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Functional Characterization of the α- and β-Subunits of a Group II Chaperonin from *Aeropyrum pernix* K1

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We isolated and functionally characterized the α- and β-subunits (ApCpnA and ApCpnB) of a chaperonin from *Aeropyrum pernix* K1. The constructed vectors pET3d-*ApCpnA* and pET21a-*ApCpnB* were transformed into *E. coli* Rosetta (DE3), BL21 (DE3), or CodonPlus (DE3) cells. The expression of ApCpnA (60.7 kDa) and ApCpnB (61.2 kDa) was confirmed by SDS-PAGE analysis. Recombinant ApCpnA and ApCpnB were purified by heat-shock treatment and anion-exchange chromatography. ApCpnA and ApCpnB were able to hydrolyze not only ATP, but also CTP, GTP, and UTP, albeit with different efficacies. Purified ApCpnA and ApCpnB showed the highest ATPase, CTPase, UTPase, and GTPase activities at 80°C. Furthermore, the addition of ApCpnA and ApCpnB effectively protected citrate synthase (CS) and alcohol dehydrogenase (ADH) from thermal aggregation and inactivation at 43°C and 50°C, respectively. In particular, the addition of ATP or CTP to ApCpnA and ApCpnB resulted in the most effective prevention of thermal aggregation and inactivation of CS and ADH. The ATPase activity of the two chaperonin subunits was dependent on the salt concentration. Among the ions we examined, potassium ions were the most effective at enhancing the ATP hydrolysis activity of ApCpnA and ApCpnB.

Key words: *Aeropyrum pernix*, ATP hydrolysis activity, chaperonin, thermal aggregation, thermal inactivation

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Chaperonins are ubiquitous chaperones that are required for correct protein folding, assembly, and degradation [6, 18]. They are classified into two distant groups [16]. Group I chaperonins are found in bacteria and endosymbiotic organelles (mitochondria and chloroplasts), and there are subsets of bacterially related homologs in methanogens. Group II chaperonins are found in archaea and the eukaryotic cytosol (chaperonin-containing t-complex polypeptide-1, or TCP-1, ring complex) [1, 9, 11]. Group II chaperonins exist as an 8- or 9-membered rotationally symmetrical double-ring in a toridal structure composed of homologous subunits of about 60 kDa. Each ring has a large central cavity in which a non-native protein can undergo productive folding in an ATP-dependent manner [3, 11, 24]. A unique structural feature, termed the helical protrusion, is thought to act as a built-in lid to seal off the central cavity of group II chaperonins during folding [14, 15, 16]. Opening and closing of the folding chamber is controlled by a conformational cycle driven by ATP binding and hydrolysis. All chaperonins share a similar subunit architecture consisting of three distinct domains as follows: an ATP-binding equatorial domain, a distal apical domain harboring the polypeptide-binding sites, and an intermediate hinge domain [25].

Group II chaperonins have been reported from various thermophilic archaea, such as *Sulfolobus shibatae* TF55 [27], *Pyrodictium occultum* [21], *Sulfolobus solfataricus* [19], *Thermoplasma acidophilum* [12, 28], *Methanopyrus kandleri* [22], *Pyrococcus kodakaraensis* KOD1 [13, 30], *Thermococcus* strain KS-1 [31], *Methanococcus thermolithotrophicus* [7], *Pyrococcus horikoshii* OT3 [23], *Aeropyrum pernix* K1 [26], and *Pyrococcus furiosus* [5]. Despite numerous reports about the functions of archaeal chaperonins, the functional characteristics of the subunits of the chaperonin from *A. pernix* K1 have not been investigated in detail. In

the present study, we isolated and functionally characterized the α- and β-subunits (ApCpnA and ApCpnB) of a chaperonin from *A. pernix* K1. We examined the effect and nucleotide dependencies of ApCpnA and ApCpnB on the thermal aggregation and inactivation of the foreign model proteins citrate synthase (CS) and alcohol dehydrogenase (ADH). We also investigated the salt dependency of the ATPase activities of ApCpnA and ApCpnB.

