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ORIGINAL PAPER

Construction and characterization of the hetero-oligomer of the group II chaperonin from the hyperthermophilic archaeon, *Thermococcus* sp. strain KS-1

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Abstract The hyperthermophilic archaeon *Thermococcus* sp. strain KS-1 (*T.* KS-1) expresses two different chaperonin subunits, α and β, for the folding of its proteins. The composition of the subunits in the hexadecameric double ring changes with temperature. The content of the β subunit significantly increases according to the increase in temperature. The homo-oligomer of the β subunit, Cpnβ, is more thermostable than that of the α subunit, Cpnα. Since Cpnα and Cpnβ also have different protein folding activities and interactions with prefoldin, the hetero-oligomer is thought to exhibit different characteristics according to the content of subunits. The hetero-oligomer of the *T.* KS-1 chaperonin has not been studied, however, because the α and β subunits form hetero-oligomers of varying compositions when they are expressed simultaneously. In this study, we characterized the *T.* KS-1 chaperonin hetero-oligomer, Cpnαβ, containing both α and β in the alternate order, which was constructed by the expression of α and β subunits in a coordinated fashion and protease digestion. Cpnαβ protected citrate synthase from thermal aggregation, promoted the folding of acid-denatured GFP in an ATP-dependent manner, and exhibited an ATP-dependent conformational change. The yield of refolded GFP generated by Cpnαβ was almost equivalent to that generated by Cpnβ but lower than that generated by Cpnα. In contrast, Cpnαβ exhibited almost the same level of thermal stability as Cpnα, which was lower than that of Cpnβ. The affinity of Cpnαβ to prefoldin was found to be between those of Cpnα and Cpnβ, as expected.

Keywords *Thermococcus* chaperonin · Hetero-oligomer · Thermal stability · Interaction with prefoldin

Abbreviations

T. KS-1	Hyperthermophilic archaeon *Thermococcus* sp. strain KS-1
CCT	Chaperonin containing t-complex-polypeptide 1
PhPFD	*Pyrococcus horikoshii* prefoldin
CS	Citrate synthase from porcine heart
GFP	Green fluorescence protein
CD	Circular dichroism spectroscopy
SPR	Surface plasmon resonance
PAGE	Polyacrylamide gel electrophoresis
Cpnα	*T.* KS-1 chaperonin α homo-oligomer
Cpnβ	*T.* KS-1 chaperonin β homo-oligomer
Cpnαβ	*T.* KS-1 chaperonin hetero-oligomer containing α and β, alternately

Introduction

Chaperonins, a ubiquitous class of molecular chaperones, are double-ring assemblies of about 60 kDa subunits. Each ring has a large central cavity, in which, a non-native protein can undergo productive folding in an ATP-dependent manner (Bukau and Horwich 1998; Ranson et al. 1998). Based on the protein sequence similarity and the features of their structures, chaperonins can be separated into two groups, group I and group II. Whereas group I chaperonins, represented by GroEL of *Escherichia coli*, are found in eubacteria, eukaryotic organelles, mitochondria,

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and chloroplasts; group II chaperonins are found in archaea and in the cytoplasm of eukaryotic cells (Dunn et al. 2001; Hartl and Hayer-Hartl 2002; Klumpp and Baumeister 1998). The group I chaperonin (GroEL) is built by 14 identical copies of 57 kDa subunits, organized into two heptameric rings that create two open cavities. To promote protein folding, GroEL cooperates with a heptameric ring of GroES, a co-chaperone of 10 kDa (Bukau and Horwich 1998; Ranson et al. 1998). GroES acts as a detachable lid for the cavity of group I chaperonins. Group II chaperonins are weakly homologous to group I chaperonins and function without an analogous co-factor. The eukaryotic chaperonin containing TCP-1 (CCT), also termed TRiC, assists in the folding of a small set of proteins (Dunn et al. 2001). CCT is also built by two rings; each ring contains eight different, albeit homologous, subunits (30% identity).

The archaeal chaperonins are double-ring complexes of identical or diverse subunits. Two chaperonin genes are present in most complete archaeal genomes. Some thermophilic methanogens [e.g. *Methanopyrus kandleri*, *Methanococcus jannaschii* (*Methanocaldococcus jannaschii*), and *Methanococcus thermolithrotrophicus*] have chaperonins with identical subunits (Andra et al. 1996; Furutani et al. 1998; Kowalski et al. 1998). Some archaea are equipped with more than three chaperonin subunits. *Sulfolobus* spp. and *M. burtonii* contain three different subunits. Recently, it was found that there are five chaperonin subunits (Hsp60-1, -2, -3, -4 and -5) in *Methanosarcina acetivorans* (Maeder et al. 2005). Among them, Hsp60-1, -2, and -3 have orthologs in *Methanosarcinae*, but others, Hsp60-4 and -5, occur only in *M. acetivorans*. In most archaeal group II chaperonins, the subunit composition is thought to be constant. However, it changes according to growth temperature in several archaeal chaperonins (e.g. *Sulfolobus shibatae*, *Thermococcus* sp. strain KS-1, *Thermococcus kodakaraensis*) (Kagawa et al. 2003; Yoshida et al. 2001; Izumi et al. 2001).

