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ORIGINAL PAPER

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Contribution of the *C*-terminal region to the thermostability of the archaeal group II chaperonin from *Thermococcus* sp. strain KS-1

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Abstract Chaperonin is a double ring-shaped oligomeric protein complex, which captures a protein in the folding intermediate state and assists its folding in an ATP-dependent manner. The chaperonin from a hyperthermophilic archaeum, *Thermococcus* sp. strain KS-1, is a group II chaperonin and is composed of two distinct subunits, α and β. Although these subunits are highly homologous in sequence, the homo-oligomer of the β-subunit is more thermostable than that of the α-subunit. To identify the region responsible for this difference in thermostability, we constructed domain-exchange mutants. The mutants containing the equatorial domain of the β-subunit were more resistant to thermal dissociation than the mutants with that of the α-subunit. Thermostability of a β-subunit mutant whose *C*-terminal 22 residues were replaced with those of the α-subunit decreased to the comparable level of that of the α-subunit homo-oligomer. These results indicate that the difference in thermostability between α and β-subunits mainly originates in the *C*-terminal residues in the equatorial domain, only where they exhibit substantial sequence difference.

Keywords Archaea · Group II chaperonin · Thermostability

Abbreviations CPNααα: α-Subunit homo-oligomer · CPNβββ: β-Subunit homo-oligomer · CPNββα: Domain-exchange subunit homo-oligomer whose apical, intermediate, and equatorial domains are derived from β, β, and α, respectively · CPNααβ, CPNβαα, CPNαββ, CPNαβα, and CPNβαβ: Homo-oligomers of the domain-exchange subunit named in the same manner as CPNββα · CPNβcα: β-Subunit homo-oligomer in which the *C*-terminal 22 amino acid residues of β were replaced by those of α · CD: Circular dichroism

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Introduction

Protein folding in the intracellular environment is highly dependent on the assistance of molecular chaperones (Hartl and Hayer-Hartl 2002; Young et al. 2004). Chaperonins are essential molecular chaperones that are involved in protein folding in the cell. They capture non-native proteins and promote their folding in vivo and in vitro in an ATP-dependent manner (Bukau and Horwich 1998; Hartl and Hayer-Hartl 2002). Based on their amino acid sequences and oligomeric structures, they are divided into two groups. Group I chaperonins are found in bacteria, organelles

(mitochondria and chloroplasts), and some archaea, while group II chaperonins exist in archaea and eukaryotic cytosol (Kim et al. 1994; Kubota et al. 1995; Conway de Macario et al. 2003; Klunker et al. 2003). Both chaperonins form double ring structures composed of about 60-kDa subunits, which are folded into three domains: apical, intermediate, and equatorial domains (Braig et al. 1994; Ditzel et al. 1998).

Group I chaperonins are represented by the *Escherichia coli* chaperonin, GroEL. GroEL is composed of 14 identical subunits arranged in two heptameric rings stacked back to back, and facilitates protein folding with a heptameric GroES complex, in an ATP-dependent manner (Bukau and Horwich 1998; Hartl and Hayer-Hartl 2002). On the other hand, group II chaperonins form toroidal double rings with an eight- or ninefold rotary symmetry, and function without a cofactor like GroES. The eukaryotic group II chaperonin forms a hexadecameric complex composed of eight different subunits, whereas archaeal chaperonins form hexadecamaeric or octadecameric structures consisting of one to three different subunits(Gutsche et al. 1999; Kubota 2001). Electron microscopic studies have revealed that the specific subunits of eukaryotic group II chaperonin contribute to the binding of substrate proteins (Llorca et al. 1999, 2000). The content of particular subunits of eukaryotic group II chaperonin, CCT, changes along with the cell cycle (Yokota et al. 2001). The subunit compositions of several archaeal chaperonins also vary in response to ambient temperature (Izumi et al. 2001; Yoshida et al. 2001; Kagawa et al. 2003).

The chaperonin from the hyperthermophilic archaeum *Thermococcus* sp. strain KS-1 (*T*. KS-1) is composed of two distinct subunits, α and β. Both subunits can assemble to form double ring homo-oligomers and are able to capture denatured proteins and fold them in an ATP-dependent manner in vitro (Yoshida et al. 1997, 2000, 2002a). These subunits are highly homologous to each other throughout their length, except for about 20 amino acid residues in the *C*-terminal region (Yoshida et al. 1997). The natural *T*. KS-1 chaperonin exists as a hetero-oligomer with variable subunit composition (Yoshida et al. 2001). Expression of the α and β-subunits is regulated differently, and only the β-subunit is thermally inducible (Yoshida et al. 2001). Thus, the proportion of the β-subunit in *T*. KS-1 chaperonin increases with temperature, and β-subunit-rich chaperonin is more thermostable than α subunit-rich-chaperonin (Yoshida et al. 2002b). However, the mechanism underlying the thermostability of the β-subunit is still unclear. To clarify this issue, we constructed domain-exchange mutants and a *C*-terminal-exchange mutant, and tested their thermostability. We revealed that the 22 amino acid residues at the *C*-terminus in the equatorial domain contribute to the difference in thermostability between *T*. KS-1 chaperonin oligomers.