Materials and Methods

Bacterial Strains, Plasmids, and Reagents

We used *E. coli* DH5α to prepare plasmids and *E. coli* Rosetta (DE3) and BL21 Codonplus (DE3) cells to express ApCpnA and ApCpnB. Shotgun clones of *A. pernix* K1 containing the α- and β-subunits of the chaperonin of interest (ORF APE0907, *ApCpnA*; and ORF APE2072, *ApCpnB*) were purchased from NITE Biological Resource Center (NBRC, Chiba, Japan). The pET3d plasmid used to express ApCpnA and the pET21a plasmid used to express ApCpnB were purchased from Novagen Inc. (San Diego, CA, USA). 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. 1.1.1.37) and nucleotide triphosphates (NTPs) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Expression and Purification of ApCpnA and ApCpnB

E. coli Rosetta and Codonplus (DE3) cells were transformed with pET3d-*ApCpnA* and pET21a-*ApCpnB*, respectively, precultured in 10 ml of LB medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl) with 50 μg/ml ampicillin for 12 h at 37°C, and then 1 mM IPTG was added. After an 8 h induction, cells were harvested by centrifugation at 9,800 ×*g* for 10 min at 4°C and stored at −80°C. The collected *E. coli* Rosetta/pET3d-*ApCpnA* cells were resuspended in buffer A (50 mM Tris-HCl buffer, pH 7.5, containing 15 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT), whereas *E. coli* BL21 Codonplus/pET21a-*ApCpnB* cells were resuspended in buffer B (50 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol, 20 mM NaCl, and 1 mM DTT). The suspended cells were 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.

To purify ApCpnA and ApCpnB, soluble fractions were heated at 90°C for 20 min and denatured proteins were removed by centrifugation at 9,800 ×*g* for 10 min at 4°C. The supernatant fractions were loaded by fast protein liquid chromatography (FPLC) on a HiTrap Q column (Amersham Biosciences, Piscataway, NJ, USA) equilibrated in the above-mentioned buffer and the bound proteins were eluted with a linear gradient of NaCl (20 mM~1 M in the same buffer). The protein bands on the gels were scanned using the Gel-Pro Analyzer (Gel-Pro Analyzer 3.1 program; ING PLUS, Korea) and quantified by densitometric analysis.

Measurement of NTPase Activity

The NTPase activities of recombinants ApCpnA and ApCpnB were measured by colorimetrical quantitation of released phosphate (Pi) with a malachite green assay as described previously [20]. A standard assay was performed with 150 μl of buffer C (50 mM BisTris-HCl buffer, pH 5.0, containing 2 mM NTPs, 100 mM KCl, 5 mM $MnCl_2$, and 8 μg/ml ApCpnA or ApCpnB). The reaction of purified ApCpnA and ApCpnB was initiated by adding 2 mM NTPs, and allowed to proceed at 37−85°C for 10 min with 8 μg/ml ApCpnA or ApCpnB. After the reaction, the samples were placed on ice and briefly centrifuged. After the addition of 800 μl of malachite green reagent and 100 μl of 34% (v/w) trisodium citrate, 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 for every assay.

Measurement of Thermal Aggregation of CS and ADH

The thermal aggregation of CS from porcine heart was monitored at 43°C in the absence or presence of ApCpnA and ApCpnB. CS at a concentration 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 the chaperonin subunits (ApCpnA and ApCpnB) and 2 mM NTPs. The turbidity of thermally aggregated CS was monitored at 500 nm for 20 min with a UV-visible spectrophotometer. In the same manner, the thermal aggregation of ADH from *Saccharomyces cerevisiae* was monitored by measuring light scattering at 500 nm at 50°C for 20 min. Monitoring was started when 0.025 μM ADH was added to 50 mM phosphate buffer (pH 7.0), which was preincubated at 50°C with or without chaperonins (final concentrations of 0.005 μM) and 2 mM NTPs.

Measuring the Thermal Inactivation of CS and ADH

CS catalyzes the conversion of oxaloacetic acid to citric acid. CS activity was measured as the amount of enzyme that catalyzed the synthesis of 1 μM of citrate per minute at 412 nm. CS at a concentration of 0.15 μM was incubated for 20 min at 43°C in the absence or presence of chaperonins (0.005 μM) and ATP (2 mM). After the incubation, CS activity was measured in buffer D (50 mM TE buffer, pH 8.0, 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 with the help of β-NAD. ADH activity was monitored by recording the amount of NADH produced by measuring the absorbance at 340 nm. ADH at a concentration of 0.025 μM was incubated for 20 min at 50°C in the absence or presence of chaperonins (0.005 μM) and NTPs (2 mM). During the incubation, the ADH activity in buffer E (100 mM glycine-NaOH buffer, pH 8.8, containing 1 mM β-NAD, and 100 mM ethanol) was followed by recording the absorbance at 340 nm at 25°C.

