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ORIGINAL PAPER

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Comparative analysis of the protein folding activities of two chaperonin subunits of *Thermococcus* strain KS-1: the effects of beryllium fluoride

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Abstract We conducted a comparative analysis of the effects of beryllium fluoride (BeFx) on protein folding mediated by the α- and β-subunit homooligomers (α16mer or β16mer) from the hyperthermophilic archaeum *Thermococcus* strain KS-1. BeFx inhibited the ATPase activities of both α16mer and β16mer with equal efficiency. This indicated that BeFx replaces the γ-phosphate of chaperonin-bound ATP, thereby forming a stable chaperonin–ADP–BeFx complex. In the presence of ATP and BeFx, both of the two chaperonin subunits mediated green fluorescent protein (GFP) folding. Gel filtration chromatography revealed that the refolded GFP was retained by both chaperonins. Protease digestion and electron microscopic analyses showed that both chaperonin–ADP–BeFx complexes of the two subunits adopted a symmetric closed conformation with the built-in lids of both rings closed and that protein folding took place in their central cavities. These data indicated that basic protein folding mechanisms of α16mer and β16mer are likely similar although there were some apparent differences. While β16mer-mediated GFP refolding in the presence of ATP–BeFx that proceeded more rapidly than in the presence of ATP alone and reached a twofold higher plateau than that achieved with AMP–PNP, the folding mediated by the α16mer that proceeded with much lower yields. A mutant of α16mer, trapα, which traps the unfolded and partially folded substrate protein, did not affect the ATP–BeFx-dependent GFP folding by β16mer but it suppressed that mediated by α16mer to the level of spontaneous folding. These results suggested that β16mer differed from the α16mer in nucleotide binding affinity or the rate of nucleotide hydrolysis.

Keywords Archaea · Group II chaperonin · Beryllium fluoride · Protein folding mechanism

Abbreviation

CCT	Chaperonin-containing *t*-complex polypeptide-1
TriC	TCP-1 ring complex
α16mer	The wild-type α-subunit chaperonin homooligomer from *Thermococcus* strain KS-1
β16mer	The wild-type β-subunit chaperonin homooligomer from *Thermococcus* strain KS-1
DTT	Dithiothreitol
BeFx	Beryllium fluoride
HPLC	High performance liquid chromatography
CS	Citrate synthase
GFP	Green fluorescence protein

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Introduction

Chaperonins are known to be involved in protein folding in the three domains of organisms: Bacteria, Archaea and Eukarya (Hartl and Hayer-Hartl 2002). Between the two types of chaperonins, group I chaperonins are found in bacteria, while group II chaperonins are found in archaea and the cytoplasm of eukaryotes (Conway de Macario and Macario 2003; Kim et al. 1994; Klunker et al. 2003; Kubota et al. 1995). Archaeal group II chaperonins, which consist of 1–3 types of homologous subunits, form assemblages of an 8 or 9 rotationally symmetrical double-ring toroidal structure (Archibald et al. 1999; Gutsche et al. 1999). Although no Gro-ES-like co-chaperonin has been found for group II chaperonins, the latter do interact with a recently identified heterohexameric protein complex, prefoldin/GimC (Geissler et al. 1998; Leroux et al. 1999; Vainberg et al. 1998).

The chaperonin from the hyperthermophilic archaeum *Thermococcus* strain KS-1 is composed of two distinct but similar subunits, α and β (Yoshida et al. 1997, 2000, 2001, 2002a). ATP-dependent protein folding occurs in the α and β subunit homooligomers (α16mer and β16mer) as well as in the native chaperonin (Yoshida et al. 1997, 2000, 2002b, 2002c). Archaeal chaperonins are composed of apical, intermediate and equatorial domains (Ditzel et al. 1998; Shomura et al. 2004), and a long helical protrusion in the apical domain functions as a built-in lid to close the central cavity (Ditzel et al. 1998; Iizuka et al. 2004; Shomura et al. 2004). ATP binding and hydrolysis induce a conformational change, from the open conformation with opened built-in-lids, which is the substrate binding conformation, to the closed conformation with the closed built-in lids for encapsulating unfolded proteins in the central cavity (Ditzel et al. 1998; Gutsche et al. 2001; Llorca et al. 2001; Meyer et al. 2003; Yoshida et al. 2002c). The built-in lid of the eukaryotic chaperonin CCT/TRiC is not closed by ATP binding but by ATP hydrolysis (Meyer et al. 2003). In contrast, ATP binding by the α16mer without hydrolysis induces the built-in lid to close the central cavity (Iizuka et al. 2003). This indicates that the trigger for lid closure is ATP binding (Gutsche et al. 2001; Iizuka et al. 2004). Beryllium fluoride (BeFx) stabilizes a transient state of ATP hydrolysis, thereby freezing the ATPase reaction, and was used to study the mechanism of ATP hydrolysis by group I and group II chaperonins (Chaudhry et al. 2003; Iizuka et al. 2005; Inobe et al. 2003; Melki et al. 1997; Melki and Cowan 1994; Meyer et al. 2003; Taguchi et al. 2004). In archaeal chaperonins, ATP hydrolysis may be required to reopen the chaperonin cavity and thus to release the folded protein into the environment.

