart was monitored at 43°C in the absence or presence of ApCpnB. CS of 0.15 μM in 40 mM *N*-2-hydoxyethlypiperazine-*N'* 2-ethanesulfonic acid (HEPES, pH 7.5) was heated at 43°C in the absence or presence of ApCpnB (0.15 μM as the final concentration) without or with 2 mM ATP. The turbidity of thermal aggregated CS was monitored at 500 nm for 20 min with a UV–visible spectrophotometer.

In the same manner, the thermal aggregation of alcohol dehydrogenase from *Saccharomyces cerevisiae* was monitored by measuring the light scattering at 500 nm at 50°C for 20 min. The monitoring was started by adding 0.025 μM ADH to 50 mM phosphate buffer (pH 7.0), which was preincubated at 50°C without or with ApCpnB (0.005 μM as the final concentration) and 2 mM ATP.

Measurement of Thermal Inactivation of CS, ADH, and MDH

CS catalyzes the reaction of oxaloacetic acid to citric acid. CS activity was measured as the amount of enzyme that catalyzes the synthesis of 1 μM of citrate per 1 min at 412 nm. CS of 0.15 μM was incubated for 20 min at 43°C in the absence or presence of ApCpnB (0.15 μM) and ATP (2 mM). After the incubation, CS activity was measured in the buffer C (50 mM TE buffer, pH 8.0, containing 0.1 mM oxaloacetic acid, 0.1 mM DTNB, and 0.15 mM acetyl-coA) by monitoring the absorbance at 412 nm at 25°C.

ADH catalyzes the conversion of ethanol to acetaldehyde by aid of β-NAD. ADH activity was monitored by recording the increased amount of β-NADH$^+$, which has a unique absorbance at 340 nm. ADH of 0.025 μM was incubated for 20 min at 50°C in the absence or presence of ApCpnB (0.005 μM) and ATP (2 mM). During the incubation, ADH activity was chased in the buffer D (100 mM glycine-NaOH buffer, pH 8.8, containing 1 mM β-NAD, and 100 mM ethanol) by recording the absorbance at 340 nm at 25°C.

The enzymatic activity of malate dehydrogenase from *Thermus flavus* was measured following the standard procedure of MDH assay [19]. An aliquot of MDH solution was added to buffer E (100 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM oxaloacetate and 0.25 mM β-NADH$^+$). The final concentrations of MDH and ApCpnB were 0.4 μM. The incubation proceeded for 30 min at 85°C in the absence or presence of ApCpnB and ATP (2 mM). The MDH activity was monitored by measuring the absorbance at 340 nm at 25°C. The activities of CS, ADH, and MDH at the incubation time of zero were taken as 100%.

Coexpression of Pro-CPB and Measurement of Pro-CPB Activity

For coexpression of pro-CPB together with the molecular chaperonin ApCpnB in *E. coli*, *E. coli* BL21 Codonplus (DE3) was cotransformed with plasmids designed to permit coexpression of pro-CPB together with molecular chaperonin ApCpnB. Transformant *E. coli* Codonplus/[pET24a-Mpcb+pET21a-ApCpnB] was grown at 37°C, 200 rpm, and 0.5 mM IPTG was added at the mid-exponential phase.

Before measuring the activity of pro-CPB, the pro region of pro-CPB was cleaved off by trypsin treatment. The trypsin treatment was performed at 37°C for 1 h in 900 μl of activation buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl) containing the sample (100 μl) and trypsin (10 μl). Then, the reaction was stopped by addition of 0.1 mM PMSF. Under these conditions, the amount of CPB required to decompose 1 mM hippuryl-L-arg (1 μM) as a substrate per minute at 25°C was defined as 1.0 unit of activity. Absorbance at 254 nm was measured at 25°C for 2 min with a spectrophotometer (Shimadzu, Japan). The detailed assay condition was previously described [32].