Results and Discussion

NTPase Activities of Recombinants ApCpnA and ApCpnB

We expressed and purified the α- and β-subunits of a chaperonin from *A. pernix* K1. *ApCpnA* and *ApCpnB* were amplified by PCR from the *A. pernix* K1 shotgun clones, and transformed into *E. coli* Rosetta or Codonplus (DE3) cells, respectively. As shown in Fig. 1, expression of ApCpnA

(60.7 kDa) and ApCpnB (61.2 kDa) was confirmed by SDS-PAGE analysis. Recombinants ApCpnA and ApCpnB were purified to 92% and 94% purity, respectively, by heat-shock treatment and anion-exchange chromatography. In this study, the NTPase activities of the recombinants ApCpnA and ApCpnB were assayed by colorimetrical quantitation of released phosphate (Pi) using the malachite green assay. Enzyme-dependent NTP hydrolysis was maximal in the temperature range of 70–85°C. As shown in Fig. 2, ApCpnA and ApCpnB were able to hydrolyze not only ATP, but also CTP, GTP, and UTP. Purified ApCpnA and ApCpnB hydrolyzed the nucleotides with the following efficacy (from highest to lowest): ATP > CTP > UTP > GTP at 80°C, which is close to the optimal growth temperature of *A. pernix*. We also investigated the salt dependency of the ATPase activities of ApCpnA and ApCpnB. ApCpnA and ApCpnB had ATPase activity in the presence of various salts at the indicated concentrations.

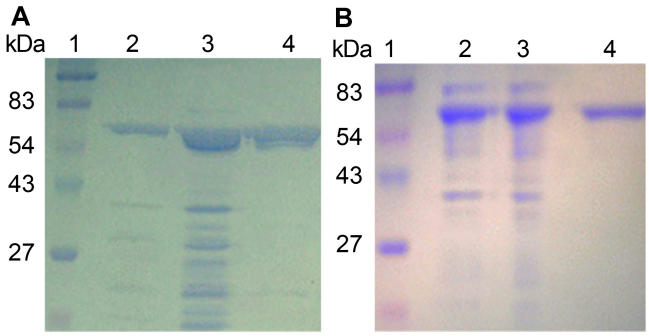

Fig. 1. Purification of recombinants ApCpnA (**A**) and ApCpnB (**B**).
1, Protein molecular weight marker; 2, Soluble faction after heat shock at 85°C for 20 min; 3, Soluble faction after dialysis; 4, Hitrap Q column peak fraction for ApCpnA (**A**) and ApCpnB (**B**).

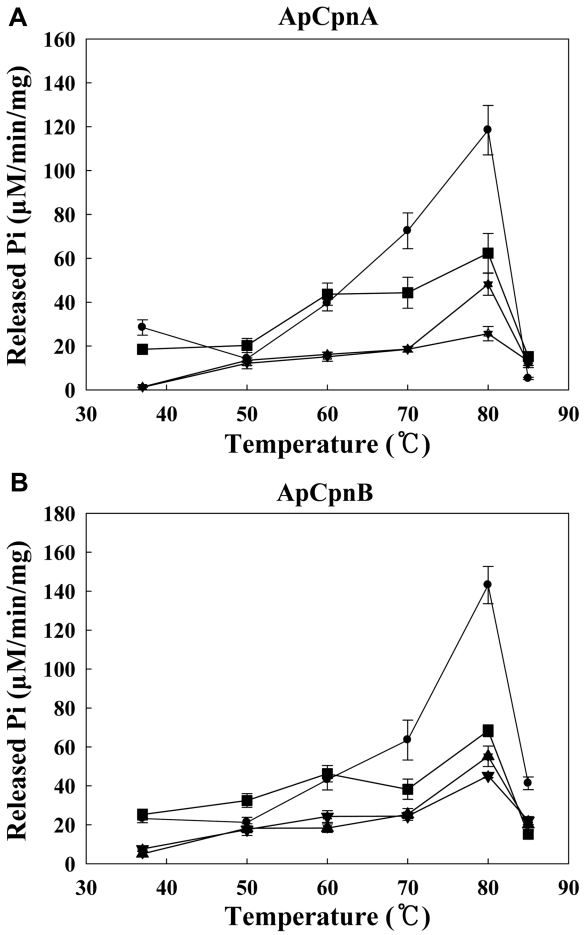

Fig. 2. NTPase activity of partially purified ApcpnA (**A**) and ApcpnB (**B**).
The partially purified soluble ApCpnA and ApCpnB fractions (20 μg/ml) obtained by heat-shock treatment were reacted with 2 mM NTPs and the reaction was allowed to proceed at different temperatures. (●) ATPase; (■) CTPase; (▲) GTPase; (▼) UTPase.