Materials and methods

Bacterial strains, plasmids and reagents

The *E. coli* strains used in this study were JM109 or DH5α for plasmid preparation, and BL21(DE3) for protein expression. Plasmids for the expression of domain-exchange mutants were constructed using pKCα201 and pKCβ201 as templates, which were made by the following procedure. Full-length α- and β-subunit genes were amplified by PCR using 5'-TTCCATGG CACAGCTTAGTGGACAGCCG-3' (*Nco* I site underlined) and 5'- GGGATCCACTAGTTCACATG CCCATGTCCATTC-3' (*Bam* HI site underlined) as primers for the α-subunit gene; and 5'-GGCCATGG CCCAGCTTGCAGGCCAGCCA-3' (*Nco* I site underlined) and 5'-GGGATCCACTAGTTCAGTCG AGATCGCTTCCG-3' (*Bam* HI site underlined) as primers for the β-subunit gene. PCR products were digested with *Nco* I and *Bam* HI, and cloned into the *Nco* I/*Bam*H I site of pET21d (Novagen). The plasmids pK1Eα and pK1Eβ (Yoshida et al. 1997) were used in the construction of the *C*-terminal-exchange mutant of the β-subunit. DNA polymerase and restriction endonucleases were products of Takara Bio Inc. (Shiga, Japan) or TOYOBO (Osaka, Japan). ATP was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Construction of domain-exchange mutants

Site-directed mutagenesis was performed using a QuikChange site-directed mutagenesis kit (Stratagene, CA) to introduce the same restriction enzyme digestion sites into the domain junctions of both subunits. The primer sets used are listed in Table 1. Introduced restriction enzyme sites (sites A–D) in both the α and β-subunits and the constructs of domain-exchange mutants are shown in Fig. 1. Although the mutation did not induce any amino acid substitution in the α-subunit, a mutation of Ser-419 to Ala occurred in the β-subunit. The chaperonin function and thermostability of CPNβββ exhibited the same as the wild-type (data not shown). For construction of the CPNβαα and CPNαββ mutants, gene fragments corresponding to the apical domain regions of the resultant α- and β-subunits were obtained by restriction digestion using *Ban* III and *Kpn* I, and were ligated into the opposite subunit genes (Fig. 1b). Other mutants were constructed using the same procedure. All constructs were verified by DNA sequencing.

Construction of C-terminal-exchange mutant

Site-directed mutagenesis was conducted using a QuikChange site-directed mutagenesis kit to create

Table 1 Primers used for constructing mutants

Primer name	Sequence	Enzyme
Primer sets for the domain-exchange mutants		
α site A (+)	5'-CATCGCTGGAGAACTCCTTCGAAAGGCTGAGGAGCTTC-3'	*Nsp* V
α site A (-)	5'-GAAGCTCCTCAGCCTTTCGAAGGAGTTCTCCAGCGATG-3'	*Nsp* V
α site B (+)	5'-CGGTGTCGTCATCGATAAGGAAGTTGTCCACC-3'	*Ban* III
α site B (-)	5'-GGTGGACAACTTCCTTATCGATGACGACACCG-3'	*Ban* III
α site C (+)	5'-CCTCATCCGCGGTGGTACCGAGCACGTCATTG-3'	*Kpn* I
α site C (-)	5'-CAATGACGTGCTCGGTACCACCGCGGATGAGG-3'	*Kpn* I
α site D (+)	5'-CTCCAGAGATCGAGCTAGCCATCAGGCTCGAC-3'	*Nhe* I
α site D (-)	5'-GTCGAGCCTGATGGCTAGCTCGATCTCTGGAG-3'	*Nhe* I
β site A (+)	5'-CGGTTGTTATTGCTGGTGAGCTTCTTCGAAAGGCTGAGGAGC-3'	*Nsp* V
β site A (-)	5'-GCTCCTCAGCCTTTCGAAGAAGCTCACCAGCAATAACAACCG-3'	*Nsp* V
β site B (+)	5'-GGGTGTCGTCATCGATAAGGAGGTCGTCCACC-3'	*Ban* III
β site B (-)	5'-GGTGGACGACCTCCTTATCGATGACGACACCC-3'	*Ban* III
β site D (+)	5'-GTGCTCCGGAGATCGAGCTAGCCATCAGGCTCGACGAG-3'	*Nhe* I
β site D (-)	5'-CTCGTCGAGCCTGATGGCTAGCTCGATCTCCGGAGCAC-3'	*Nhe* I
Primer sets for the C-terminal-exchange mutant		
α site E (+)	5'-GCAGCTATAATGATCCTTCGAATCGCAGATGTC-3'	*Nsp* V
α site E (-)	5'-GACATCGTCGATTCGAAGGATCATTATAGCTGC-3'	*Nsp* V
β site E (+)	5'-GCCGCCATAATGATCCTTCGAATCGACGACGTCATC-3'	*Nsp* V
β site E (-)	5'-GATGACGTCGTCGATTCGAAGGATCATTATGCCGCC-3'	*Nsp* V

