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Cooperativity of α- and β-Subunits of Group II Chaperonin from the Hyperthermophilic Archaeum *Aeropyrum pernix* K1

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α- and β-subunits (*ApCpnA* and *ApCpnB*) are group II chaperonins from the hyperthermophilic archaeum *Aeropyrum pernix* K1, specialized in preventing the aggregation and inactivation of substrate proteins under conditions of transient heat stress. In the present study, the cooperativity of α- and β-subunits from the *A. pernix* K1 was investigated. The *ApCpnA* and *ApCpnB* chaperonin genes were overexpressed in *E. coli* Rosetta and Codonplus (DE3), respectively. Each of the recombinant α- and β-subunits was purified to 92% and 94% by using anion-exchange chromatography. The cooperative activity between purified α- and β-subunits was examined using citrate synthase (CS), alcohol dehydrogenase (ADH), and malate dehydrogenase (MDH) as substrate proteins. The addition of both α- and β-subunits could effectively protect CS and ADH from thermal aggregation and inactivation at 43°C and 50°C, respectively, and MDH from thermal inactivation at 80°C and 85°C. Moreover, in the presence of ATP, the protective effects of α- and β-subunits on CS from thermal aggregation and inactivation, and ADH from thermal aggregation, were more enhanced, whereas cooperation between chaperonins and ATP in protection activity on ADH and MDH (at 85°C) from thermal inactivation was not observed. Specifically, the presence of both α- and β-subunits could effectively protect MDH from thermal inactivation at 80°C in an ATP-dependent manner.

Keywords: Chaperonin, hyperthermophilic archaeum, protein folding, *Aeropyrum pernix*

Molecular chaperonins are ubiquitous chaperones that are required for the correct folding, assembly, transport, and degradation of proteins within the cell [6, 9, 12, 16, 24, 26, 33, 34]. The chaperonins are seven- to nine-membered double-ring complexes, which can capture non-native proteins in their central cavity and mediate correct folding to the biologically active state in an ATP-dependent manner [1, 5, 9, 28, 35]. They are classified into two types [8]. Group I chaperonins are found in eubacteria, mitochondria, and chloroplasts, and group II chaperonins are found in archaea and eukaryotic cytosol [5, 21, 23, 35]. Group II chaperonins in archaea are also called thermosomes for their bearing higher temperature [27]. The group I chaperonins are a complex of a tetradecamer that is capped by the heptameric co-chaperone GroES [1, 10, 15, 22, 31]. 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 [13, 28, 31]. Every subunit of both groups of chaperonins shares a similar three-domain arrangement composed of an equatorial domain that contains an ATP binding site, apical domain that forms the opening of the central cavity, and intermediate domain that connects the apical and equatorial domains [4, 7]. Recently, the group II chaperonin was found to cooperate with a novel chaperone such as prefoldin [2, 3, 11, 14, 17, 25, 32], whereas the interaction and functional cooperation between chaperonin and prefoldin are not well understood. In addition, there is not much information on the cooperativity of group II chaperonins, especially concerning archaeal chaperonins. We have already reported that *ApCpnA* and *ApCpnB*, group II chaperonins from *A. pernix* K1, efficiently prevent the thermal aggregation and inactivation of foreign model proteins, such as citrate synthase (CS), alcohol dehydrogenase (ADH), and malate dehydrogenase (MDH) [29, 30]. Our recent study has reported that *PhCpn*, the group II chaperonin from the hyperthermophilic archaeum *Pyrococcus horikoshii* OT3, prevents protein aggregation and refolds denatured substrate in the presence of divalent metal ions such as Mg^{2+} ion [20].

The complete genome sequence of a hyperthermophilic archaeum *A. pernix* K1 revealed that this strain has two kinds of thermosome subunit genes (α- and β-subunits) [19]. In the present study, the cooperativity of the α- and β-

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subunits of group II chaperonins from the hyperthermophilic archaeum *A. pernix* K1 was investigated. We have expressed and purified the α- and β-subunits of chaperonin from the *A. pernix* K1. The cooperative activity between the purified α- and β-subunits as molecular chaperones was estimated using CS, ADH, and MDH as substrate proteins. The recent report showed that chaperonin required ATP to prevent protein aggregation and refold denatured substrate [18, 20, 29, 30]. Therefore, this study also examined whether cooperation between purified chaperonin and ATP can effectively inhibit the thermal aggregation and inactivation of CS, ADH, and MDH.