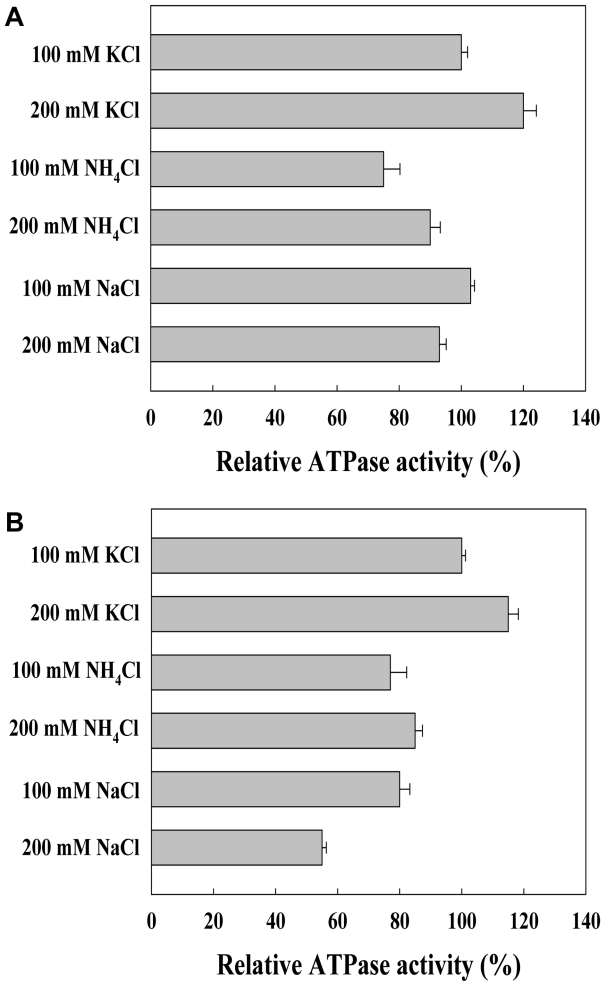

Fig. 3. Salt dependence of the ATPase activities of ApCpnA and ApCpnB.
ApCpnA (**A**) and ApCpnB (**B**) were incubated in buffers with different salts at the indicated concentrations. The initial rate of ATP hydrolysis was determined as described in the Materials and Methods section. Activity in 100 mM KCl was set to 100%.

As shown in Fig. 3, potassium ions were the most effective at stimulating the ATP hydrolysis activity of ApCpnA and ApCpnB. Among the archaea, potassium strongly stimulates the ATPase activity of the thermosome from *Sulfolobus solfataricus* [8] and has a mild stimulatory effect on the thermosome from *Thermoplasma acidophilum* [10]. The ATPase activities of ApCpnA and ApCpnB were found to vary strongly with the concentration of potassium ions (Fig. 4). Activity was maximal at 200 mM K^+.

ApCpnA and ApCpnB Prevent Thermal Aggregation and Inactivation of CS

To confirm that ApCpnA and ApCpnB have chaperonin activity, we investigated whether they protected CS from thermal aggregation and inactivation. The denaturation and renaturation of CS have been well documented [33], and CS has been used to investigate the molecular chaperone activities of heat-shock protein 90 (HSP90) and