Nsp V restriction sites in the *C*-terminal region (site E, see Fig. 1a) of the α- and β-subunit genes. The oligonucleotides were used as mutagenic primers: α site E (+) and α site E (-) for the α-subunit gene; β site E (+) and β site E (-) for the β-subunit gene (Table 1). The resultant mutant gene of the β-subunit was digested by *Nde* I and *Nsp* V, and the fragment was ligated into the *Nde* I and *Nsp* V digest of the expression vector encoding the mutated α-subunit gene (Fig. 1b). The construct was verified by DNA sequencing.

Expression and purification of wild-type and mutant chaperonins

Wild-type and mutant chaperonins were expressed in *E. coli* BL21 (DE3) cells with the expression vectors. The cells were grown aerobically overnight at 37°C in 2× YT medium (1.6% Tryptone, 1% yeast extract, and 0.5% NaCl) containing 100 μg/ml of ampicillin or 75 μg/ml of kanamycin. Chaperonins were purified as previously described (Yoshida et al. 2001). Purified chaperonins were concentrated by ultrafiltration, supplemented with glycerol to 20% (v/v), and stored at −80°C. Protein concentrations were measured by the Bradford method with a protein assay kit (Bio-Rad Laboratories, CA), using bovine serum albumin as the standard (Bradford 1976).

Native-PAGE analyses

Chaperonins (0.5 μg/μl) were incubated for 30 min at 80, 85, 90, or 95°C in the assay buffer (50 mM Tris-HCl pH 8.0, 25 mM $MgCl_2$, and 100 mM KCl). Aliquots of the mixture were placed on ice for more than 30 min, and then 2.5 μg of protein was applied to 3–6% polyacrylamide gels without SDS (Laemmli 1970). In the time course experiments, aliquots were taken out at specific points in time. Gels were stained with Coomassie brilliant blue R-250.

Residual ATPase activity measurements

The assay mixtures containing 50 ng/μl of chaperonin were incubated at 90°C for 10, 20, 30 and, 60 min, and then tested for ATPase activity at 80°C. The reactions were started by the addition of ATP (1 mM), and terminated by mixing in ice-cold 2% perchloric acid after incubation. The amount of inorganic phosphate produced was measured by the malachite green assay (Baykov et al. 1988; Geladopoulos et al. 1991). The rate of spontaneous hydrolysis of ATP at 80°C was measured, and the residual ATPase activity was determined. The ATPase activities at 70°C of CPNααα, CPNβββ, CPNββα, CPNααβ, CPNβαα, CPNαββ, CPNαβα, CPNβαβ were 0.041, 0.018, 0.008, 0.023, 0.022, 0.016, 0.017, and 0.024 μmol/min/mg, respectively.

Circular dichroism measurements

Thermal denaturation of chaperonins was monitored by measuring circular dichroism (CD) at 222 nm. CD measurements were carried out in a quartz cuvette of path length 0.1 cm in the assay buffer containing 0.15 mg/ml protein using a JASCO J-720 spectropolarimeter (JASCO, Tokyo, Japan). Changes in CD at