Although two *Thermococcus* chaperonin subunits are highly homologous in sequence, their expression is distinct. The cellular content of the α-subunit was lower at higher temperatures, with the minimum at 90–93°C, whereas that of the β-subunit increased with increasing temperature up to 93°C (Yoshida et al. 2001). The optimal temperature for the ATPase activity of β16mer (>90°C) was higher than that for α16mer (approx. 60°C). The natural *Thermococcus* chaperonin exists as a heterooligomer with variable subunit composition (Yoshida et al. 2001). Expressions of the α and β subunits are regulated differentially, and only the β subunit is thermally inducible as well as being thermostable (Yoshida et al. 2001, 2002a). At higher temperatures, the β-subunit may be more functional for protein folding than in the α-subunit. However, the functional difference between these two subunits in protein folding is largely unknown. We found that a nonhydrolyzable ATP analog, AMP–PNP, induced the GFP refolding mediated by β16mer but not by the α16mer (Yoshida et al. 2002c). The α16mer in the presence of ATP and BeFx forms the ADP–BeFx complex, which is thought to mimic the ATP-bound state (Iizuka et al. 2005). This complex has been shown to adopt the asymmetric conformation with one ring closed and is able to encapsulate the unfolded protein in the closed ring but does not fold it (Iizuka et al. 2005). However, the effect of BeFx on β16mer remains to be studied. To examine the differences in the protein folding mechanisms of these subunits, we conducted a comparative analysis on the effect of BeFx on the protein folding mediated by α16mer and β16mer using GFP and CS as substrates.

Materials and methods

Proteins and reagents

Both ATP and ADP were purchased from Sigma (St. Louis, MO, USA). $BeSO_4$, NADP, NaF and thermolysin were purchased from Wako Pure Chemicals (Osaka, Japan). Citrate synthase (CS) from *Thermoplasma acidophilum* and glucose-6-phosphate dehydrogenase from *Geobacillus stearothermophilus* were purchased from Sigma. ADP-dependent glucokinase from *Thermococcus litoralis* was purified as previously described (Koga et al. 2000). Protein concentrations were measured by the Bradford method using a protein assay kit (Bio-Rad Laboratories, CA, USA); bovine serum albumin served as the standard (Bradford 1976).

Purification of chaperonin subunit homooligomers and GFP

Chaperonin subunits from *Thermococcus* strain KS-1 were expressed in *Escherichia coli* strain BL21 (DE3) cells using expression plasmids pK1Eα, pK1Eα2 and pK1Eβ for the α-subunit mutant and the wild-type α- and β-subunits, respectively (Yoshida et al. 1997, 2000). The expressed chaperonin subunits formed homohexadecamers in *E. coli* cells and were designated trapα, α16mer (α-subunit) and β16mer (β-subunit). *E. coli* cells

were aerobically grown overnight at 37°C in 2× YT medium supplemented with 100 μg/ml of ampicillin or 75 μg/ml of kanamycin. Cells were harvested by centrifugation for 20 min at 6,510*g* and stored at −80°C until used. *Thermococcus* chaperonins were purified as previously described (Yoshida et al. 2001), and they were pooled and concentrated by ultrafiltration (Centriprep 10, Amicon, MA, USA). They were confirmed to be nucleotide-free by analysis of bound nucleotides (Yoshida et al. 2002b, 2002c).

A heat stable GFP mutant was used to assess chaperonin-mediated protein folding (Yoshida et al. 2002c). The protein was expressed in recombinant *E. coli* strain BL21 (DE3) using the expression vector pET21C-GFP (His) as previously described (Yoshida et al. 2002c), after which GFP was purified, pooled and concentrated by ultrafiltration (Centriprep 10).