RESULTS AND DISCUSSION

Overexpression and Purification of ApCpnB

In order to investigate the expression of the chaperonin B gene (*ApCpnB*) from *A. pernix* K1 in *E. coli*, *ApCpnB* was cloned into vector pET21a and expressed in *E. coli* BL21 Codonplus (DE3). Overexpression of ApCpnB was induced by the addition of 0.5 mM IPTG for 8 h. To purify the recombinant ApCpnB, cells with overexpressed ApCpnB were disrupted and centrifuged as described in Materials and Methods. Most of the ApCpnB in the soluble fraction (supernatant) was partially purified by heat treatment at 90°C for 20 min. Large amounts of the impurities from

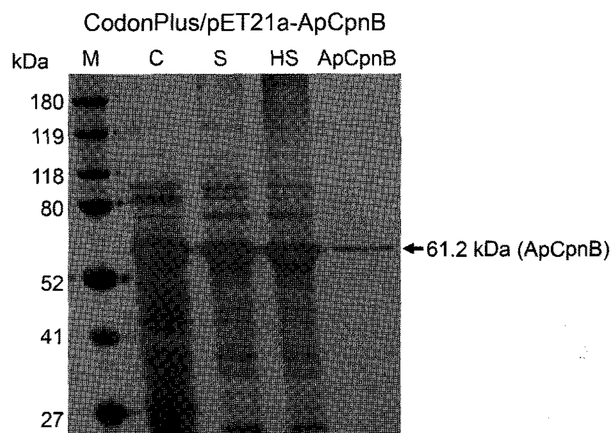

Fig. 1. Purification of ApCpnB from the *E. coli* Codonplus/pET21a-ApCpnB.

The transformant *E. coli* Codonplus/pET21a-ApCpnB was collected by centrifugation at 9,800 ×g for 10 min at 4°C and resuspended in buffer A (50 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol, 20 mM NaCl, and 1 mM DTT). The collected cells were then disrupted by sonication on ice and the debris was removed by centrifugation at 9,800 ×g for 10 min at 4°C. In order to purify the expressed ApCpnB, the supernatant fraction was purified by using heat-shock treatment at 90°C for 20 min and fast protein liquid chromatography on a HiTrap Q column step. The soluble fraction was analyzed by SDS–PAGE (14% gel). M, Protein molecular mass marker; C, Crude extract of induced cells; S, Soluble fraction of crude cell extract; HS, Soluble fraction after heat treatment at 90°C for 20 min; ApCpnB, Hitrap Q column peak fraction.

the soluble fraction were aggregated after heating and removed by centrifugation. In this study, the ApCpnB was further purified by anion-exchange chromatography with a HiTrap Q column. After anion-exchange chromatography with the HiTrap Q column, 94% purity and 63.7% yield were finally obtained (Fig. 1). The purified ApCpnB was sufficiently pure for the study of the chaperone activity.

ATPase Activity of Purified ApCpnB

The ATPase activity of the recombinant ApCpnB was assayed at different temperatures between 37°C and 85°C for 10 min with 8 μg/ml ApCpnB concentration. The ATPase activity of the recombinant ApCpnB increased in a temperature-dependent manner and reached a maximum at 80°C, but sharply declined with further increase of temperature (Fig. 2). Moreover, the ATPase activity of the recombinant ApCpnB was significantly increased in an ApCpnB concentration-dependent manner (data not shown). According to several reports, the chaperonins from *M. thermolithotrophicus* (65°C), *Thermococcus* strain KS-1 (60°C), and *T. acidophilum* (50~90°C) were reported to exhibit ATPase activity with the hydrolysis rate [9, 30, 34]. Furthermore, enzyme-dependent ATP cleavage of another archaeal *M. kandleri* chaperonin was observed from 70°C to 115°C with an optimum at 102°C [1, 2]. In our study, the optimal temperature for ATPase activity was found to be 80°C. The thermostability of chaperonin has been the subject of considerable interest for academic research and industrial applications [7, 26]. Therefore, the thermostable ApCpnB will provide us with an interesting model system for studying the protein folding mechanism, such as the holdase and foldase activities, at the elevated temperature.