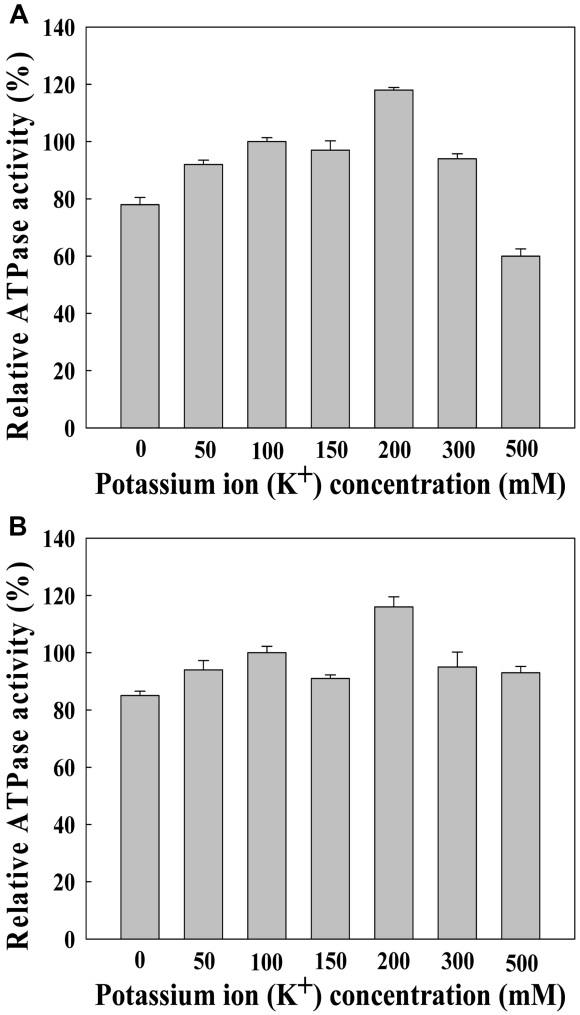

Fig. 4. Salt dependence of the ATPase activities of ApCpnA and ApCpnB.
ApCpnA (**A**) and ApCpnB (**B**) were incubated in buffers with KCl at the indicated concentrations. Initial rates of ATP hydrolysis were determined as described in the Materials and Methods section. Activity at 100 mM KCl was set to 100%.

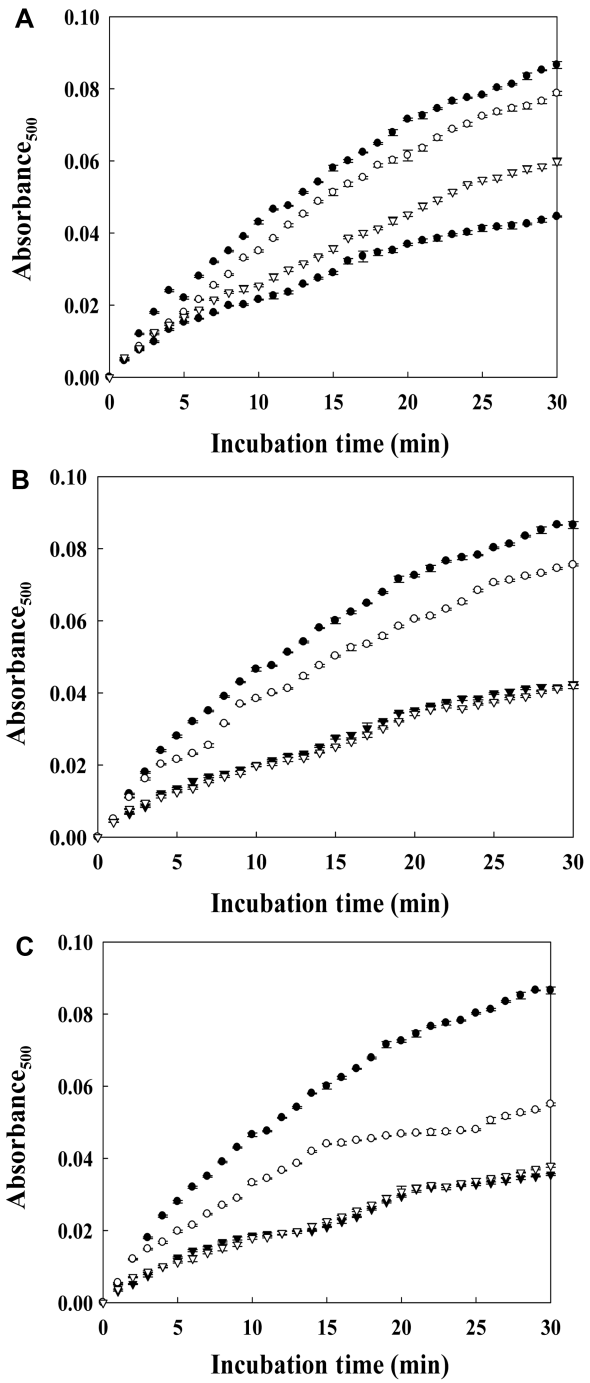

Fig. 5. Influence of the chaperonin system on the aggregation of heat-denatured CS at 43°C.
(**A**) (●) CS only; (○) CS:ApCpnA; (▼) CS:ApCpnA+ATP; (▽) CS:ApCpnA +CTP. (**B**) (●) CS only; (○) CS:ApCpnB; (▼) CS:ApCpnB+ATP; (▽) CS:ApCpnB+CTP. (**C**) (●) CS only; (○) CS:ApCpnA+ApCpnB; (▼) CS:ApCpnA+ApCpnB+ATP; (▽) CS:ApCpnA+ApCpnB+CTP.