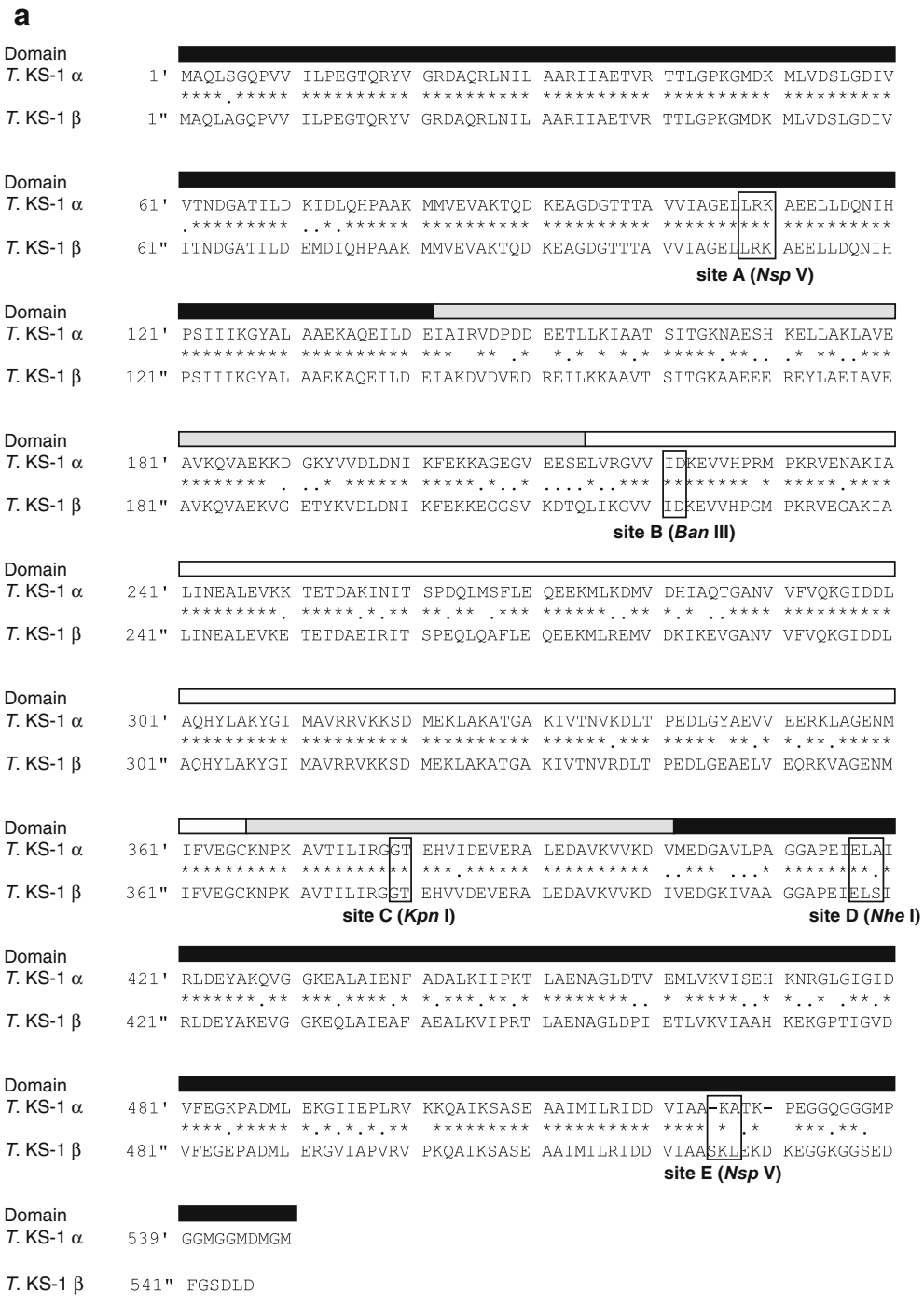

Fig. 1 Amino acid sequence alignment of *T.* KS-1 chaperonin subunits and the domain-exchange and C-terminal-exchange mutants. **a** Amino acid sequences of the α- and *β*-subunits of *T.* KS-1 chaperonin aligned with GENETYX software (Genetyx Corporation, Tokyo, Japan). Identical and similar residues are shown by *asterisks* and *periods*, respectively. The domain structure is shown above the sequence. *Black, gray* and *white lines* represent the equatorial domain, intermediate domain and apical domain, respectively. *Boxed residues* indicate the digestion site introduced for constructing domain-exchange mutants. **b** The construct of the domain-exchange mutants and *C*-terminal-exchange mutant. *Ap* apical domain, *Int* intermediate domain, *Eq* equatorial domain

222 nm were scanned at 1°C/min from 60 to 99°C with a peltier temperature controller. The CD data for the unfolded chaperonin were measured for the same concentration of the oligomer, which was incubated in assay buffer containing 6 M guanidine-HCl. To indicate the degree of denaturation of chaperonins, the CD data were normalized to values obtained with and without 6 M guanidine-HCl at 60°C. A value of 1.0 corresponds to the form denatured by guanidine-HCl and 0, to the native form.