The hyperthermophilic archaeon, *Thermococcus* sp. strain KS-1 (*T.* KS-1), has two homologous chaperonin subunits, α and β (Yoshida et al. 1997). The natural composition of subunits in the hexadecameric double ring changes with temperature (Yoshida et al. 2001). The β subunit is significantly more abundant at the higher temperature than the α subunit. The homo-oligomer of the β subunit (Cpnβ) is also more thermostable than the homo-oligomer of the α subunit (Cpnα) (Yoshida et al. 2002a). It has been suggested that the stability can be attributed to differences in 20 amino acids at the C-terminal end (Yoshida et al. 2006). Although Cpnα and Cpnβ have been well studied (Iizuka et al. 2003, 2004, 2005; Shomura et al. 2004; Yoshida et al. 1997, 2002a, b), the hetero-oligomer of the *T.* KS-1 chaperonin has not yet been studied. Because of the high homology, the α and β subunits form hetero-oligomers with varying compositions when the α and β subunits are simultaneously expressed. It has been predicted that thermal stability of the hetero-oligomer of the *T.* KS-1 chaperonin is between Cpnα and Cpnβ. In addition, our recent study has indicated that Cpnβ exhibits a stronger interaction with prefoldin than Cpnα (Zako et al. 2006).

In this study, we characterized the *T.* KS-1 chaperonin hetero-oligomer, Cpnαβ, containing both α and β; this complex was constructed by a coordinated expression of the α and β subunits and protease digestion.

Materials and methods

Enzymes, proteins, and reagents

The restriction endonucleases were purchased from Takara Bio (Shiga, Japan). Thrombin protease was purchased from GE Healthcare (Buckinghamshire, UK). *Pyrococcus horikosii* prefoldin was prepared as described (Okochi et al. 2004). The GFP used in this study was a thermostable mutant. It was expressed and purified as described previously (Iizuka et al. 2001). Citrate synthase (CS) from porcine heart was obtained from Sigma (St. Louis, MO). The ammonium sulfate suspension of CS was desalted on a NAP-5 column (GE Healthcare) before use. ATP was purchased from Wako Chemicals (Tokyo, Japan). Other reagents were also products of Wako Chemicals.

Construction of the plasmid for expressing the chaperonin hetero-complex, Cpnαβ, composed of connected α and β subunits

PCR amplification was performed on the plasmid containing the wild-type α subunit gene using the primer set (5′-CAT ATG GCA CAG CTT AGT GGA CAG CCG G-3′, 5′-CGG GAT CCA TTA CCA CGA GGA TAC ATG CCC ATT CCG CCG GG-3′). The amplified DNA fragment contains an *Nde*I digestion site at the N terminus and a *Bam*HI digestion site at the C terminus; it also encodes the α subunit from the N-terminal initiation codon, but the C-terminal sequence is replaced with the thrombin protease sequence and does not contain a deletion of the translation stop codon. The β subunit gene was also amplified to introduce a *Bgl*II digestion site at the N terminus without the initiation codon and a *Bam*HI site at the C terminus using the primer set (5′-GAA GAT CTG CCC AGC TTG CAG GCC AG-3′, 5′-CGG GAT CCT CAG TCG AGA TCG CTT CCG AAG TC-3′). The amplified fragments were sub-cloned into the pT7Blue T-vector (Novagen, Madison, WI). After sequence confirmation, the inserted DNA fragments were excised with *Nde*I/*Bam*HI or *Bgl*II/

*Bam*HI and then excised from the gel and purified. The fragment containing the α subunit gene was sub-cloned into the *Nde*I/*Bam*HI site of the pET9a vector (Novagen). And then, the β subunit gene was sub-cloned into the *Bam*HI site of pET9a containing the α subunit gene.

Purification of *T.* KS-1 chaperonin complexes

T. KS-1 chaperonin homo-oligomers, Cpnα and Cpnβ, and the hetero-oligomer, Cpnαβ, were expressed in the *E. coli* strain BL21(DE3) and BL21*pRare, respectively. They were cultured at 30°C in 2× YT medium (1.6% tryptone, 1% yeast extract, and 0.5% NaCl) containing 75 μg/ml kanamycin. Cells were harvested by centrifugation and stored at −80°C until use. The harvested cells were thawed in 50 mM Tris–HCl pH 7.5 and disrupted by sonication. The chaperonin complexes were purified as described (Iizuka et al. 2001). In the case of Cpnαβ, the purified chaperonin complex was subjected to cleavage by incubation with thrombin (1 mg/100 unit thrombin) at 22°C for 40 h. Then, the thrombin was removed by gel filtration using HiLoad 26/60 superdex 200 prep grade (GE Healthcare) with buffer A (50 mM Tris–HCl pH 7.5, 25 mM $MgCl_2$, and 1 mM DTT). Purified chaperonins were concentrated by ultrafiltration (Centriprep YM-10, Millipore, MA).