Materials and Methods

Bacterial Strains, Plasmids, and Reagents

In this study, *E. coli* DH5a was used for the preparation of plasmids, and *E. coli* Rosetta and Codonplus (DE3) [F$^-$, *ompT*, rB$^-$, mB$^-$] were used for the expression of *ApCpnA* and *ApCpnB*, respectively. Shotgun clones of *A. pernix* K1 containing a chaperonin ORF, APE0907 (*ApCpnA*) and APE2072 (*ApCpnB*), were purchased from NITE Biological Resource Center (NBRC, Chiba, Japan). The pET3d and pET21a plasmids for expressing *ApCpnA* and *ApCpnB* were purchased from Novagen Inc. (San Diego, CA, USA). Restriction enzymes, *ExTaq* DNA polymerase, and other reagents for gene manipulation were purchased from TaKaRa Shuzo (Kyoto, Japan). Citrate synthase (CS, EC 4.1.3.7) from porcine heart was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Alcohol dehydrogenase (ADH, EC 1.1.1.1), malate dehydrogenase (MDH, EC 1.1.1.37), and ATP were obtained from Sigma-Aldrich (MO, USA).

Construction and Transformation of Expression Plasmids for *ApCpnA* and *ApCpnB*

For expression of *ApCpnA*, a shotgun clone of *A. pernix* K1 containing a chaperonin ORF, APE0907, was used as a template for PCR amplification of the chaperonin gene. Oligonucleotide primers (5'-CCATGGTTGAGATGGCTGCGACGGGA-3' and 5'-GATT GGATCCTTAGAACTCGAACTTGCTGC-3'; the underlined sequences denote the *Bam*HI and *Nco*I restriction enzyme sites, respectively) were designed to add *Bam*HI and *Nco*I restriction digestion sites. The amplified DNA fragment was subcloned in a pT7Blue T vector (Novagen, WI, USA). After sequence confirmation, the ORF was digested by *Bam*HI and *Nco*I, and cloned into pET3d. After the confirmation by sequencing, the constructed pET3d-*ApCpnA* (6.1 kb) was transformed into *E. coli* Rosetta.

For expression of *ApCpnB*, a shotgun clone of *A. pernix* K1 containing a chaperonin ORF, APE2072, was used as a template for PCR amplification of the chaperonin gene. Oligonucleotide primers (5'-CCGGAACATATGGTTGACCGTGTGATCGA-3' and 5'-GATT GGATCCTTAGAACTCGAACTTGCTGC-3'; the underlined sequences denote the *Nde*I and *Bam*HI restriction enzyme sites, respectively) were designed to add *Nde*I and *Bam*HI restriction digestion sites. The amplified DNA fragment was subcloned in a pT7Blue T vector (Novagen, WI, USA). After sequence confirmation, the ORF was digested by *Nde*I and *Bam*HI, and subsequently cloned into pET21a. After the conformation by sequencing, the constructed pET21a-*ApCpnB* (6.9 kb) was transformed into *E. coli* Codonplus (DE3).

Expression and Purification of the Recombinant α- and β-Subunits

Each of *E. coli* Rosetta and Codonplus (DE3) cells, which were transformed with pET3d-*ApCpnA* and pET21a-*ApCpnB*, respectively was 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 8 h induction by IPTG, 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 for the separation into soluble and insoluble fractions.

In order to purify the expressed α- and β-subunits, the 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 band densities were scanned and quantified using the Gel-Pro Analyzer. The protein bands on the gels were scanned by the Gel-Pro Analyzer (Gel-Pro Analyzer 3.1 program; ING PLUS, Korea) and quantified by a densitometer.

Measurement of *In Vitro* Thermal Aggregation of CS and ADH

Thermal aggregation of CS from porcine heart was monitored by measuring the absorbance at 500 nm with a spectrophotometer (Shimadzu, Japan) at 43°C with continuous stirring. Monitoring started with the addition of CS (0.15 μM) to 40 mM HEPES-KOH (pH 7.5), preincubated for 15 min at 43°C, with or without both α- and β-subunits (final concentrations, 0.15 μM). To examine the chaperone activity, α- and β-subunits were added to the dilution buffer at a molar ratio of 1:1:1 (CS:α-subunit:β-subunit) with or without 2 mM ATP.