ATP hydrolysis and binding assays

Thermococcus chaperonins (0.25 μg/μl) were preincubated in folding buffer [50 mM Tris–Cl (pH7.5), 100 mM KCl, 25 mM $MgCl_2$ and 5 mM DTT] for 5 min at 60°C, with or without 5 mM NaF and 1 mM $BeSO_4$. The ATP hydrolysis reaction was started by adding ATP to a final concentration of 1 mM, and was terminated by adding 2% (w/v; final concentration) perchloric acid. Liberated Pi was measured using the malachite green method (Baykov et al. 1988; Geladopoulos et al. 1991). The rate of the spontaneous ATP hydrolysis in the absence of chaperonin was measured at each temperature and subtracted from the total ATPase activity to yield the chaparonin-ATPase activity.

Chaperonin-bound nucleotide was analyzed by HPLC. *Thermococcus* chaperonins (1 μM) were incubated 5 min at 60°C in folding buffer containing 5 mM NaF, 1 mM $BeSO_4$ and 1 mM ATP. After incubation, the reaction mixture was loaded on a gel filtration spin column (Bio-Spin 30, Bio-Rad Laboratories, CA, USA) equilibrated with folding buffer in the presence of 5 mM NaF and 1 mM $BeSO_4$ and subjected to centrifugation at 60°C. Perchloric acid (final concentration, 2%) was then added to the eluate, which was then incubated for 10 min at 0°C. After removing the precipitated proteins by centrifugation, the supernatant was neutralized and loaded onto a reverse phase column (TSKgel ODS-80T_M, Tosoh Co., Tokyo) equilibrated with 100 mM sodium phosphate buffer (pH 6.9), and the eluted nucleotide was detected by its UV-absorbance.

Measurement of ADP released from *Thermococcus* chaperonins using ADP-dependent glucokinase

The ADP-dependent (AMP-forming) glucokinase from the hyperthermophilic archaeum *T. litoralis* catalyzes the phosphorylation of glucose using ADP as an essential phosphoryl group donor (Koga et al. 2000). This enabled chaperonin ATPase activity to be assayed spectrophotometrically by measuring ADP release using ADP-dependent glucokinase and NADP-dependent glucose-6-phosphate dehydrogenase as previously described (Koga et al. 2000). The reaction mixture (1.5 ml) contained 50 mM Tris–HCl (pH 7.5), 20 mM glucose, 2 mM $MgCl_2$, 300 mM KCl, 0.67 U/ml glucose-6-phosphate dehydrogenase and 1 mM NADP, with or without 5 mM NaF and 1 mM $BeSO_4$. The reaction mixture without NADP was preincubated for 1 min at 60°C, and the reaction was started by the addition of NADP. After 1 min, 1 mM ATP was added and was followed by the addition of 0.25 μM chaperonin 3 min later. The ADP produced was continuously monitored by absorbance of NADPH at 340 nm. One unit of enzyme activity was defined as that which converted 1 μmol of glucose per min at 37°C.

Analysis of association of refolded GFP with *Thermococcus* chaperonins

The heat stable GFP mutant containing three amino acid substitutions (F99S, M153T and V163A), which has an apparent melting temperature of 81.9°C (Topell et al. 1999), was used for the protein folding analysis (Sakikawa et al. 1999; Yoshida et al. 2002c). The GFP (10 μM) was first denatured by incubation in 12.5 mM HCl and 5 mM DTT at room temperature for 30 min, after which refolding of the GFP was carried out at 60°C. When starting the protein folding reaction, the denatured GFP solution was diluted 300-fold (final GFP concentration, 0.033 μM) with the folding buffer containing 0.05 μM chaperonin homooligomer in the presence or absence of 5 mM NaF and 1 mM $BeSO_4$. The molar GFP:chaperonin ratio was 1:1.5 in the folding mixture. The folding mixture was preincubated for 10 min at 60°C, after which ATP (final concentration, 1 mM) was added at appropriate times. The reaction mixture was continuously stirred throughout the folding reaction, and the 510 nm fluorescence elicited from folded GFP by excitation at 396 nm was continuously monitored with a fluorometer (FP-777, JASCO, Tokyo). As a control, native GFP was diluted with folding buffer without chaperonin homooligomer, and the intensity of its fluorescence was taken as 100%.

To analyze the association of folded GFP with chaperonin, the folding reaction mixture was subjected to gel filtration chromatography at 60°C. After 10 and 30 min at 60°C, the folding mixture (250 μl) was applied to a gel filtration column (Asahipack GF510HQ, Shodex Co., Tokyo), and the proteins were eluted with folding buffer without DTT at a flow rate of 1 ml/min. The elution of the proteins was monitored using an on-line UV-spectrophotometer at 280 nm (UV-8010, Tosoh Co., Tokyo), while GFP fluorescence (excitation, 396 nm; emission, 510 nm) was simultaneously detected using an on-line fluorometer (FS-8010, Tosoh Co.).