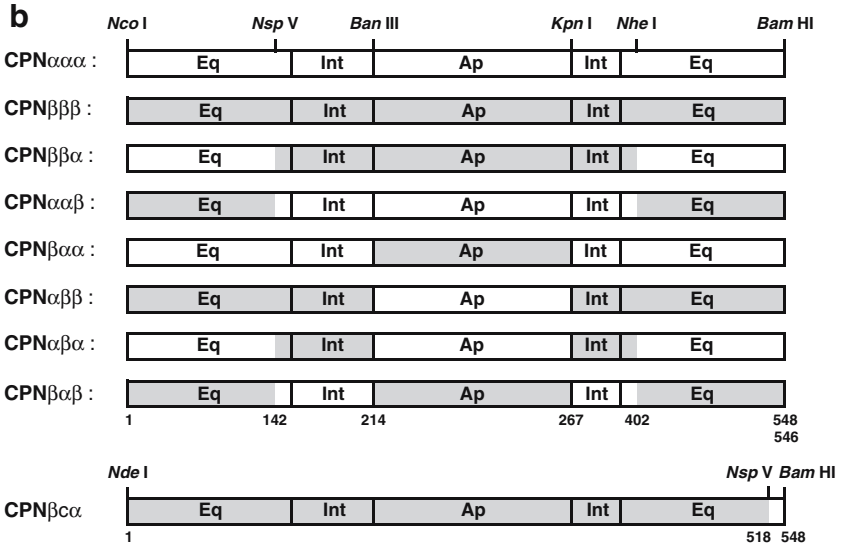

Fig. 1 (Contd.)

Results and discussion

Preparation of domain-exchange mutants

The chaperonin from *Thermococcus* sp. strain KS-1 (*T.* KS-1) is composed of two highly homologous subunits, α and *β*, which form functional homo- and hetero-oligomers. Previously, we have revealed that *β*-subunit-rich chaperonin is more thermostable than the α-subunit-rich chaperonin (Yoshida et al. 2002b). Judging from this high sequence identity (Fig. 1a), the difference in stability is expected to be attributable to a difference in a specific region. To identify this region, domain-exchange mutants were constructed as shown in Fig. 1b. The homo-oligomers of the α-subunit with the apical, intermediate, or equatorial domains of the *β*-subunit are referred to as CPN*βαα*, CPN*αβα*, and CPN*ααβ*, respectively. The oligomers of the *β*-subunit mutants and the corresponding α-subunit domain substitutions are named similarly. The α- and *β*-subunit homo-oligomers are abbreviated to CPNααα and CPN*βββ* respectively. Although the *β*-subunit with the amino acid substitution of Ser-419 to Ala was used in this study for constructing domain-exchange mutants, the chaperonin function and thermostability of CPN*βββ* were the same as those of the wild-type.

Thermostability of domain-exchange mutants

First, the stability of the domain-exchange mutants was analyzed by Native-PAGE (Figs. 2 and 3). Wild-type *T.* KS-1 chaperonin homo-oligomers migrated as smeared multiple bands near the top of the gel (open arrowheads), which is likely to reflect the single double-ring homo-oligomers and their multimers (Yoshida et al. 2002b). Before heat treatment, all mutants migrated in the native gel in the same way (open arrowheads) indicating that they form oligomers like the wild-type chaperonins. After 30-min incubation at 80–95°C, the bands corresponding to the oligomers disappeared, and fast migrating bands (closed arrowheads) became predominant. As previously demonstrated (Yoshida et al. 2002b), these new bands correspond to monomers. The oligomers CPNααα (A), CPN*ββα* (C), CPN*βαα* (E), and CPN*αβα* (G) dissociated completely after the incubation at 95°C. On the other hand, CPN*βββ* (B), CPN*ααβ* (D), CPN*αββ* (F), and CPN*βαβ* (H) remained after incubation at 95°C though their amounts decreased.

Then, the time course of dissociation of the mutants at 90°C was examined (Fig. 3). The oligomers (open arrowheads) were dissociated gradually, and the amount of monomers (closed arrowheads) increased as the incubation proceeded. CPN*βββ* (B), CPN*ααβ* (D), CPN*αββ* (F), and CPN*βαβ* (H) were more resistant to thermal dissociation than CPNααα (A), CPN*ββα* (C), CPN*βαα* (E), and CPN*αβα* (G). These results suggest that the domain-exchange mutant containing the equatorial domain from the *β*-subunit is more thermostable than the mutant with that of the α-subunit.

Because ATPase activity is lost when *T.* KS-1 chaperonin dissociates (Yoshida et al. 2002b), thermostability of the domain-exchange mutants was also compared in terms of ATPase activity. The half-lives of the domain-exchange mutants (Table 2) containing the equatorial domain from the *β*-subunit (CPN*βββ* CPN*ααβ*, CPN*αββ*, and CPN*βαβ*) were similar, ranging from 14.1 to 14.7 min, which were longer than those of the mutants with the equatorial domain of the α-subunit (6.6–7.9 min). The result is consistent with the results of the Native-PAGE analyses. We conclude that the