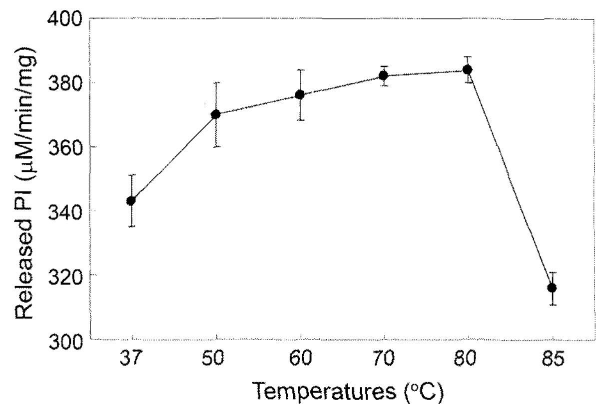

Fig. 2. Effects of temperature on the ATPase activity of ApCpnB.
The purified soluble ApCpnB was reacted by the addition of ATP to 2 mM and the reaction was allowed to proceed at 37~85°C for 10 min.

Chaperonin Activity of ApCpnB on Thermal Aggregation and Inactivation of CS

To confirm the chaperonin activity of purified ApCpnB, its effects on the thermal aggregation and inactivation of CS were investigated. The denaturation and renaturation of CS have been well documented [37], and CS has been used for investigating the molecular chaperone activities of heat-shock protein 90 (HSP90) and associated proteins [31], GroEL [4], and small heat-shock proteins (sHSPs) [6]. In the absence of any substrates, CS is readily inactivated on incubation at higher temperatures, with a midpoint of transition at more than 43°C [36]. As shown in Fig. 3A, CS was rapidly aggregated during incubation for 20 min at 43°C. This is based on unspecific hydrophobic interactions

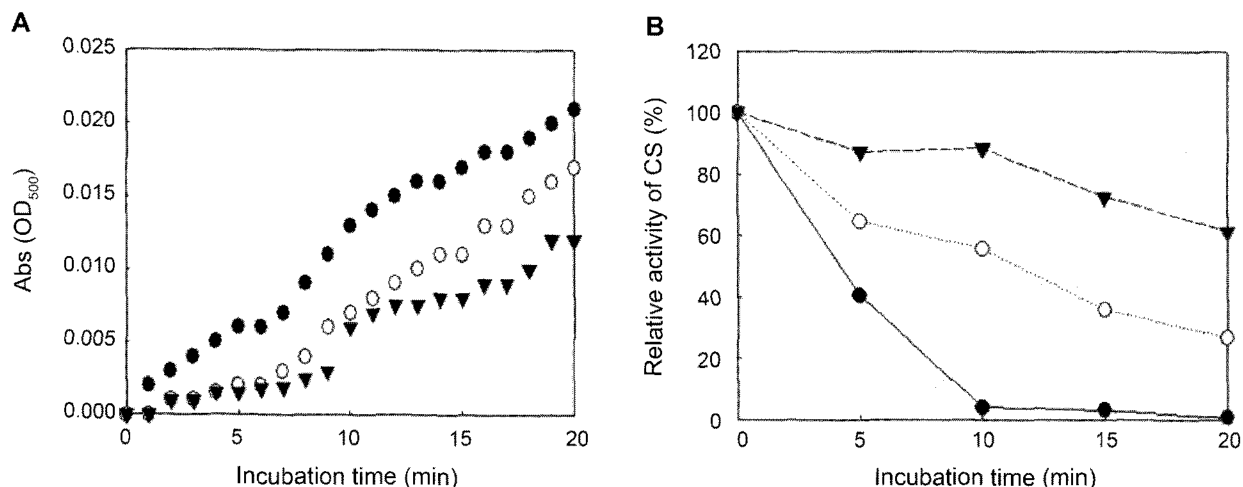

Fig. 3. Influences of ApCpnB on the thermal aggregation and inactivation of CS.
A. The prevention kinetics of ApCpnB (0.15 μM) on the thermal aggregation of CS (0.15 μM) was monitored by light scattering of CS. The CS enzyme was incubated at 43°C in HEPES buffer (pH 7.5) in the absence or presence of ApCpnB with ATP. **B.** The remaining CS activity at 43°C was measured at the times indicated. Symbols: (●) CS only; (○) CS+ApCpnB; (▼) CS+ApCpnB+ATP.