Under these conditions, β16mer was eluted as two separate peaks with two close positions.

Folding of citrate synthase

The dimeric thermophilic enzyme, citrate synthase (CS), from *Thermoplasma acidophilum* also was used as the substrate for the protein-folding analysis. The CS was denatured by incubation in 50 mM HEPES–KOH (pH 7.5) containing 6 M guanidine HCl and 5 mM DTT for 30 min at 50°C. The denatured CS was diluted 60-fold (to a final concentration of 0.33 μM) with the CS folding buffer [50 mM HEPES–KOH (pH 7.5), 100 mM KCl, 25 mM $MgCl_2$] containing 0.33 μM chaperonin homooligomer at 50°C in the presence or absence of 5 mM NaF and 1 mM $BeSO_4$, with or without 1 mM ATP. The molar ratio of monomeric CS to the chaperonin oligomer was 1:1 in the folding mixture. The folding buffer was preincubated for 10 min at 50°C before the dilution, and 1 mM ATP was added 2 min after dilution. After incubation for an additional 15 min, the CS activity in the folding mixture was determined as previously described (Furutani et al. 1998). As a control, native CS was diluted in the folding buffer without chaperonin homooligomer, and the activity of the native CS was set as 100%.

Protease sensitivity assays

Thermococcus chaperonins (50 nM) were incubated in the assay buffer [50 mM Tris–HCl (pH 7.5), 25 mM $MgCl_2$, and 100 mM KCl] with or without 1 mM ATP for 10 min at 60°C. The chaperonin–ADP–BeFx complex was generated by preincubation with 1 mM ATP, 5 mM NaF and 1 mM $BeSO_4$. Proteolytic digestion of the chaperonin was carried out with thermolysin (1 ng/μl for α16mer and 3 ng/μl for β16mer) for 10 min at 60°C. An aliquot of the reaction mixture was then precipitated with 30% (w/v) trichloroacetic acid and placed on ice for at least 5 min. After centrifugation (17,000*g* for 20 min at 4°C), the precipitate was washed with ice-cold water, boiled for 5 min in sample buffer and subjected to SDS-PAGE (12% polyacrylamide gel, 1% SDS) (Laemmli 1970). The resultant gel was stained with Coomassie brilliant blue R-250.

Electron microscopy and image processing

Thermococcus chaperonins in folding buffer with or without 5 mM NaF, 1 mM $BeSO_4$ 1 mM ATP were adsorbed onto a carbon-coated grid on a hot plate at 60°C and stained with 2% uranyl acetate. The grid was then observed in a transmission electron microscope (EF-2000, Hitachi, Tokyo) operated at an accelerating voltage of 200 kV and using an energy-filtering device to obtain zero-loss images. The electron micrographs were taken using a CCD camera with a pixel size of 24 μm at a magnification of 140,000; the sampling steps were 0.17 nm.

Chaperonin particles (n = 183, 146, 179 and 157) were extracted from the electron micrographs under the following conditions: α16mer alone, α16mer with ATP and BeFx, β16mer alone and β16mer with ATP and BeFx. The particles were classified into 5–6 clusters using a hierarchical clustering method considering translation and rotation. After the rotational and translational fitting parameters were refined and particles with low correlation functions were discarded, the remaining particles with better correlation values (>0.5) were averaged. The statistical significance of rotational symmetry of the chaperonin was analyzed by comparing original averaged images with their 180° rotational images around the center of gravity using the Student's t test. In addition, a model of the closed conformation of the α16mer (Shomura et al. 2004) was created from projections of its atomic coordinates. Using this model, the orientations of averaged particles (side view or top view) were judged by eye. All the procedures were performed using a package of Eos (Yasunaga and Wakabayashi 1996).

Results

Inhibition of the ATPase activity of *Thermococcus* chaperonin subunits by BeFx

We first measured the ATPase activities of α16mer and β16mer using released Pi as an index. In the absence of BeFx, the ATPase activities of α16mer and β16mer were calculated to be 21.3 and 10.7 nmol/mg/min at 60°C, respectively. Activities of both α16mer and β16mer were similarly inhibited by BeFx in a concentration-dependent manner (Fig. 1a). With increasing BeFx concentrations, ATPase activities decreased steeply until reaching a plateau (10–20% of an intact ATPase reaction) at BeFx concentrations greater than 500 μM. NaF had no effect on ATPase activity, while $BeSO_4$ slightly inhibited the ATPase activity of β16mer (data not shown).