Thermal aggregation of ADH from *Saccharomyces cerevisiae* was monitored by measuring the absorbance at 500 nm with a spectrophotometer (Shimadzu, Japan) at 50°C with continuous stirring. Monitoring started with the addition of ADH (0.025 μM) to 50 mM phosphate buffer (pH 7.0), preincubated for 20 min at 50°C, with or without both α- and β-subunits (final concentrations, 0.005 μM). To investigate the chaperone activity, α- and β-subunits were added to the dilution buffer at a molar ratio of 5:1:1 (ADH:α-subunit:β-subunit) with or without 2 mM ATP.

Measurement of *In Vitro* Thermal Inactivation of CS, ADH, and MDH

CS, ADH, and MDH are often used in chaperone assays since these thermosensitive enzymes aggregate at moderately increased temperatures. CS catalyzes the reaction of oxaloacetate to citric acid. CS activity was measured as the amount of enzyme that catalyzed the synthesis of 1 μmole of citrate per 1 minute at 412 nm. To measure the thermal inactivation of CS (0.15 μM), a reaction mixture

containing a molar ratio of 1:1:1 (CS:α-subunit:β-subunit) with or without 2 mM ATP was incubated for 20 min at 43°C. Thermal inactivation of CS was monitored at 412 nm with a spectrophotometer (Shimadzu, Japan) at 25°C in buffer D (50 mM TE, pH 8.0, 0.1 mM oxaloacetic acid, 0.1 mM DTNB, and 0.15 mM acetyl-coA). The activity of native CS was calculated and taken to be 100%. The rest of the data were described as relative percentage.

In the same way, ADH catalyzes the reaction of ethanol to acetaldehyde. ADH activity was measured *via* increasing β-$NADH^+$ with absorption at 340 nm. To measure the thermal inactivation of ADH (0.025 μM), a reaction mixture containing a molar ratio of 5:1:1 (ADH:α-subunit:β-subunit) with or without 2 mM ATP was incubated at 50°C for 20 min. Thermal inactivation of ADH was monitored at 340 nm with a spectrophotometer (Shimadzu, Japan) at 25°C in buffer E (100 mM glycine-NaOH, pH 8.8, 1 mM β-$NADH^+$, and 100 mM ethanol). The activity of native ADH was calculated and taken to be 100%. The rest of the data were described as relative percentage.

MDH catalyzes the reaction of malate to oxaloacetate. To measure the thermal inactivation of MDH from *Thermus flavus*, MDH (0.4 μM) was incubated at 80°C and 85°C for 30 min, respectively. Additionally, a reaction mixture containing a molar ratio of 1:1:1 (MDH:α-subunit:β-subunit) with or without 2 mM ATP was incubated for 30 min at 80°C and 85°C, respectively. The final concentrations of α- and β-subunits were 0.4 μM. Thermal inactivation of MDH was monitored at 340 nm with a spectrophotometer (Shimadzu, Japan) at 25°C in buffer F (100 mM potassium phosphate, pH 7.5, 0.25 mM β-NAD, and 0.5 mM malate). The activity of native MDH was calculated and taken to be 100%. The rest of the data were described as relative percentage.

RESULTS AND DISCUSSION

Prevention of *In Vitro* Thermal Aggregation of CS and ADH by Cooperation between α- and β-Subunits

In our previous study, we expressed and purified α- and β-subunits of chaperonin from *A. pernix* K1. *ApCpnA* and *ApCpnB*, chaperonin genes from *A. pernix* K1, were amplified by PCR from the *A. pernix* K1 genomic DNA, and expressed in *E. coli* Rosetta and Codonplus (DE3), respectively. Each of the recombinant α- and β-subunits was purified to 92% and 94% by using anion-exchange chromatography [29, 30].