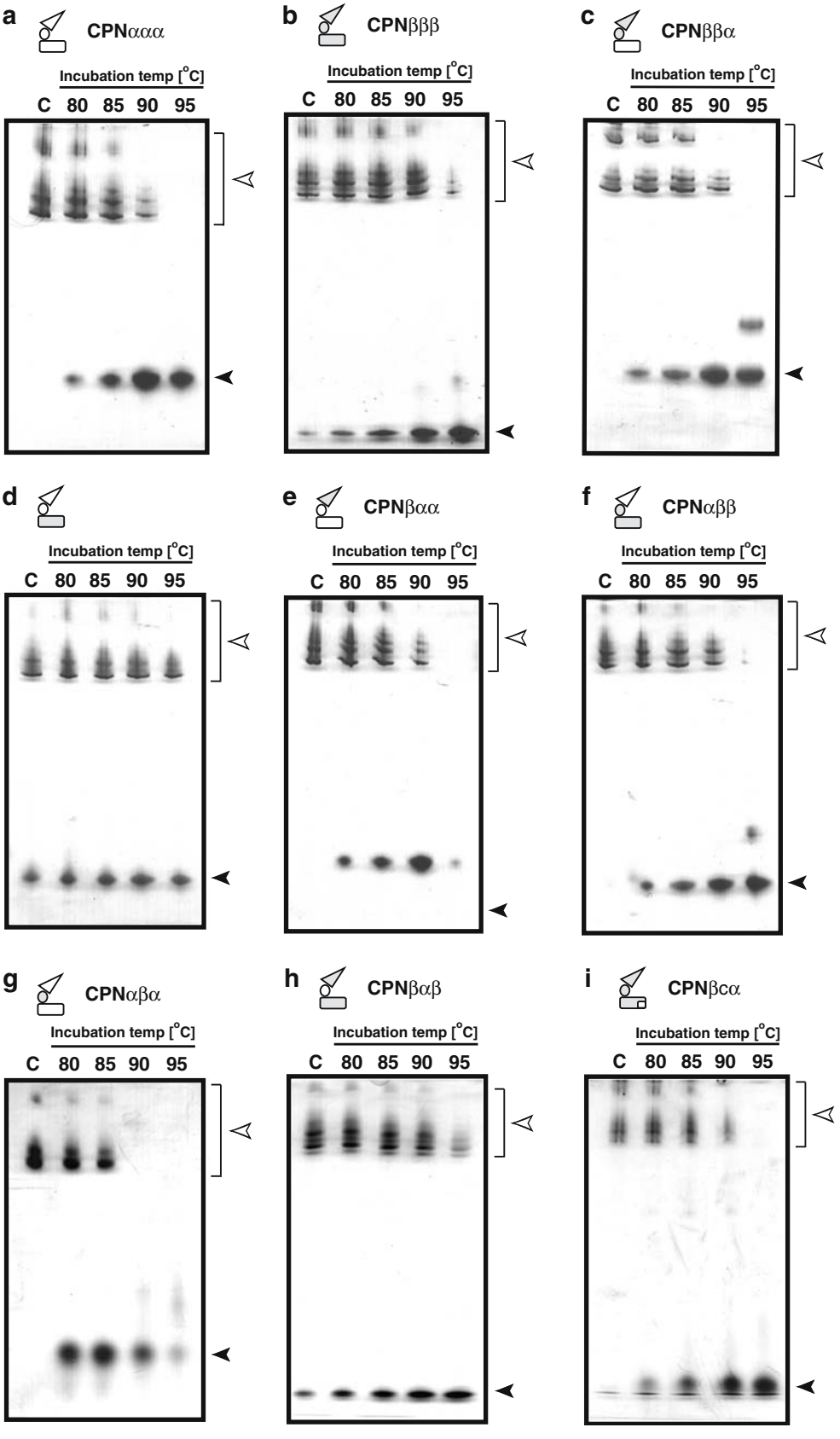

Fig. 2 Thermal dissociation of wild-type and mutant chaperonin oligomers. The wild-type and mutant chaperonins were incubated at the indicated temperature for 30 min. The protein was analyzed on 3–6% polyacryamide gels. Each lane contained 2.5 μg of protein. Gels were stained with Coomassie brilliant blue R-250. *Open arrowheads* show the band corresponding to the oligomer. *Closed arrows* show the band corresponding to the monomer. Chaperonin subunits are shown as *models*. The right side is the central cavity. The apical, intermediate, and equatorial domains are represented by a *triangle*, *circle*, and *square*, respectively. The domains derived from α- and *β*-subunits are shown in *white* and *gray*, respectively. *A* CPNααα; *B* CPN*βββ*; *C* CPN*ββ*α; *D* CPNα*αβ*; *E* CPN*β*αα; *F* CPNα*ββ*; *G* CPNα*β*α; *H* CPN*β*α*β*; *I* CPN*β*cα