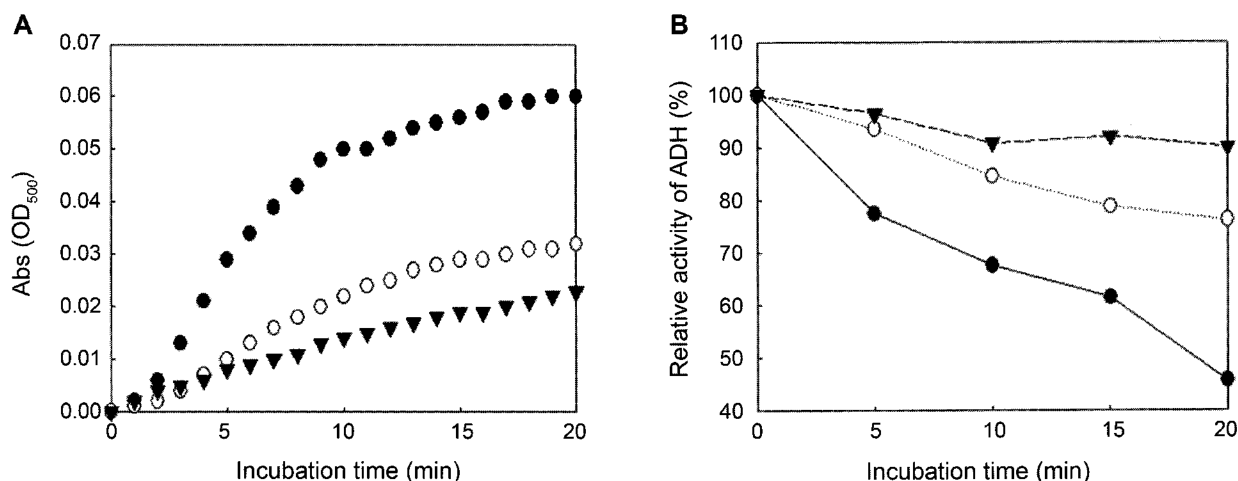

Fig. 4. Influences of ApCpnB on the thermal aggregation and inactivation of ADH.
A. The prevention kinetics of ApCpnB (0.005 μM) on the thermal aggregation of ADH (0.025 μM) monitored by light scattering of ADH. The enzyme was incubated at 50°C in 50 mM phosphate buffer (pH 7.0) in the absence or presence of ApCpnB with ATP. **B.** The remaining ADH activity at 50°C was measured at the times indicated. Symbols: (●) ADH only; (○) ADH+ApCpnB; (▼) ADH+ApCpnB+ATP.

between unfolding intermediates. In quantitative terms, at a 1:1 molar ratio of the ApCpnB relative to CS, the thermal aggregation of CS showed a slight decline to about 20% of its spontaneous value. Furthermore, the CS aggregation level was prevented more than 40% by the presence of ATP (Fig. 3A). After heat treatment for 20 min at 43°C, the residual activity of CS was measured in the buffer C (Fig. 3B). The extent and kinetics of thermal inactivation of CS was lessened with the ApCpnB including ATP. Thus, these results indicate that the unfolding intermediate of CS was protected from inactivation by the chaperone-like activity of ApCpnB in the presence of ATP.

Chaperonin Activity of ApCpnB on Thermal Aggregation and Inactivation of ADH

The denaturation and renaturation of ADH have been used for investigating the molecular chaperone activities of *Thermococcus* sp. strain KS-1 [19] and *Pyrococcus* sp. strain KOD1 [33]. ADH was used as a model system to study the influence of ApCpnB on heat-induced activity *in vitro*. The effect of ApCpnB on the thermal aggregation of ADH at 50°C has been tested and the results are shown in Fig. 4A. The ADH aggregation level was prevented almost 65% by ApCpnB including ATP, whereas at 5:1 molar ratio of ADH to pure ApCpnB, prevention of ADH aggregation was only 50% (Fig. 4A). To prove the effect of ApCpnB on the thermal inactivation of ADH, after heat treatment for 20 min at 50°C, the residual activity of ADH was measured in the buffer D (Fig. 4B). In the presence of ApCpnB including ATP, the extent of thermal inactivation of ADH was lessened compared with the control obtained without the ApCpnB. These results clearly demonstrate that the ApCpnB might have bound to the thermally unfolded substrate protein and prevented its denaturation, and possesses chaperone-like activity as well.