associated proteins [29], GroEL [2], and small heat-shock proteins (sHSPs) [4]. In the absence of any substrate, CS is readily inactivated upon incubation at higher temperatures with a mid-point of transition at more than 43°C [32]. In the presence of ApCpnA and ApCpnB, the thermal aggregation of CS was significantly reduced compared with the control (Fig. 5). The addition of either the α- or β-subunit of the chaperonin decreased the thermal aggregation of CS by up to 20% and 30%, respectively. When both α- and β-subunits were added, thermal aggregation of CS was decreased by up to 70%. Furthermore, the addition of ATP or CTP further prevented thermal aggregation of CS. These data imply that the recombinants ApCpnA and ApCpnB bound to thermal aggregation-susceptible CS, resulting in reduction of the unfolding intermediate of CS. Furthermore, ApCpnA and ApCpnB may have diverted aggregated polypeptides towards the chaperonin-assisted folding pathway. To further investigate the effects of ApCpnA and ApCpnB on the thermal inactivation of CS, CS was heat treated for 20 min at 43°C, and then the residual activity of CS was measured in buffer C. CS was rapidly inactivated during incubation. However, the presence of ApCpnA and ApCpnB protected CS from thermal inactivation (Fig. 6). Addition of either the α- or β-subunit decreased thermal inactivation of CS by up to 40% and 30%, respectively. When both subunits were added, thermal inactivation of CS decreased by up to 80%. In particular, addition of ATP or CTP to ApCpnA and ApCpnB protected CS from thermal inactivation to an even greater extent than was observed in the absence of nucleotides. These results indicate that the unfolding intermediate of CS was protected from inactivation by the chaperone-like activity of ApCpnA and ApCpnB in the presence of ATP or CTP.

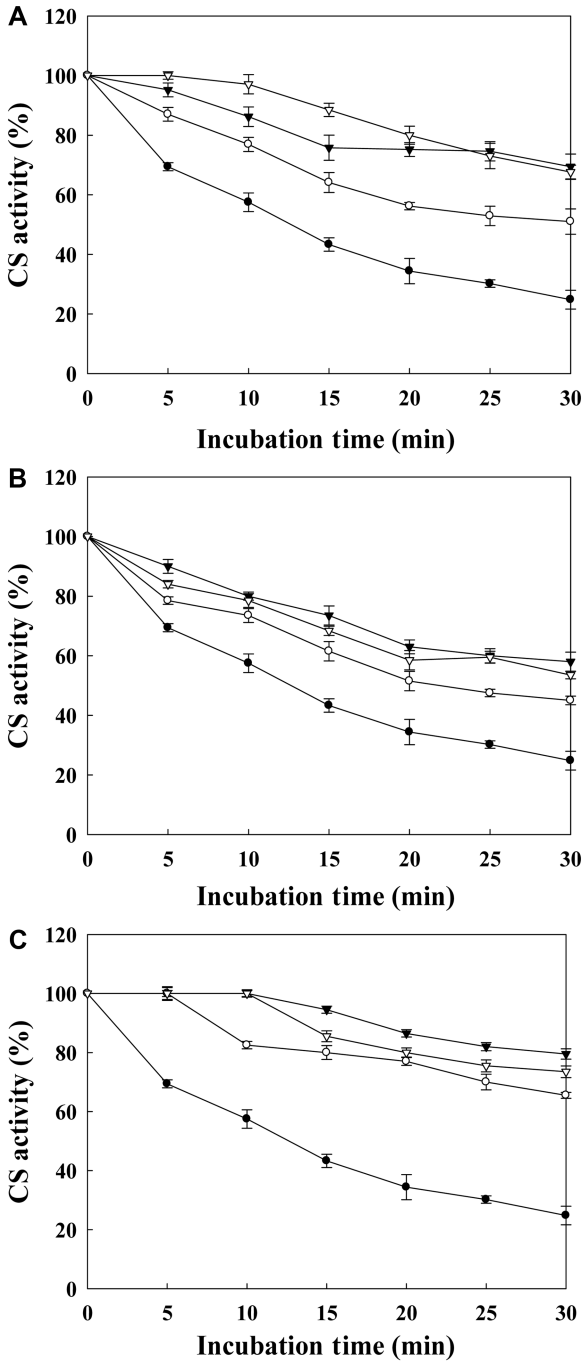

Fig. 6. Inactivation kinetics of CS at 43°C in the presence of ApCpnA and ApCpnB.
(**A**) (●) CS only; (○) CS:ApCpnA; (▼) CS:ApCpnA+ATP; (▽) CS:ApCpnA +CTP. (**B**) (●) CS only; (○) CS:ApCpnB; (▼) CS:ApCpnB+ATP; (▽) CS:ApCpnB+CTP. (**C**) (●) CS only; (○) CS:ApCpnA+ApCpnB; (▼) CS:ApCpnA+ApCpnB+ATP; (▽) CS:ApCpnA+ApCpnB+CTP.