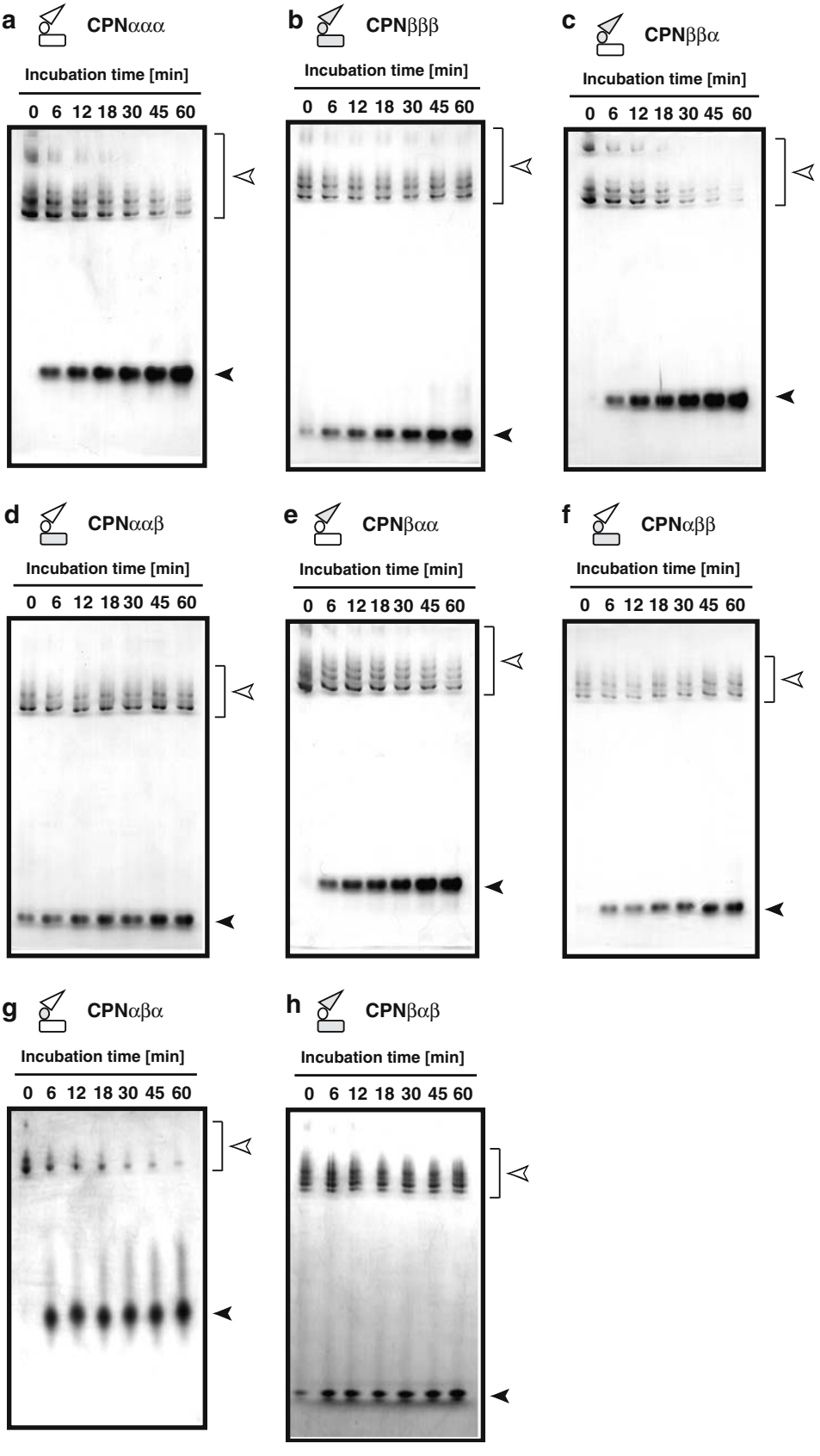

Fig. 3 Time-course of thermal dissociation of wild-type and domain-exchange mutants. The wild-type and domain-exchange mutants were incubated at 90°C for the period indicated. Aliquots were then applied to 3–6 % polyacryamide gels. Each lane contained 2.5 μg of protein. Gels were stained with Coomassie brilliant blue R-250. *Open arrowheads* show the band corresponding to the oligomer. *Closed arrowheads* show the band corresponding to the monomer. *A* CPNααα; *B* CPN*βββ*; *C* CPN*ββα*; *D* CPNαα*β*; *E* CPN*β*αα; *F* CPNα*ββ*; *G* CPNα*β*α; *H* CPN*β*α*β*