In this work, the cooperative activity between purified α- and β-subunits as molecular chaperones was estimated by using CS, ADH, and MDH as substrate proteins. When CS and ADH were preincubated at 43°C and 50°C, respectively, their thermal aggregation was observed (Fig. 1). However, this thermal aggregation of CS and ADH could be inhibited by adding α- or β-subunits at 43°C and 50°C, respectively [29, 30]. In particular, the addition of both α- and β-subunits remarkably inhibited thermal aggregation of CS (Fig. 1A) and ADH (Fig. 1B). In Fig. 1A, the single addition of α- or β-subunits attenuated thermal aggregation of CS up to 50% and 80%, respectively. Based on these data, the α-subunit more effectively protected CS from thermal aggregation than did the β-subunit. In the presence of both α- and β-subunits (at a molar ratio of 1:1:1 with CS:α-subunit:β-subunit), thermal aggregation of CS was dramatically inhibited up to 90% compared with reaction without both α- and β-subunits. Moreover, the addition of 2 mM ATP with both α- and β-subunits more efficiently prevented thermal aggregation of CS (Fig. 1A). In a recent study, it was reported that chaperonin requires ATP to prevent protein aggregation and refold denatured substrate [18, 20, 29, 30]. Our result is consistent with the recent reports in which chaperonin could prevent protein aggregation and refold denatured substrate in the presence of ATP [20, 29, 30]. These results show that cooperation between α- and β-subunits can enhance protection activity from thermal aggregation on CS at 43°C in an ATP-dependent manner (Fig. 1A). As shown in Fig. 1B, the

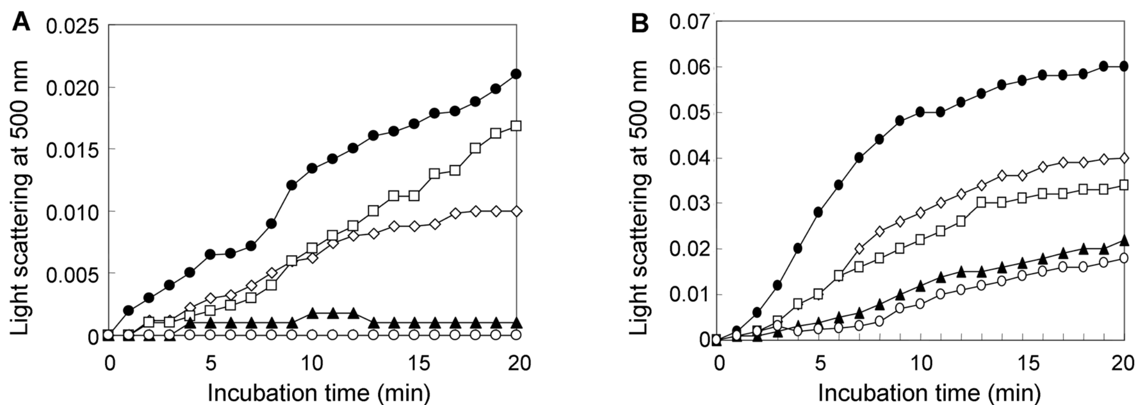

Fig. 1. Prevention of thermal aggregation of CS and ADH by cooperation between α- and β-subunits of group II chaperonin from *A. pernix* K1.

A. Inhibition of thermal aggregation of CS at 43°C in the presence of both α- and β-subunits. Symbols: (●), CS only; (◇), CS:α-subunit (1:1); (□), CS:β-subunit (1:1); (▲), CS:α-subunit:β-subunit (1:1:1); (○), CS:α-subunit:β-subunit (1:1:1)+ATP. **B**. Inhibition of thermal aggregation of ADH at 50°C in the presence of both α- and β-subunits. Symbols: (●), ADH only; (◇), ADH:α-subunit (5:1); (□), ADH:β-subunit (5:1); (▲), ADH:α-subunit:β-subunit (5:1:1); (○), ADH:α-subunit:β-subunit (1:1:1)+ATP.

single addition of α- or β-subunits decreased thermal aggregation of ADH up to 60% and 50%, respectively. The β-subunit protected ADH from thermal aggregation slightly more effectively than did the α-subunit. In the case of the addition of both α- and β-subunits (at a molar ratio of 5:1:1 with ADH:α-subunit:β-subunit), thermal aggregation of ADH was more remarkably attenuated up to 30%. Moreover, the addition of 2 mM ATP with both α- and β-subunits more efficiently prevented thermal aggregation of ADH.

In conclusion, our results indicate that addition of both α- and β-subunits can effectively protect CS and ADH from thermal aggregation at 43°C and 50°C, respectively. Moreover, in the presence of ATP, the addition of both α- and β-subunits enhances protection activity on CS and ADH from thermal aggregation. Therefore, these results suggest that the cooperation of α- and β-subunits from the *A. pernix* K1 is effective in enhancement of the protein holding activity. The holding activity of the α- and β-subunits from *A. pernix* K1 will provide a reliable and relevant strategy for prolonging the process stability of recombinant biocatalysts.