We confirmed the inhibitory effect of BeFx on the ATPase activity of *Thermococcus* chaperonins by measuring released ADP (Fig. 1b). When ATP was added to the reaction mixture without chaperonin, absorbance at 340 nm was increased slightly indicating the presence of trace quantities of ADP in the biological grade ATP. After *Thermococcus* chaperonins were added to the mixture in the absence of ADP, the absorbance increased linearly, and this effect was significantly inhibited by BeFx (Fig. 1b). Using this method, we determined that BeFx reduced the ATPase activities of *Thermococcus* chaperonins to 10% of control, which is comparable to the results obtained by measuring the Pi release. After removing free nucleotides from the reaction medium by gel filtration, chaperonin-bound

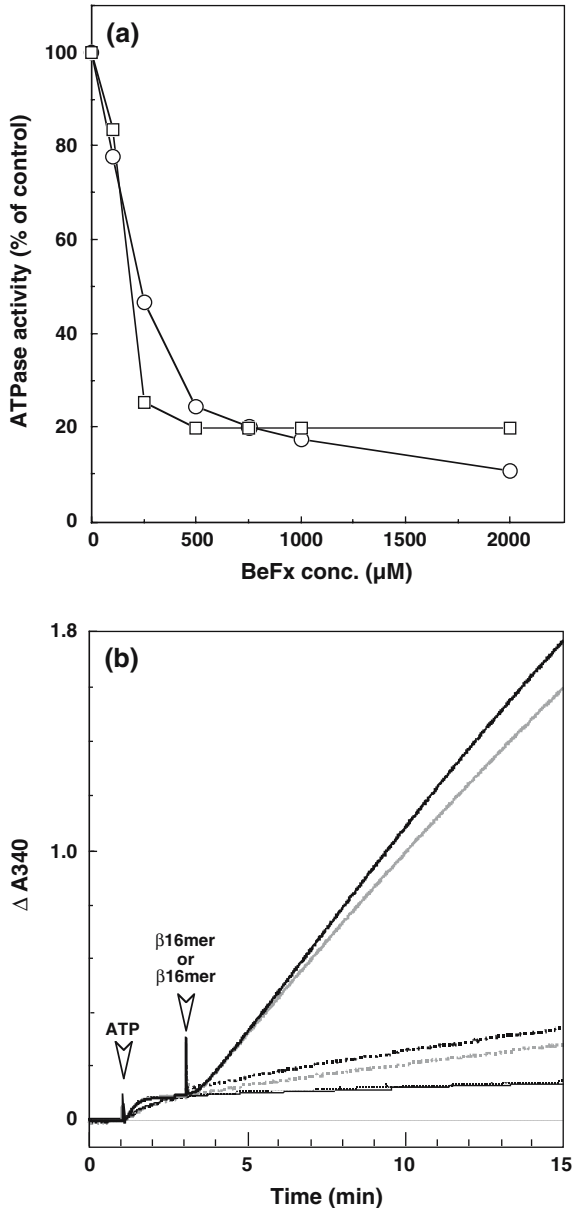

Fig. 1 Effect of BeFx on the ATPase activity of *Thermococcus* chaperonins. **a** Concentration-dependent effect of BeFx on residual ATPase activity in the reaction medium. ATPase activity with and without BeFx was assayed using Pi release as an index, as described in the Materials and methods. The ATPase activity of α16mer (*open circles*) or β16mer (*open squares*) without BeFx was defined as 100%. **b** ATPase activities of α16mer and β16mer, with and without BeFx, using time-dependent release of ADP as an index. Released ADP was measured using an ADP-dependent glucokinase assay, as described in the text. The reaction was started at time 0 by the addition of NADP (final concentration, 1 mM). At 1 min (*open arrow head*), ATP (final concentration, 1 mM) was added, and then chaperonin (final concentration, 0.25 μM) at 3 min (*closed arrow head*). The quantity of NADPH formed was continuously monitored as a change in absorbance at 340 nm. The quantities of ADP in the buffer with (*thin black line*) and without (*thin black dotted line*) BeFx served as controls. Measured were the quantities of ADP released by α16mer without BeFx (*thick black line*), α16mer with BeFx (*thick black dotted line*), β16mer without BeFx (*thick gray line*) and β16mer with BeFx (*thick gray dotted line*)

nucleotides were analyzed with HPLC and identified mainly as ADP. This result was consistent with that reported by Iizuka et al. 2005. Taken together, these results indicate that BeFx inhibits release of both ADP and Pi from *Thermococcus* chaperonin and that a stable chaperonin–ADP–BeFx complex was produced in the presence of ATP and BeFx. The inhibitory activity of BeFx in both chaperonin subunits was approximately the same.