ApCpnA and ApCpnB Prevent the Thermal Aggregation and Inactivation of ADH

The denaturation and renaturation of ADH have been used to investigate the molecular chaperone activities of *Thermococcus* sp. strain KS-1 [17] and *Pyrococcus* sp. strain KOD1 [30]. In this study, we used ADH as a second model protein to study the chaperonin activity of ApCpnA and ApCpnB. We evaluated the effects of ApCpnA and ApCpnB on the thermal aggregation of ADH at 50°C (Fig. 7). ADH aggregated rapidly during a 20 min incubation at 50°C. However, the addition of ApCpnA and ApCpnB effectively reduced the thermal aggregation of ADH (Fig. 7). Furthermore, the addition of ATP or CTP to ApCpnA and ApCpnB further prevented thermal aggregation of ADH compared with the control. The addition of either the α- or β-subunit decreased the thermal aggregation of ADH by up to 80% and 50%, respectively. When both α- and β-subunits were added, the thermal aggregation of ADH was decreased by up to 90%. To confirm the effects of ApCpnA and ApCpnB on the thermal inactivation of ADH, the residual activity of ADH was measured in buffer D after heat treatment for 20 min at 50°C. As shown in Fig. 8, ADH was rapidly inactivated during incubation. However, the presence of ApCpnA and ApCpnB significantly protected

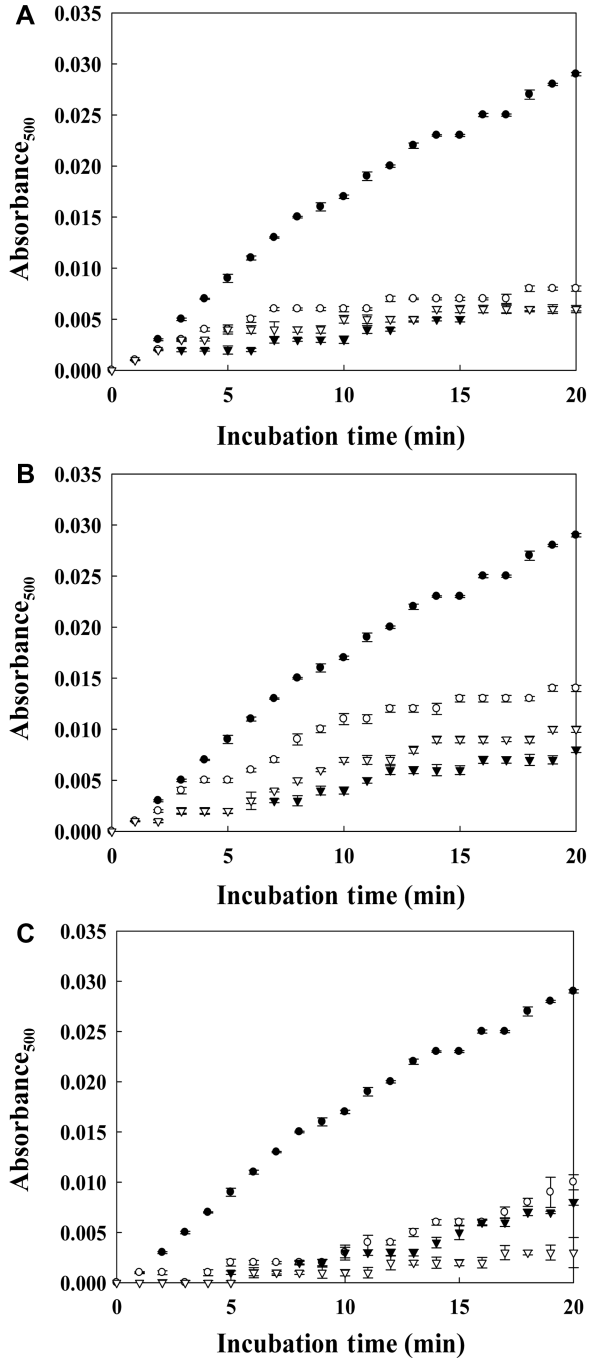

Fig. 7. Influence of the chaperonin system on aggregation of heat-denatured ADH at 50°C.
(**A**) (●) ADH only; (○) ADH:ApCpnA; (▼) ADH:ApCpnA+ATP; (▽) ADH:ApCpnA+CTP. (**B**) (●) ADH only; (○) ADH:ApCpnB; (▼) ADH:ApCpnB+ATP; (▽) ADH:ApCpnB+CTP. (**C**) (●) ADH only; (○) ADH:ApCpnA+ApCpnB; (▼) ADH:ApCpnA+ApCpnB+ATP; (▽) ADH:ApCpnA+ApCpnB+CTP.