ATP hydrolysis activity

ATP hydrolysis activities were measured at several temperatures in a 300 μl reaction mixture containing 50 mM Tris–HCl pH 8.0, 300 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, with or without 15 μg chaperonins. The reaction was initiated by the addition of ATP and terminated by mixing with ice-cold 2% perchloric acid. The amount of Pi that was produced by spontaneous hydrolysis of ATP was the substrate used to calculate the ATP hydrolysis activity.

Thermal stability of the chaperonin oligomers

To examine the thermal stability of the ATPase activity of the chaperonin, chaperonin oligomers (60 μl) (final concentration of 0.5 μg/μl) were incubated at 80, 85, 90, and 95°C for 30 min. In the time course experiments, chaperonin oligomers were incubated at 95°C then aliquots were taken out at specific points in time. Then the proteins were analyzed by native PAGE and stained by Coomassie brilliant blue.

Circular dichroism measurements

Thermal denaturation of the chaperonins was monitored by measuring circular dichroism (CD) at 222 nm. CD measurements were carried out as described (Yoshida et al. 2006).

Fluorometric monitoring of GFP refolding

GFP refolding reactions were carried out at 60°C. Acid-denatured GFP solutions (25 μM) were diluted 150-fold into the folding buffer (buffer A containing 100 mM KCl and 5 mM DTT) in the presence and absence of 0.25 μM chaperonins. ATP was added to 1 mM of the folding buffer or to the reaction mixture after a 5 min incubation. The fluorescence of GFP at 510 nm, with an excitation of 396 nm, was continuously monitored with a fluorophotometer, RF-5300PC (Shimadzu, Kyoto, Japan) with continuous stirring. As a control, native GFP was diluted into the folding buffer without the chaperonins. The fluorescence intensity of native GFP was taken as 100%.

Protection of CS from thermal aggregation

Thermal aggregation of CS was monitored with light scattering at 500 nm with a fluorophotometer at 50°C. CS (120 nM as monomer) was incubated in the assay buffer (buffer A containing 100 mM KCl and 5 mM DTT) in the presence or absence of chaperonins (120 nM). The assay buffer was pre-incubated at 50°C and continuously stirred throughout the measurement.

Protease sensitivity assay

Chaperonins were incubated with or without nucleotide (1 mM) at 60°C in TKM buffer under continuous mixing. Digestion with thermolysin (1 ng/μl) was carried out for 5 min at 60°C. Proteins in the reaction mixture were precipitated with the addition of trichloroacetic acid and then analyzed on 12% SDS gels. Gels were stained with Coomassie brilliant blue R-250.

Analysis of the prefoldin–chaperonin interaction by surface plasmon resonance

Surface plasmon resonance (SPR) experiments were performed with a Biacore J system (Biacore AB, Uppsala, Sweden) at the sensor temperature of 25°C as described. PhPFD was coupled to the sensor chip (CM5 research grade) via standard *N*-hydroxysuccinimide and *N*-ethyl-*N*-(dimethylaminopropyl)carbodiimide activation. For immobilizing PhPFD, 190 μl of 50 ng/ml prefoldin in 10 mM sodium phosphate buffer (pH 7.5) was injected on the sensor surface; 50 mM Tris–HCl buffer (pH 7.5) was then injected to quench the unreacted *N*-hydroxysuccinimide groups. The mobile phase buffer used TKM buffer (50 mM Tris–HCl pH 7.5, 100 mM KCl, 10 mM

$MgCl_2$). Analytes were injected at various concentrations and the bound analytes were degenerated by loading 50 mM Tris–HCl pH 9.0 at 300 s after injection. Kinetic constants were calculated from the sensorgrams with BIAevaluation software, version 3.1 (Biacore) using the bivalent model [first step: L + A ↔ L.A, second step: L + L.A ↔ L2.A; L, ligand (PhPFD); A, analyte (CPN)] as the best-fit model. Association constants (K_A) were calculated by the resonance unit at equilibrium using the following equation:

$$R_{eq} = R_{max}C/(C + 1/K_A)$$

where R_{eq} is equilibrium resonance units, R_{max} is the resonance signal at saturation, and C is the concentration of free analyte. Relative K_A values were calculated by dividing the K_A values by the highest K_A value.

Results and discussion

Expression and purification of the *T.* KS-1 chaperonin hetero-oligomer containing α and β subunits in the alternate order

Since *T.* KS-1 α and β chaperonin subunits are highly homologous to each other, they assemble into oligomers randomly. Therefore, we engineered genes that encode covalently linked α and β subunits to obtain the *T.* KS-1 chaperonin hetero-oligomer containing α and β subunits in the alternate order. Previously, *T.* KS-1 chaperonin homo-oligomers of two or four covalently linked α subunits were constructed by connecting the N-terminal Val 9 residue to the C-terminal Ala 526 residue (Furutani et al. 2005). Although these chaperonins formed proper double-ring structures capable of capturing unfolded proteins and exhibiting ATPase activity, they lacked ATP-dependent protein folding activity. We attributed the loss of folding activity to the decreased flexibility of the N and C termini of the subunits, which is necessary for the ATP-dependent conformational change. We then tried to obtain active chaperonin complexes by specifically digesting the linker sequence (Kanzaki et al. 2008). The C-terminal amino acid sequence of the wild-type subunit was substituted with the thrombin recognition sequence (MYPR|GN), and then two, four, or eight subunits were covalently connected with flexible linkers (Gly-Ser). The connected chaperonin complexes exhibited reduced GFP folding activity compared to the wild-type α homo-oligomer (Cpnα), but this activity was recovered to a wild-type level upon thrombin digestion.