Thermal Stabilization of MDH by ApCpnB

Hyperthermophilic chaperonins were expected to increase the thermal stability of substrate proteins because of their high thermostability. From the previous research, *Thermococcus* strain KS-1 chaperonin also prevented the thermal inactivation of MDH, even in the absence of ATP [19]. In this study, when the MDH diluted in buffer E was preincubated at

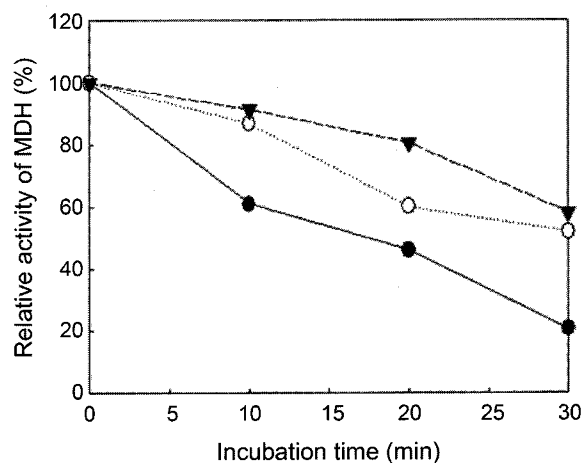

Fig. 5. Effect of ApCpnB on heat stabilization of MDH of *T. flavus*.
MDH (0.4 μM) was incubated at 85°C in the absence or presence of ApCpnB (0.4 μM) with ATP. At the times indicated, the remaining MDH activity was measured. Symbols: (●) MDH only; (○) MDH+ApCpnB; (▼) MDH+ApCpnB+ATP.

85°C, it was dramatically inactivated within 10 min. The remaining activity of MDH was keep about 60% in the presence of ApCpnB and its residual activity approached about 65% in the presence of ATP (Fig. 5). As a result, the binding of ATP is sufficient for ApCpnB to perform chaperonin function in preventing the thermal inactivation of MDH at an elevated temperature.

Effect of ApCpnB on Production of a Soluble and Active Form of pro-CPB

E. coli is one of the most popular host organisms for the high-level production of various recombinant proteins. However, the high-level expression of recombinant gene products in *E. coli* often results in the misfolding of the protein of interest and its subsequent degradation by proteases or its deposition into biologically inactive aggregates known as inclusion bodies [10, 16, 20]. Recently, in order to produce soluble and active recombinant proteins, the study of molecular chaperones and chaperonins as protein folding and holding machines is of great interest and proceeds to diverse fields, especially concerning archaeal chaperonins [23, 24]. This study has also investigated the effect of the chaperonin B from the hyperthermophilic archaeon *A. pernix* K1 on the production of soluble and active recombinant proteins in *E. coli*.

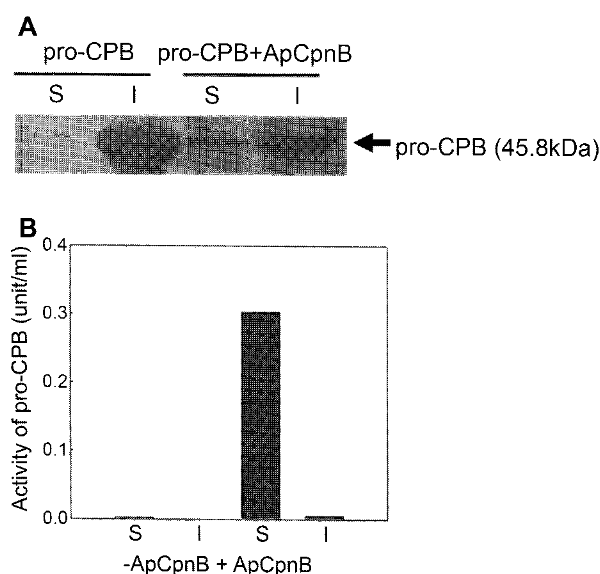

Fig. 6. Effect of ApCpnB on the production of a soluble and active form of pro-CPB in *E. coli* Codonplus/[pET24a-Mpcb+pET21a-ApCpnB].