Effect of BeFx on protein folding mediated by *Thermococcus* chaperonins

We next examined the effect of BeFx on the folding of a monomeric protein, GFP. Following denaturation, about 7.5% of denatured GFP spontaneously refolded in the folding buffer (Fig. 2a, b). When denatured GFP was diluted with folding buffer containing α16mer or β16mer, it was captured by the chaperonins, which suppressed the spontaneous folding (Fig. 2a, b). After the addition of ATP, the captured GFP was refolded by the chaperonins (Fig. 2a, b). As reported previously (Yoshida et al. 2002c), addition of the nonhydrolyzable ATP analog AMP–PNP induced refolding of captured denatured GFP by β16mer (Fig. 2b), but not the refolding by α16mer (data not shown). When ATP was added to folding reaction mixture containing BeFx, the GFP was refolded by both α16mer and β16mer (Fig. 2c, d), although the profile of the folding by β16mer seen with ATP and BeFx differed from that by α16mer. β16mer-mediated refolding of GFP proceeded more rapidly in the ATP–BeFx reaction mixture than in the ATP-only mixture and reached a plateau at a level approximately twofold higher than in the AMP–PNP reaction mixture (Fig. 2d). Notably, when the two chaperonins were preincubated with ATP and BeFx before addition of denatured GFP, neither were able to capture the denatured GFP; and GFP folding was equivalent to spontaneous folding (data not shown).

To determine whether the folded GFP was physically associated with *Thermococcus* chaperonins, the folding mixture was analyzed by gel filtration HPLC at 60°C. After incubation for 10 or 30 min after addition of ATP in the presence of BeFx, a significant quantity of GFP fluorescence (folded protein) was eluted with either of the subunits, whereas no fluorescence was detected at the position of the *Thermococcus* chaperonins in the absence of BeFx (Fig. 3a, b). In the presence of BeFx, the elution profile of the folded GFP was approximately the same after incubation for 30 min as that after 10 min (Fig. 3a, b), which indicates that the refolded GFP remained stably bound to the chaperonin–ADP–BeFx complex for at least 25 min. Approximately 3/4 of the refolded GFP was eluted as a free monomer, and only about 1/4 of the refolded GFP was eluted with chaperonin. This may reflect the fact that *Thermococcus* chaperonins release