We prepared the *T.* KS-1 chaperonin hetero-oligomer containing α and β subunits in the alternate order (Fig. 1a). To add *Nde*I digestion site to the N terminus and a *Bam*HI digestion site to the C terminus, the α subunit gene was amplified by PCR. The amplified DNA fragment encoded the α subunit from the N-terminal initiation codon, but the C-terminal sequence was replaced with the thrombin protease sequence and lacked a translational stop codon. The β subunit gene was also amplified to introduce a *Bgl*II digestion site to the N terminus, delete the initiation codon, and add a *Bam*HI site to the C terminus. The inserted DNA fragments were excised by *Nde*I/*Bam*HI or *Bgl*II/*Bam*HI digestion and then gel purified. Thus, we obtained the DNA fragments ligated, in order, into the pET9a vector at the sites of *Nde*I and *Bam*HI.

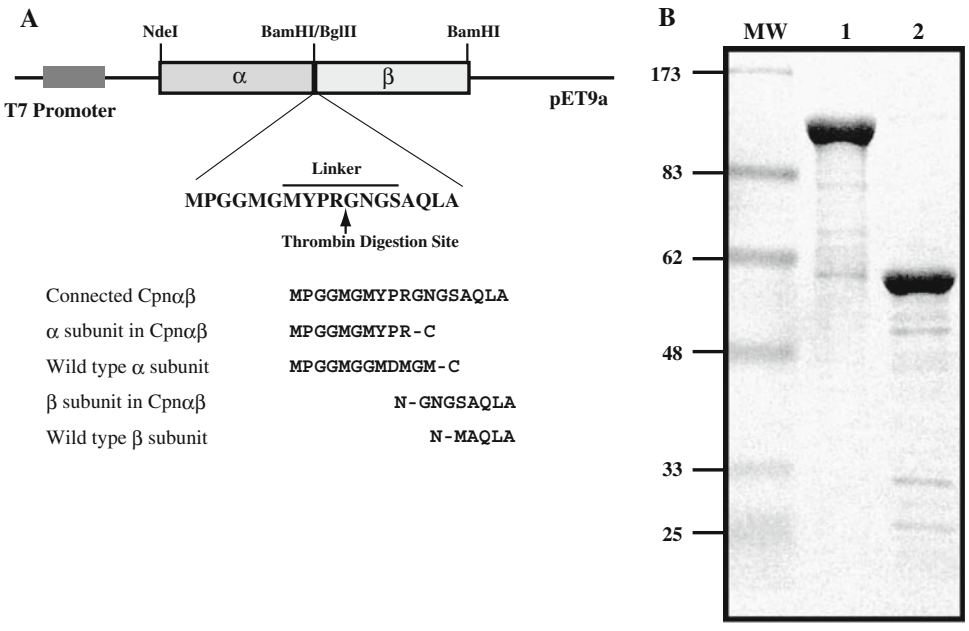

Fig. 1 Construction and purification of the recombinant Cpnα*β*. **a** A schematic image of the plasmid used to express the chaperonin hetero-complex composed of connected α and *β* subunits, Cpnα*β*. The amino acid sequences around the connected site are shown. Details of the construction are described in "Materials and methods". **b** Images of SDS-PAGE of the purified Cpnα*β* before (1) and after (2) thrombin digestion

The expression plasmid for the α–β connected chaperonin was transformed into the *E. coli* strain BL21*pRare. The expressed connected chaperonin was purified in the same manner as the wild-type chaperonin, and then the linker sequences were cleaved by the thrombin protease (Fig. 1b). This construct was designated Cpnαβ. The difference between Cpnαβ and the native complex of α and β is at the C terminus of α and the N terminus of β, as shown in Fig. 1a.

Functional characterization of Cpnαβ

First, we investigated the effects of the *T.* KS-1 chaperonin on preventing thermal aggregation of CS from porcine heart (Fig. 2a). CS denatures and aggregates at 50°C, and this process can be observed with light scattering. When chaperonins are included in the buffer, the aggregation of CS is inhibited. At 50°C, the thermal aggregation of CS is almost completely suppressed by an equal molar amount of Cpnαβ. The efficiency was almost the same as that of Cpnα and Cpnβ.