A. Western blot analysis of pro-CPB in the soluble (S) and insoluble (I) fractions when ApCpnB was coexpressed or not. **B**. Enzyme activities of pro-CPB when ApCpnB was coexpressed (+ApCpnB) or not (-ApCpnB). The recombinant *E. coli* Codonplus/[pET24a-Mpcb+pET21a-ApCpnB] was grown in 10 ml of LB medium and at the mid-exponential phase 0.5 mM IPTG was added. After 8 h induction with IPTG, the cells were harvested and sonicated. The cell lysate was separated as soluble (S) and insoluble (I).

To produce a soluble and active form of pro-CPB in *E. coli*, *E. coli* BL21 Codonplus (DE3) cells were cotransformed with plasmids designed to permit coexpression of pro-CPB together with molecular chaperone ApCpnB. Transformant *E. coli* Codonplus/[pET24a-Mpcb+pET21a-ApCpnB] was grown at 37°C, 200 rpm and 0.5 mM IPTG was added at the mid-exponential phase. After 8 h induction of IPTG, cells were coexpressed with pro-CPB (45.8 kDa) and ApCpnB. In Fig. 6A, the result of Western blot shows that the coexpression of pro-CPB together with ApCpnB had a marked effect on the yield of pro-CPB as a soluble form. In addition, Fig. 6B shows that the coexpression of pro-CPB together with ApCpnB induced about 121-folds increase of the pro-CPB activity compared with the expression of pro-CPB alone. Therefore, the coexpression of pro-CPB together with ApCpnB was effective on the production of pro-CPB as a soluble and active form, presumably through facilitating correct folding of pro-CPB protein.

In conclusion, the recombinant chaperonin B from *A. pernix* K1 was successfully expressed in *E. coli*, as a major intracellular soluble protein, and could be purified into a homogeneous protein by two simple steps of heat treatment and anion-exchange chromatography. The purified ApCpnB severely prevented the thermal aggregation of CS and ADH, especially in the presence of ATP. Moreover, the thermal inactivation of CS, ADH, and MDH was greatly reduced by the aid of ApCpnB and ATP. ATP drives the conformational change of the group II chaperonin from the open-lid substrate-binding conformation to the closed-lid conformation to encapsulate an unfolded protein in the central cavity. The detailed mechanism of this conformational change remains unknown.

Coexpression of pro-CPB and ApCpnB had a marked effect on the production of pro-CPB as a soluble and active form. These results suggest that ApCpnB has both foldase and holdase activities and can be used as a powerful molecular machinery for the production of recombinant proteins as soluble and active forms in *E. coli*.

Acknowledgments

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REFERENCES

1. Andra, S., G. Frey, M. Nitsch, W. Baumeister, and K. O. Stetter. 1996. Purification and structural characterization of the