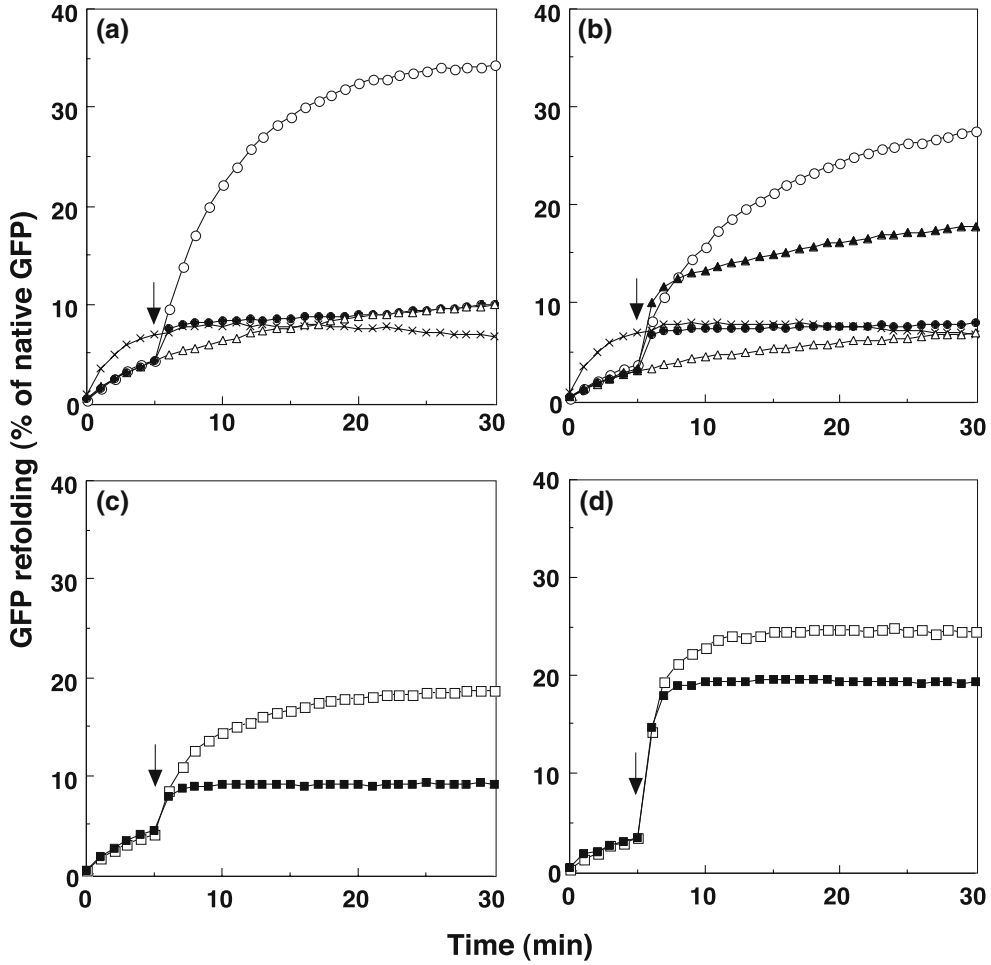

Fig. 2 *Thermococcus* chaperonin-mediated folding of GFP in the presence of both ATP and BeFx. Time course of GFP folding mediated by α16mer without (**a**) or with BeFx (**c**) and β16mer without (**b**) or with BeFx (**d**). The folding mixture was incubated at 60°C. At 0 min, denatured GFP was diluted 300-fold with folding buffer containing nucleotide-free chaperonin. At 5 min (*arrow*), ATP or AMP–PNP with or without trap-α was added to the folding mixture. The folding of GFP was continuously monitored as a change in fluorescence at 510 nm. The fluorescence intensity of native GFP at the same concentration was set as 100%. *Crosses* spontaneous GFP folding; *open triangles* GFP folding mediated by α16mer or β16mer without ATP; *open circles* α16mer or β16mer with ATP; *black circles* α16mer or β16mer with ATP and trapα; *black triangles* β16mer with AMP-PNP; *open squares* α16mer or β16mer with ATP and BeFx; *black squares* α16mer or β16mer with ATP, trapα and BeFx

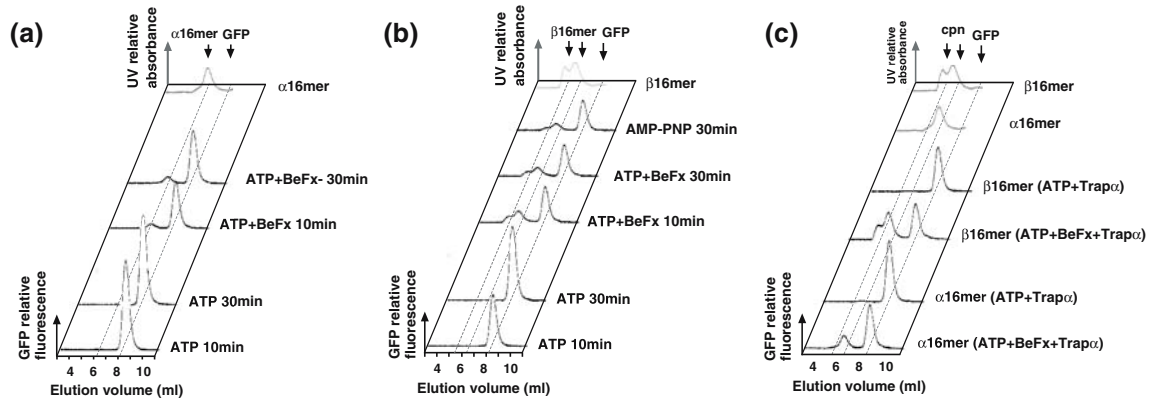

Fig. 3 HPLC analysis of *Thermococcus* chaperonin-mediated GFP folding in the presence of both ATP and BeFx. HPLC elution profiles of GFP folding mediated by α16mer (**a**) and β16mer (**b**) after incubation for 10 or 30 min, with and without BeFx. (**c**) HPLC analyses of GFP folding mediated by α16mer and β16mer after incubation for 30 min in the presence of trap-α, with or without BeFx. The folded products were analyzed by HPLC gel filtration chromatography at 60°C. The flow rate was 1 ml/min. Elution of chaperonin and GFP was separately monitored by the UV absorbance at 280 nm (*gray lines*) and the fluorescence at 510 nm with excitation at 396 nm (*black lines*). The *dotted lines* mark the elution volume of α16mer or β16mer and free GFP, as indicated

captured GFP, whether folded or not, at temperatures lower than 50°C (Yoshida et al. 2002a). Although the temperature of the HPLC column was kept at 60°C, the temperature might have fallen to less than 50°C during transfer from the fluorometer to the HPLC, which would have caused release of the folded GFP from the central cavity.