The protein folding activity was examined using acid-denatured GFP as a substrate. GFP denatures and loses fluorescence in an acidic environment. The acid-denatured GFP refolds in a neutral environment and its refolding is easily monitored by a recovery of fluorescence. When acid-denatured GFP is diluted in the refolding buffer at a neutral pH, it refolds spontaneously and the fluorescence gradually recovers. The yield of spontaneous refolding at 20 min after dilution was estimated to be ∼6%. With the presence of either of the *T.* KS-1 chaperonins in the folding mixture, spontaneous refolding of GFP is inhibited. The addition of ATP to the mixtures at 5 min induces the productive folding of GFP in each case. These results show that all of the chaperonins are able to capture unfolded GFP. Cpnα, Cpnβ, and Cpnαβ arrested the GFP in an intermediate state and enhanced its folding in an ATP-dependent manner. The yield of refolded GFP varies according to which

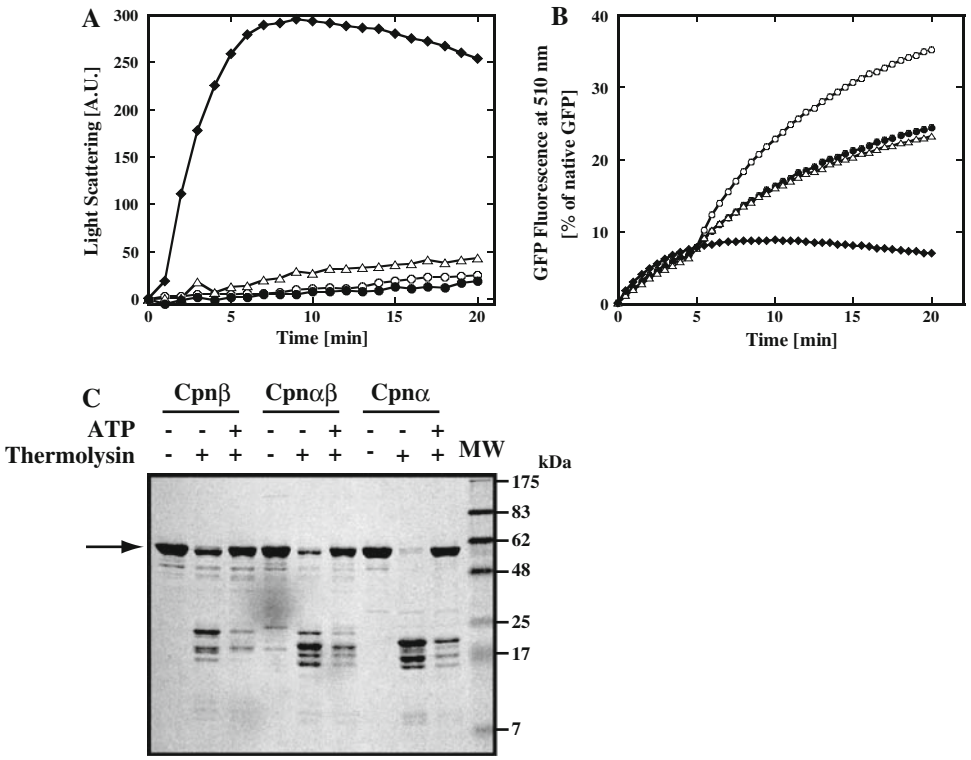

Fig. 2 Functional characterization of Cpnαβ. **a** Effect of *T.* KS-1 chaperonins on the thermal aggregation of CS. CS (120 nM) was incubated in the absence (*closed diamonds*) or presence of chaperonins, Cpnα (*open circles*), Cpnβ (*open triangles*), and Cpnαβ (*closed circles*) (120 nM) at 50°C. Details are described in "Materials and methods". **b** Refolding of GFP mediated by *T.* KS-1 chaperonins. The folding mixture was incubated at 60°C. The recovery of native GFP was continuously monitored with fluorescence at 510 nm. Fluorescence intensity of native GFP of the same concentration was taken as 100%. At 0 min, acid-denatured GFP (5 μM) was diluted 100-fold into the folding buffer containing 100 nM chaperonins, Cpnα (*open circles*), Cpnβ (*open triangles*), and Cpnαβ (*closed circles*). At 10 min after dilution, 1 mM ATP was added. Spontaneous refolding of GFP was observed upon dilution of denatured GFP into the folding buffer without chaperonin and ATP (*closed diamonds*). Details are described in "Materials and methods". **c** Protease sensitivity assay for the conformational change of chaperonins. Chaperonins were incubated with thermolysin (1 ng/μl) in the presence or absence of ATP (1 mM) and then analyzed by SDS-PAGE. Details are described in "Materials and methods"

chaperonin is present. After 25 min from the addition of ATP, about 35% GFP was refolded by Cpnα. In contrast, Cpnβ and Cpnαβ could refold GFP up to about 22% (Fig. 2b). We next examined the ATPase activities of the chaperonin complexes. The ATPase activities of Cpnα, Cpnβ, and Cpnαβ were 14.0, 8.43, and 11.0 nmol/min/mg at 60°C, respectively. They were slightly affected by the presence of GFP. The relatively low ATPase activities of Cpnβ and Cpnαβ might be correlated with the relatively low GFP refolding activity.

