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 toroidal 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 Mg²⁺ 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-
subunits of group II chaperonins from the hyperthermophilic archaeon *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) 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, *Ex*Taq 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'-CCATGGTTGATGCGTCGACGGGA-3' and 5'-GATTGATAGTACACTGACTGCTGC-3'; the underlined sequences denote the BamHI and *NcoI* restriction enzyme sites, respectively) were designed to add BamHI and *NcoI* restriction digestion sites. The amplified DNA fragment was subcloned in pET7Blue T vector (Novagen, WI, USA). After sequence confirmation, the ORF was digested by BamHI and *NcoI*, 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'-CCGGAACCAATATGTGCTCCAGCTGACGGGA-3' and 5'-GATTGATAGTACACTGACTGCTGC-3'; the underlined sequences denote the *NdeI* and BamHI restriction enzyme sites, respectively) were designed to add *NdeI* and BamHI restriction digestion sites. The amplified DNA fragment was subcloned in a pET7Blue T vector (Novagen, WI, USA). After sequence confirmation, the ORF was digested by *NdeI* and BamHI, and subsequently cloned into pET21a.

After the confirmation 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 preincubated 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 MgCl₂, 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 50°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 oxaalocetate 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