Prevention of *In Vitro* Thermal Inactivation of CS, ADH, and MDH by Cooperation between α- and β-Subunits

This study has examined whether the cooperation between purified α- and β-subunits can inhibit thermal inactivation of CS, ADH, and MDH. The cooperative activity between α- and β-subunits was estimated by measurement of thermal inactivation of CS, ADH, and MDH. When CS, ADH, and MDH were preincubated at 43°C, 50°C, and 85°C, respectively, their activities decreased (Fig. 2). However, in our previous report, it was shown that the single addition of α- or β-subunit could protect CS, ADH, and MDH from thermal inactivation [29, 30], and α-

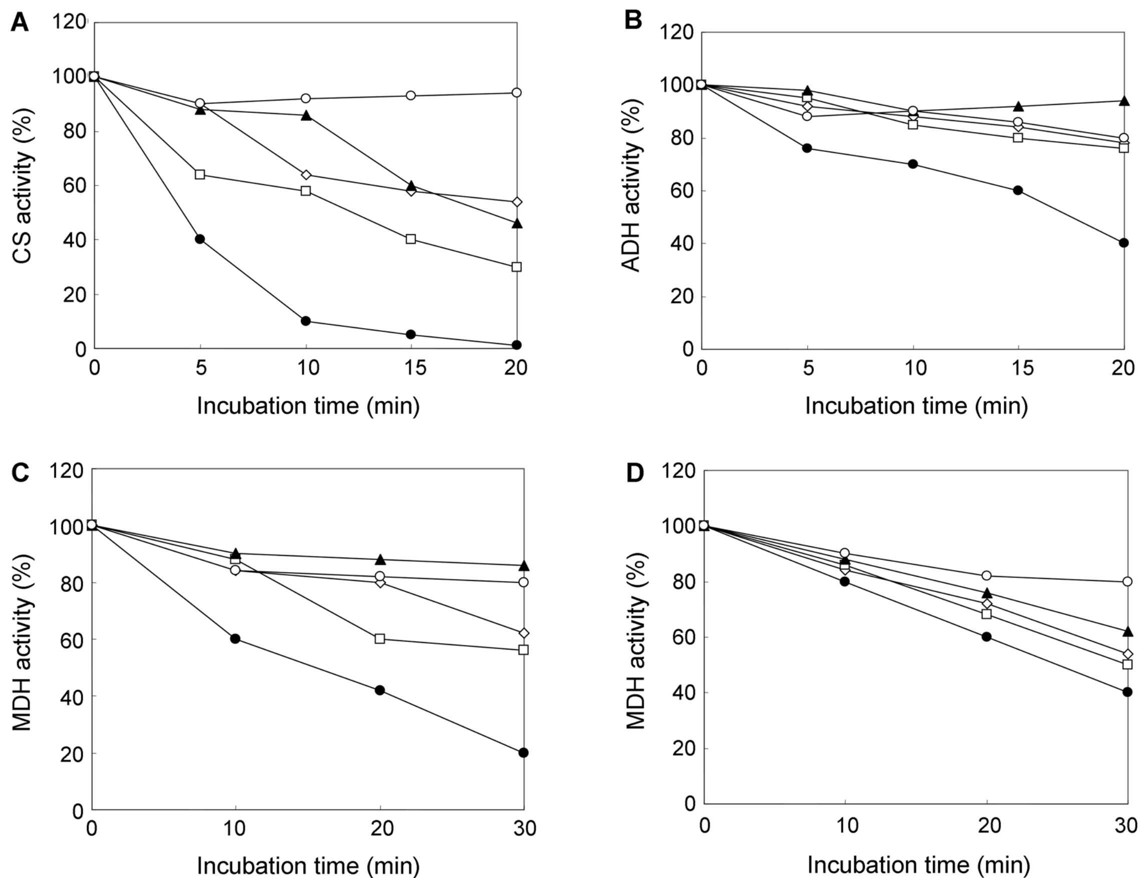

Fig. 2. Prevention of *in vitro* thermal inactivation of CS, ADH, and MDH by cooperation between α- and β-subunits of group II chaperonin from *A. pernix* K1.