The ability of ATP to induce a conformational change in the hetero-oligomer was investigated by a protein sensitivity assay (Fig. 2c). It has been previously shown that the open built-in lid is sensitive to the protease thermolysin, whereas the closed lid state is not (Iizuka et al. 2003, 2004, 2005). The chaperonins were subjected to thermolysin digestion with or without ATP in the buffer at 60°C. In the presence of ATP, the homo- and hetero-oligomers were relatively thermolysin resistant, and a polypeptide of about 60 kDa remained. In contrast, 60 kDa polypeptides were degraded nearly completely in the absence of ATP. Thus, Cpnαβ changes its conformation in an ATP-dependent manner, such as Cpnα and Cpnβ.

Thermal stability of Cpnαβ

The subunit composition of the natural *T.* KS-1 chaperonin changes with temperature. The β subunit is more abundant than the α subunit in the chaperonin complex at higher temperatures (Yoshida et al. 2001). Cpnβ was observed to be more thermostable than Cpnα (Yoshida et al. 2002a). Thus, it was expected that the hetero-oligomer would exhibit a thermal stability in between Cpnα and Cpnβ. We examined the thermal stability of Cpnαβ by several methods.

To compare the thermal stability of Cpnα and Cpnαβ, the chaperonin were incubated at several temperatures (80, 85, 90, and 95°C for 30 min) and the oligomer dissociation was analyzed by native-PAGE (Fig. 3a). The oligomers of Cpnα and Cpnαβ were dissociated completely after the incubation at 95°C consistent with the previous data that oligomer of Cpnβ remained after incubation at 95°C though their amounts decreased. Then, the time course of dissociation of the oligomer of Cpnα and Cpnαβ at 95°C was examined. After incubating for 15 min, the band corresponding to the oligomers had completely disappeared for both of the chaperonins (Fig. 3b). These data indicate that the thermal stability of Cpnαβ is almost equivalent to that of Cpnα.

Because ATPase activity almost disappears when the *T.* KS-1 chaperonin dissociates (Yoshida et al. 2002a), we also examined the thermal stability of Cpnαβ in terms of ATPase activity (Fig. 3c). The ATPase activity of Cpnα and Cpnαβ has an optimum temperature of 80°C, but the ATPase activity of Cpnβ continues to increase at temperatures above 90°C. The half-lives of the ATPase activities of Cpnα and Cpnαβ at 90°C were 9.6 and 6.0 min, respectively. In comparison, Cpnβ was stable at 90°C, with a half-life of 24.8 min. These data also indicate that the thermal stability of Cpnαβ is similar to that of Cpnα. The thermal unfolding process of the chaperonin was analyzed by far-UV CD at different temperatures 60–99°C (Fig. 3d). This result also revealed that the change in the secondary structure of the recombinant Cpnαβ is similar to that of Cpnα.

Previously, we have shown that that the difference in thermal stability between the α and β subunits can be attributed to 22 amino acids in the equatorial domain of the C terminus. As shown in Fig. 1a, the difference between Cpnαβ in the native complex of α and β is in the C terminus of α and the N terminus of β. Thus, Cpnαβ contains four β type C termini in a ring, which is thought to contribute to the thermal stability. We could not observe any influence of this ring on the thermal stability, however. A cooperative action of the β type C termini might be required to increase in the thermal stability. Another possibility is that only a partial change in the C terminus of α and the N terminus of β can induce instability into the complex.

The interaction of PhPFD with Cpnαβ

Prefoldin is a co-factor for group II chaperonins and is thought to mediate substrate transfer. Martin-Benito et al. (2002) showed through electron microscopy that eukaryotic prefoldin binds to the apical domain of CCT as "the lid of a pot". Previous results showed that prefoldin from PhPFD strongly interacts with *Pyrococcus* chaperonin or Cpnβ. In contrast, the interaction of PhPFD with Cpnα is weak. This difference is caused by the replacement of two amino acids in the helical protrusion, suggesting that the interaction site is located in the helical protrusion. However, the detailed interaction between the octameric-ring chaperonin and the hexameric prefoldin is unknown. We examined the interaction of Cpnαβ, which has 4 β subunits per ring, with PhPFD. To assess the interaction kinetics between PhPFD and *T.* KS-1 chaperonins, we monitored their binding and release by SPR using a Biacore system. The binding curves were fit using the BIAevaluation 3.1 software as described (Okochi et al. 2004). The best-fits were obtained with the bivalent analyte model throughout this study. Apparent association constant values (K_A) and association/dissociation rate constants (k_{on1}/k_{off1}) between the chaperonins and prefoldin complexes were calculated (Table 1). For comparison, the sensorgrams of Cpnα and Cpnβ at the same concentration are shown in Fig. 4.