Table 2 Half-lives of ATPase activity of wild-type and mutant chaperonins

	Half life (min)
Oligomer containing an equatorial domain derived from the α-subunit	
CPNααα	7.2
CPNβββα	6.6
CPNβαα	7.9
CPNαβα	7.0
Oligomer containing an equatorial domain derived from the β-subunit	
CPNβββ	14.1
CPNααβ	14.7
CPNαββ	14.7
CPNβαβ	14.6

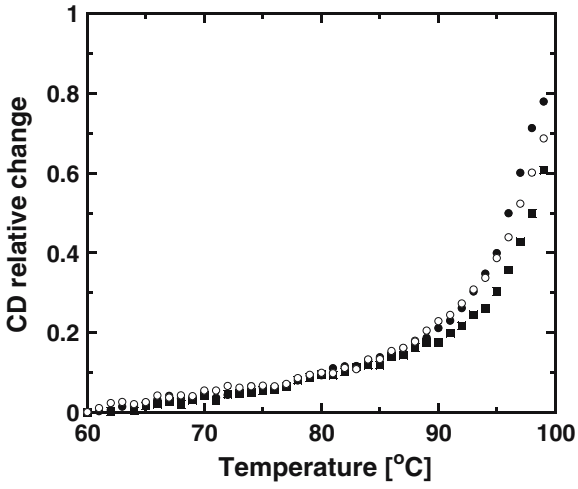

Fig. 4 Thermal denaturation of the *C*-terminal-exchange mutant. Thermal denaturation of CPNααα, CPNβββ, and CPNβcα was monitored as the change in ellipticity at 222 nm. Details are described in "Materials and methods". *Closed circles* CPNααα; *closed squares* CPNβββ; *open circles* CPNβcα

equatorial domain has an important role in the stabilization of the oligomeric structure.

Thermostability of the C-terminal-exchange mutant

In the equatorial domain, about 20 amino acid residues of the *C*-terminus are significantly different between the α- and β-subunits (Fig. 1a). It is likely that the difference in thermostability is mainly derived from the difference in this region. To investigate the contribution of the *C*-terminal sequence to oligomeric stability, a β-subunit mutant whose *C*-terminal 22 residues were replaced with those of α-subunit, was constructed (Fig. 1b). The mutant, termed CPNβcα, retained a level of ATPase activity comparable with that of CPNβββ (data not shown). Native-PAGE analysis showed that the CPNβcα oligomer dissociated almost completely at 95°C Fig. 2, site I), and that its band patterns were similar to those of CPNααα (Fig. 2a) rather than those of CPNβββ (Fig. 2b). CD analyses revealed that the change of the secondary structure in CPNβcα was intermediate between that of CPNβββ and that of CPNααα (Fig. 4). Therefore, we concluded that the difference in thermostability between α- and β-subunits was derived from their *C*-terminal sequences.

Possible structure and function of C-terminal sequences of T. KS-1 chaperonin subunits

The most significant sequence difference between the α- and β-subunits of *T*. KS-1 lies at the *C*-terminal. While the α subunit contains a typical glycine-glycine-methionine (GGM) repeat motif, the β-subunit does not (Fig. 1a). The GGM repeat sequence is conserved in group I chaperonins (McLennan et al. 1993; Brocchieri and Karlin 2000) and also in some archaeal group II chaperonins (Yoshida et al. 1997; Furutani et al. 1998; Kuo et al. 1997; Izumi et al. 1999; Kusmierczyk and Martin 2003). Interestingly, *Pyrococcus* species that are closely related to *Thermococcus* hyperthermophiles but grow at higher temperatures, have been shown to have only one chaperonin subunit, which is similar to the *Thermococcus* β-subunit lacking the GGM repeat (Archibald et al. 1999)

Among the three chaperonin domains, the equatorial domain, which is composed of *N*- and *C*-terminal regions, is involved in intra- and inter-ring contacts (Braig et al. 1994; Ditzel et al. 1998). In all crystal structures of chaperonins including *T*. KS-1 α-subunit homo-oligomers, about 20 amino acid residues of the *C*-terminus are unresolved (e.g., Braig et al. 1994; Ditzel et al. 1998; Shomura et al. 2004). These residues appear to project from the equatorial domain into the inside of the ring (Braig et al. 1994; Ditzel et al. 1998). In GroEL, truncation of the *C*-terminal by proteinase K has no effect on its oligomeric structure and protein folding activity at ambient temperature (Langer et al. 1992). Besides, McLennan et al. (1993) reported that the *C*-terminal-truncated GroEL is ineffective at high growth temperature due to its reduced thermostability. From these findings, it is likely that the *C*-terminal sequence of a chaperonin governs its oligomeric structure at elevated temperatures. The *C*-terminal sequence of the *T*. KS-1 α-subunit is glycine-rich, and it is more flexible than that of the β-subunit. We conclude that this flexibility leads to the thermal dissociation of α-subunit homo-oligomers at lower temperature.

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