A. Inhibition of thermal inactivation of CS at 43°C in the presence of both α- and β-subunits. Symbols: (●), CS only; (◇), CS:α-subunit (1:1); (□), CS:β-subunit (1:1); (▲), CS:α-subunit:β-subunit (1:1:1); (○), CS:α-subunit:β-subunit (1:1:1)+ATP. **B**. Inhibition of thermal inactivation of ADH at 50°C in the presence of both α- and β-subunits. Symbols: (●), ADH only; (◇), ADH:α-subunit (5:1); (□), ADH:β-subunit (5:1); (▲), ADH:α-subunit:β-subunit (5:1:1); (○), ADH:α-subunit:β-subunit (5:1:1)+ATP. **C**, **D**. Inhibition of thermal inactivation of MDH at 85°C (**C**) and 80°C (**D**) in the presence of both α- and β-subunits. Symbols: (●), MDH only; (◇), MDH:α-subunit (1:1); (□), MDH:β-subunit (1:1); (▲), MDH:α-subunit:β-subunit (1:1:1); (○), MDH:α-subunit:β-subunit (1:1:1)+ATP.

subunit more effectively prevents their thermal inactivation than does the β-subunit. Moreover, in the present study, the addition of both α- and β-subunits significantly prevented thermal inactivation of CS, ADH, and MDH (Fig. 2).

In Fig. 2A, the CS activity was increased up to 50% and 30% by the single addition of α- or β-subunits, respectively. The presence of both α- and β-subunits (at a molar ratio of 1:1:1 with CS:α-subunit:β-subunit) also inhibited thermal inactivation of CS more extensively than the reaction without both α- and β-subunits (Fig. 2A). The CS activity was 46% recovered efficiently by adding α- and β-subunits. Furthermore, CS activity was more increased (up to 94%) by adding 2 mM ATP, compared with the reaction with both α- and β-subunits (Fig. 2A). However, recovery of the CS activity was only slightly induced by the addition of ATP without α- and β-subunits (data not shown). Therefore, our results show that cooperation between chaperonin and ATP causes effective protection of CS from thermal inactivation. The recovery pattern of thermal inactivation of ADH was different from CS (Fig. 2B). As shown in Fig. 2B, the activity of ADH at 50°C for 20 min rapidly decreased to 40%. On the other hand, ADH activity was increased up to 85% and 80% by the single addition of α- or β-subunits, respectively. In particular, the presence of both α- and β-subunits (at a molar ratio of 5:1:1 with ADH:α-subunit:β-subunit) effectively increased ADH activity up to 98%. The addition of 2 mM ATP with both α- and β-subunits increased ADH activity up to 86%, similar to the results of the single addition of α- or β-subunits. As shown in Fig. 2C, MDH activity at 85°C for 30 min rapidly decreased to 20%, whereas the single addition of α- or β-subunits increased MDH activity up to 70% and 60%, respectively. Moreover, the addition of 2 mM ATP with both α- and β-subunits increased thermal inactivation of ADH up to 86%. In particular, the addition of both α- and β-subunits (at a molar ratio of 1:1:1 with MDH:α-subunit:β-subunit) had a more remarkably induced thermal protection effect on MDH. In the case of protection activity on ADH and MDH (at 85°C), cooperation between chaperonin and ATP was not observed. Because the ATPase activity of α- or β-subunit was reduced at 85°C in our previous study [29, 30], this study investigated whether the addition of both α- and β-subunits can enhance the protection activity on MDH from thermal inactivation at 80°C, which was close to the optimal temperature of *A. pernix* K1 (Fig. 2D). As shown in Fig. 2D, MDH activity at 80°C rapidly reduced to 40%, whereas the single addition of α- or β-subunits increased MDH activity up to 62% and 60%, respectively. The addition of both α- and β-subunits (at a molar ratio of 1:1:1 with MDH:α-subunit:β-subunit) more remarkably induced the thermal protection effect on MDH in an ATP-dependent manner similar to that of CS.

In conclusion, our results indicate that both α- and β-subunits can effectively protect CS, ADH, and MDH from thermal inactivation. Furthermore, the addition of both α- and β-subunits enhances protection activity on CS and MDH (at 80°C) from thermal inactivation in an ATP-dependent manner, whereas cooperation between chaperonin and ATP in protection activity on ADH and MDH (at 85°C) from thermal inactivation was not observed. Therefore, these results suggest that the protein holding activity is enhanced by the cooperation of α- and β-subunits from *A. pernix* K1. Based on these results, we propose that the α- and β-subunits from *A. pernix* K1 can be utilized to enhance the durability and cost-effectiveness of high-temperature biocatalysts.

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