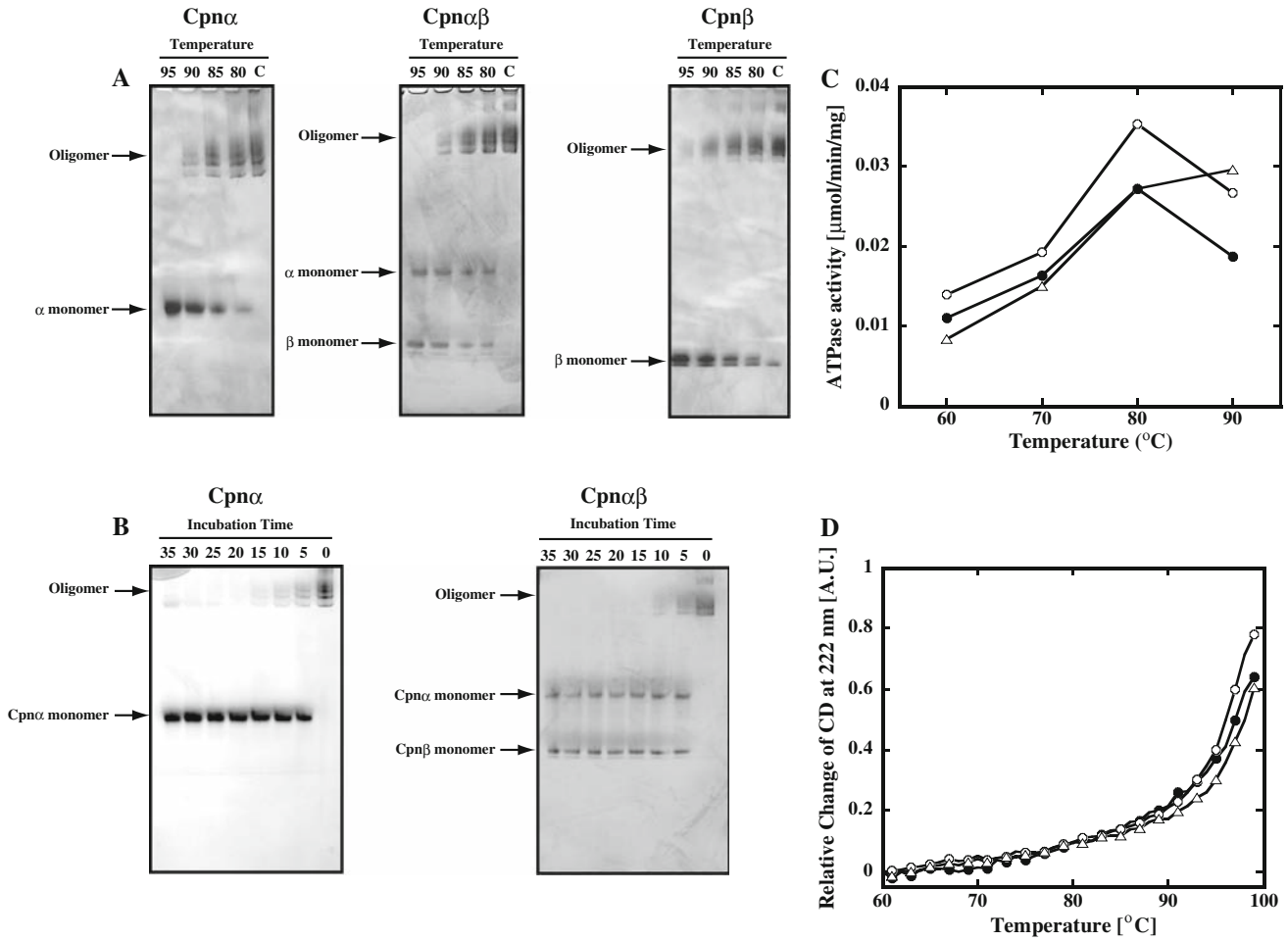

Fig. 3 Thermal stability of Cpnα*β*. **a** Thermal dissociation of Cpnα and Cpnα*β*. The chaperonins were incubated at 80, 85, 90, and 95°C for 30 min. Aliquots were then applied to 3–6% polyacrylamide gels. Each lane contained 2.5 μg of proteins. Gels were stained with Coomassie brilliant blue R-250. **b** Time course of thermal dissociation of Cpnα and Cpnα*β*. The chaperonins were incubated at 95°C for the period indicated. Aliquots were then applied to 3–6% polyacrylamide gels. **c** Temperature dependence of the ATPase activity of Cpnα, Cpn*β*, and Cpnα*β*. The chaperonins were incubated in an ATPase assay mixture for 30 min at the indicated temperature, and liberated Pi was measured by the malachite green method as described in "Materials and methods". The ATPase activity is shown: Cpnα (*open circles*), Cpn*β* (*open triangles*), and Cpnα*β* (*closed circles*). **d** Thermal denaturation of *T.* KS-1 chaperonin complexes. Thermal denaturation process of Cpnα (*open circles*), Cpn*β* (*open triangles*), and Cpnα*β* (*closed circles*) were monitored as the change in ellipticity at 222 nm. Details are described in "Materials and methods"