thermosome from the hyperthermophilic archaeum *Methanopyrus kandleri*. *FEBS Lett.* **379:** 127–131.
2. Andra, S., G. Frey, R. Jaenicke, and K. O. Stetter. 1998. The thermosome from *Methanopyrus kandleri* possesses an NH_4^+-dependent ATPase activity. *Eur. J. Biochem.* **255:** 93–99.
3. Archibald, J. M., J. M. Logsdon, and W. F. Doolittle. 1999. Recurrent paralogy in the evolution of archaeal chaperonins. *Curr. Opin. Biotechnol.* **9:** 1053–1056.
4. Buchner, J., M. Schmidt, M. Fuchs, R. Jaenicke, R. Rudolph, F. X. Schmid, and T. Kiefhaber. 1991. GroE facilitates refolding of citrate synthase by suppressing aggregation. *Biochemistry* **30:** 1586–1591.
5. Bult, C. J., O. White, G. J. Olsen, L. Zhou, R. D. Fleischmann, G. G. Sutton, *et al.* 1996. Complete genome sequence of the methanogenic archaeon *Methanococcus jannaschii*. *Science* **273:** 1058–1073.
6. Chang, Z., T. Primm, J. Jakana, I. H. Lee, I. Serysheva, W. Chiu, H. F. Gilbert, and F. A. Cuiocho. 1996. *Mycobacterium tuberculosis* 16-kDa antigen (Hsp 16.3) functions as an oligomeric structure *in vitro* to suppress thermal aggregation. *J. Biol. Chem.* **271:** 7218–7223.
7. Choi, J. J., J. W. Park, H. K. Shim, S. C. Lee, M. S. Kwon, J. S. Yang, H. O. Hwang, and S. T. Kwon. 2006. Cloning, expression, and characterization of a hyperalkaline phosphatase from the thermophilic bacterium *Thermus* sp. T351. *J. Microbiol. Biotechnol.* **16:** 272–279.
8. Ditzel, L., J. Lowe, D. Stock, K. O. Stetter, H. Huber, R. Huber, and S. Steinbacher. 1998. Crystal structure of the thermosome, the archaeal chaperonin and homolog of CCT. *Cell* **93:** 125–138.
9. Furutani, M., T. Iida, T. Yoshida, and T. Maruyama. 1998. Group II chaperonin in a thermophilic methanogen *Methanococcus thermolithotrophicus*. Chaperone activity and filament-forming ability. *J. Biol. Chem.* **273:** 28399–28407.
10. Guan, Y. X., H. X. Pan, Y. G. Gao, S. J. Yao, and M. G. Cho. 2005. Refolding and purification of recombinant human interferon-γ expressed as inclusion bodies in *Escherichia coli* using size exclusion chromatography. *Biotechnol. Bioprocess Eng.* **10:** 122–127.
11. Gutsche, I., L. O. Essen, and W. Baumeister. 1999. Group II chaperonins: New TRiC(k)s and turns of a protein folding machine. *J. Mol. Biol.* **293:** 295–312.
12. Hartl, F. U. 1996. Molecular chaperones in cellular protein folding. *Nature* **381:** 571–580.
13. Hoffmann, J. H., K. Linke, P. C. Graf, H. Lilie, and U. Jakob. 2004. Identification of a redox-regulated chaperone network. *EMBO J.* **23:** 160–168.
14. Isumi, M., S. Fuiwara, M. Takagi, S. Kanaya, and T. Imanaka. 1999. Isolation and characterization of a second subunit of molecular chaperonin from *Pyrococcus kodakaraensis* KOD1: Analysis of an ATPase-deficient mutant enzyme. *Appl. Environ. Microbiol.* **65:** 1801–1805.
15. Kawarabayasi, Y., Y. Hino, H. Horikawa, S. Yamazaki, Y. Haikawa, K. Jin-no, *et al.* 1999. Complete genome sequence of an aerobic hyper-thermophilic crenarchaeon, *Aeropyrum pernix* K1. *DNA Res.* **6:** 83–101, 145–152.
16. Kim, H. and I. H. Kim. 2005. Refolding of fusion ferritin by gel filtration chromatography (GFC). *Biotechnol. Bioprocess Eng.* **10:** 500–504.
17. Kim, S., K. R. Willson, and A. L. Horwich. 1994. Cytosolic chaperonin subunits have a conserved ATPase domain but diverged polypeptide-binding domains. *Trends Biochem. Sci.* **19:** 543–548.
18. Knapp, S., I. Schhmidt-Krey, H. Hebert, T. Bergman, H. Jornvall, and R. Ladenstein. 1994. The molecular chaperonin TF55 from the thermophilic archaeon *Sulfolobus solfataricus*. A biochemical and structural characterization. *J. Mol. Biol.* **242:** 397–407.
19. Kohda, J., H. Kawanishi, K. Suehara, Y. Nakano, and T. Yano. 2006. Stabilization of free and immobilized enzyme using hyperthermophilic chaperonin. *J. Biosci. Bioeng.* **101:** 131–136.
20. Kohda, J., Y. Endo, N. Okumura, Y. Kurokawa, K. Nishihara, H. Yanagi, T. Yura, H. Fukuda, and A. Kondo. 2002. Improvement of productivity of active form of glutamate racemase in *Escherichia coli* by coexpression of folding accessory proteins. *Biochem. Eng. J.* **10:** 39–45.
21. Kubota, H., G. Hynes, and K. Willson. 1995. The chaperonin containing t-complex polypeptide 1 (TCP-1) multisubunit machinery assisting in protein folding and assembly in the eukaryotic cytosol. *Eur. J. Biochem.* **230:** 3–16.
22. Kwak, Y. H., S. J. Kim, K. Y. Lee, and H. B. Kim. 2000. Stress response of the *Escherichia coli groE* promoter. *J. Microbiol. Biotechnol.* **10:** 63–68.
23. Kwon, M. J., S. L. Rark, S. K. Kim, and S. W. Nam. 2002. Overproduction of *Bacillus macerans* cyclodextrin glucanotransferase in *E. coli* by coexpression of GroEL/ES chaperone. *J. Microbiol. Biotechnol.* **12:** 1002–1005.
24. Lamark, T., M. Ingebrigtsen, C. Bjornstad, T. Melkko, T. Mollens, and E. Nielsen. 2001. Expression of active human C1 inhibitor serpin domain in *Escherichia coli*. *Prot. Expr. Purif.* **22:** 349–359.
25. Langer, T., G. Pfeifer, J. Martin, W. Baumeister, and F. U. Hartl. 1992. Chaperonin mediated protein folding: GroES binds to one end of the GroEL cylinder, which accommodates the protein substrate within its central cavity. *EMBO J.* **11:** 4757–4765.
26. Mayhew, M., A. C. da Silva, J. Martin, H. Erdjument-Bromage, P. Tempst, and F. U. Hartl. 1996. Protein folding in the central cavity of the GroEL–GroES chaperonin complex. *Nature* **379:** 420–426.
27. Minuth, T., G. Frey, P. Lindner, R. Rachel, K. O. Stetter, and R. Jaenicke. 1998. Recombinant homo- and hetero-oligomers of an ultrastable chaperonin from the archaeon *Pyrodictium occultum* show chaperone activity *in vitro*. *Eur. J. Biochem.* **258:** 837–845.
28. Son, H. J., E. J. Shin, S. W. Nam, D. E. Kim, and S. J. Jeon. 2006. Properties of the α subunit of a chaperonin from the hyperthermophilic crenarchaeon *Aeropyrum pernix* K1. *FEMS Microbiol. Lett.* **266:** 103–109.
29. Trent, J. D., E. Nimmesgern, J. S. Wall, F. U. Hartl, and A. L. Horwich. 1991. A molecular chaperone from a thermophilic archaebacterium is related to the eukaryotic protein t-complex polypeptide-1. *Nature* **354:** 490–493.
30. Waldmann, T., E. Nimmesgern, M. Nitsch, J. Peters, G. Pfeifer, S. Muller, *et al.* 1995. The thermosome of *Thermoplasma acidophilum* and its relationship to the eukaryotic chaperonin TRiC. *Eur. J. Biochem.* **227:** 848–856.
31. Wiech, H., J. Buchner, R. Zimmermann, and U. Jakob. 1992. Hsp90 chaperones protein folding *in vitro*. *Nature* **358:** 169–170.