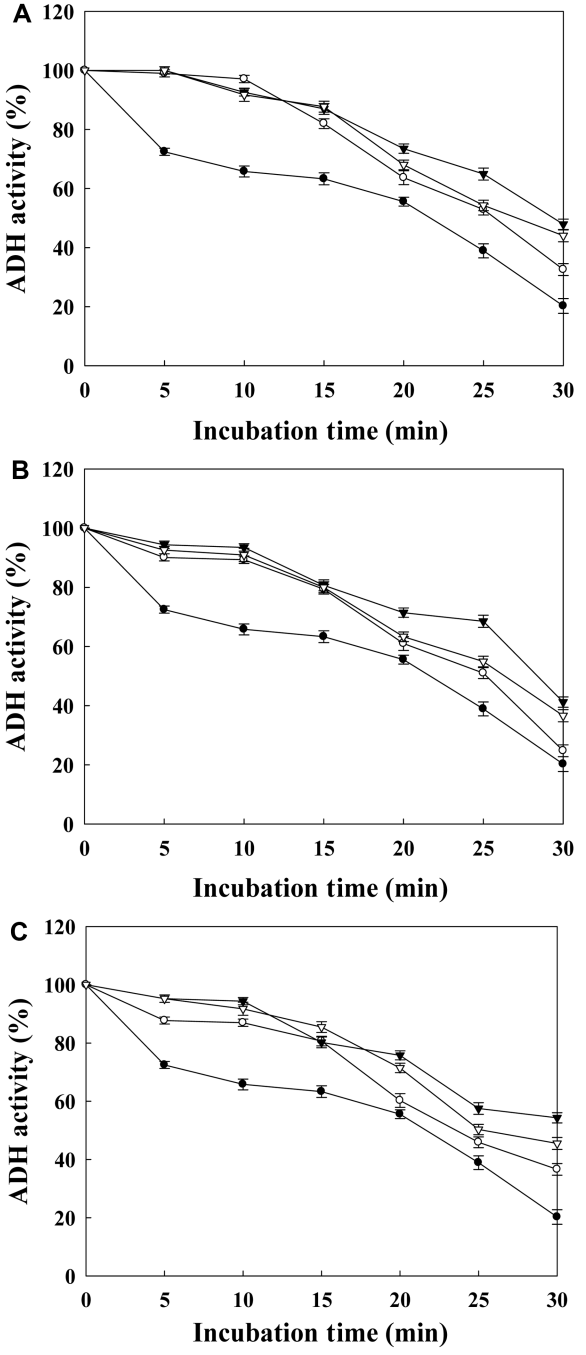

Fig. 8. Inactivation kinetics of ADH at 50°C in the presence of ApCpnA and ApCpnB.
(A): (●) ADH only; (○) ADH:ApCpnA; (▼) ADH:ApCpnA+ATP; (▽) ADH:ApCpnA+CTP. (B): (●) ADH only; (○) ADH:ApCpnB; (▼) ADH:ApCpnB+ATP; (▽) ADH:ApCpnB+CTP. (C): (●) ADH only; (○) ADH:ApCpnA+ApCpnB; (▼) ADH:ApCpnA+ApCpnB+ATP; (▽) ADH:ApCpnA+ApCpnB+CTP.

ADH activity. Furthermore, ADH activity was increased when ATP was added together with the chaperonins compared with addition of the chaperonins alone. These results suggested that ApCpnA and ApCpnB bound to the thermally unfolded substrate protein and prevented its denaturation by cooperating with ATP or CTP. Addition of

either the α- or β-subunit decreased the thermal inactivation of ADH up to 10% and 5%, respectively. When both the α- and β-subunits were added, the thermal inactivation of ADH was decreased by up to 90%.

In conclusion, the purified subunits of a *A. pernix* K1 chaperonin (ApCpnA and ApCpnB) prevented the thermal aggregation of CS and ADH, especially in the presence of ATP or CTP. Moreover, the thermal inactivation of CS and ADH was greatly reduced by the two chaperonin subunits, especially in the presence of ATP or CTP. Our results also suggested that protein folding activity is enhanced by cooperation between the α- and β-subunits of the *A. pernix* K1 chaperonin. ATP and CTP drive the conformational change of group II chaperonins from the open-lid substrate-binding conformation to the closed-lid conformation, thereby encapsulating an unfolded protein in the central cavity. The detailed mechanism of this conformational change remains unknown.

Acknowledgments

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