Table 1 Association and dissociation rate constants between PhPFD and various chaperonins obtained by surface plasmon resonance

	k_{a1} (1/Ms)	k_{d1} (1/s)	K_A (M^{-1})	Relative K_A
Cpn*β*	8.78×10^4	9.09×10^{-4}	4.72×10^7	1.00
Cpnα*β*	2.16×10^4	7.95×10^{-4}	7.17×10^6	0.15
Cpnα	7.96×10^3	7.64×10^{-4}	3.06×10^6	0.06

The kinetic constants (k_{on1} and k_{off1} for the first step; k_{on2} and k_{off2} for the second step) were calculated from the sensorgram with BIA-evaluation software, version 3.1 (Biacore) using the bivalent model [first step: L + A ↔ L.A, second step: L.A + L ↔ L2.A, ligand (PhPFD); analyte (CPN)] as the best-fitted model. The association constants (K_A) were calculated by the resonance unit at equilibrium, and relative K_A values were calculated by dividing the K_A values by the highest K_A value (4.72×10^7 M^{-1}) for Cpn*β*

Association rate constant (k_{on1}) of Cpnα*β* about threefold higher than Cpnα, but lower than Cpn*β* and the association constant value (K_A) of Cpnα*β* to PhPFD was also in between that of Cpnα and Cpn*β*. The four *β* subunits in the ring are likely to contribute to the increase in the association rate constant by about 9% compared to Cpnα (Table 1).

The detailed localization of the prefoldin-interaction site in the group II chaperonin has been examined with various chaperonin mutants. It was found that group II chaperonin binds to prefoldin through its helical protrusion in the apical domain. However, it is unknown how many monomers in the octameric ring of the chaperonin interact with the hexameric prefoldin. Here, we identified the affinity for prefoldin of the hetero-oligomer, which has four *β* subunits

that combine with four α subunits. We show that the β subunits can increase the affinity of the complex for prefoldin (Fig. 4).

The archaeal chaperonins are double-ring complexes of identical or diverse subunits. The hyperthermophilic archaeon, *Thermococcus* sp. strain KS-1 (*T.* KS-1), has two homologous chaperonin subunits, α and β. Natural composition of subunit in the hexadecameric double ring changes with the temperature. The β subunit was significantly more abundant in the higher temperature than α subunit. The homo-oligomer of β subunit (Cpnβ) was also more thermostable than the homo-oligomer of α subunit (Cpnα). As predicted, the thermal stability of the hetero-oligomer of *T.* KS-1 chaperonin was intermediate of between Cpnα and Cpnβ. In addition, our recent study indicated that Cpnβ exhibits more strong interaction with prefoldin than Cpnα. In this study, we characterized *T.* KS-1 chaperonin hetero-oligomer containing α and β alternately, Cpnαβ, which was constructed by connected expression of α and β subunits and protease digestion. Cpnαβ could protect CS from thermal aggregation, promoted folding of acid-denatured GFP in an ATP-dependent manner, and exhibited ATP-dependent conformation change. GFP refolding yield was almost same as that of Cpnβ, lower than that of Cpnα. On the contrary, Cpnαβ exhibited almost same thermal stability as Cpnα, lower than that of Cpnβ. The cooperative action of the β type C termini might be required for the increase of the thermal stability. Another possibility is that the only partly change in the C terminus of α and the N terminus of β might induce the instability of the complex. The affinity of Cpnαβ to prefoldin was compared with those of other chaperonin complexes. It was between those of Cpnα and Cpnβ, as expected. This result opens the opportunity to study the subunits arrangement of octameric-ring chaperonin that are necessary to interact with prefoldin by using four connected mutants with several composition and arrangement of Cpnα and Cpnβ.

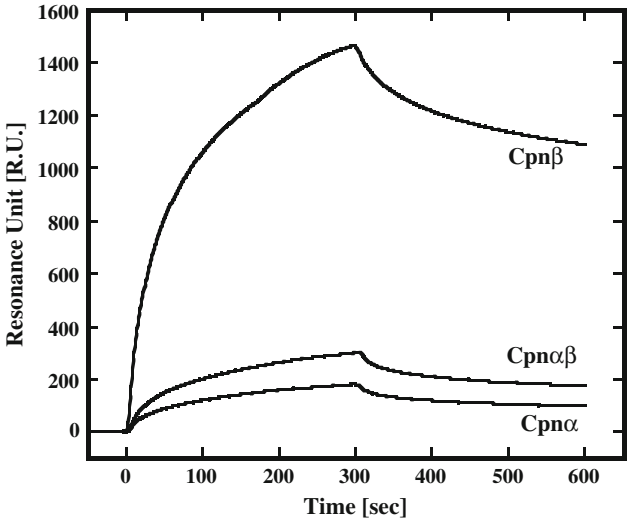

Fig. 4 Interaction of Cpnαβ and *Pyrococcus* prefoldin. Sensorgram of the measured affinities between *Pyrococcus horikosii* prefoldin (PhPFD) and various chaperonins by surface plasmon spectroscopy. PhPFD was immobilized on a Biacore biosensor chip to be 8013 RU. Cpnα, Cpnβ, and Cpnαβ injected as analytes at 50 nM are shown for comparison

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