32. Winter, J., K. Linke, A. Jatzek, and U. Jakob. 2005. Severe oxidative stress causes inactivation of DnaK and activation of the redox-regulated chaperone Hsp33. *Mol. Cell.* **17:** 381–392.
33. Yan, Z., S. Fujiwara, K. Kohda, M. Takagi, and T. Imanaka. 1997. *In vitro* stabilization and *in vivo* solubilization of foreign proteins by the beta subunit of a chaperonin from the hyperthermophilic archaeon *Pyrococcus* sp. strain KOD1. *Appl. Environ. Microbiol.* **63:** 785–789.
34. Yoshida, T., M. Yohda, T. Iida, T. Maruyama, H. Taguchi, K. Yazaki, *et al.* 1997. Structural and functional characterization of homo-oligomeric complexes of alpha and beta chaperonin subunits from the hyperthermophilic archaeum *Thermococcus* strain KS-1. *J. Mol. Biol.* **273:** 635–645.
35. Yoshida, T., R. Kawaguchi, H. Taguchi, M. Yoshida, T. Wakabayashi, M. Yohda, and T. Maruyama. 2002. Archaeal group II chaperonin mediates protein folding in the *cis*-cavity without a detachable GroES-like co-chaperonin. *J. Mol. Biol.* **315:** 73–85.
36. Zhi, W., P. Srere, and C. T. Evans. 1991. Conformational stability of pig citrate synthase and some active-site mutants. *Biochemistry* **30:** 9281–9286.
37. Zhi, W., S. J. Landry, L. M. Gierasch, and P. A. Srere. 1992. Renaturation of citrate synthase: Influence of denaturant and folding assistants. *Prot. Sci.* **1:** 522–529.