Enclosure of refolded substrate proteins within the central cavity in the presence of ATP and BeFx

Trapα is a mutant (G65C and I125T) α-subunit chaperonin homooligomer that is incapable of mediating protein folding and has been used previously to trap the unfolded and partially folded forms of GFP (Iizuka et al. 2001; Yoshida et al. 2000, 2002c). We have found that trapα binds unfolded GFP and does not release it, even in the presence of ATP (Yoshida et al. 2002c). ATP-dependent GFP folding mediated by α16mer or β16mer was inhibited by simultaneous addition of trapα (Fig. 2a, b). The mutant chaparonin did not affect the ATP–BeFx-dependent folding by β16mer, but it suppressed the folding mediated by α16mer to the level of the spontaneous folding (Fig. 2c, d). In the presence of trapα, ATP and BeFx, the folded GFP eluted with α16mer or β16mer, whereas no fluorescence was detected at the position of *Thermococcus* chaperonins in the presence of trapα and ATP only (Fig. 3c). Apparently the refolded GFP was retained within the central cavity of the stable chaperonin–ADP–BeFx complex of these subunits.

Effect of BeFx on folding of the dimeric enzyme CS by *Thermococcus* chaperonin subunits

Thermococcus chaperonins, α16mer and β16mer, captured nonnative CS and suppressed the recovery of its activity to levels lower than those obtained with spontaneous folding (Fig. 4). When ATP was added to the folding mixture, the chaperonins mediated a substantial increase in CS folding. However, CS refolding activity was inhibited in the presence of ATP and BeFx. It is noteworthy that CS is active only when it forms a dimeric complex (Smith et al. 1987). Given that ATP-dependent protein folding by chaperonin proceeds with multiple rounds of binding and release (Farr et al. 1997; Weissman et al. 1994; Yoshida et al. 2002c), this suggests that, in the presence of ATP and BeFx, CS folding mediated by *Thermococcus* chaperonins proceeded within its central cavity, but the folded enzyme was not released to the environment where it could form a dimer, and as a result showed no activity.

Conformation of *Thermococcus* chaperonin subunit homooligomers in the presence of ATP and BeFx

To study the conformation of α16mer and β16mer in the presence of ATP and BeFx, we carried out protease

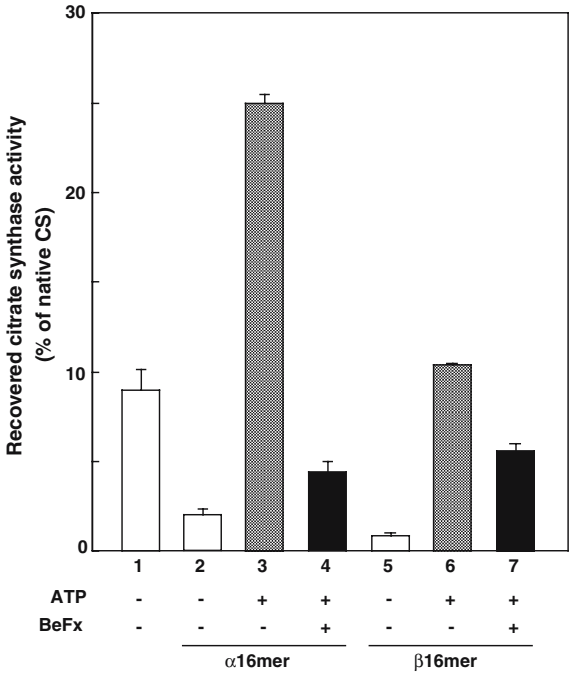

Fig. 4 Refolding of the dimeric enzyme CS mediated by *Thermococcus* chaperonins in the presence of ATP, with and without BeFx. The concentrations of CS, α16mer and β16mer were 0.33 μM. At 0 min, the denatured enzyme was diluted 60-fold in folding buffer containing the indicated chaperonin. Then after 1 min, ATP was added to a final concentration of 1 mM, with or without BeFx, as indicated. The activity of the same concentration of native enzyme was defined as 100%. *Lane 1* spontaneous CS folding; *lane 2* CS folding mediated by α16mer without addition of ATP and BeFx; *lane 3* CS folding mediated by α16mer with addition of only ATP; *lane 4* CS folding mediated by α16mer in the presence of BeFx with addition of ATP; *lane 5* CS folding mediated by β16mer without addition of ATP and BeFx; *lane 6* CS folding mediated by β16mer with addition of only ATP; *lane 7* CS folding mediated by β16mer in the presence of BeFx with addition of ATP

digestion and electron microscopic analyses. 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). We found that in the absence of ATP, both chaperonin subunit homooligomers were susceptible to digestion by thermolysin, but in the presence of ATP they were resistant (Fig. 5). Moreover, in the presence of both ATP and BeFx, both α16mer and β16mer were even more resistant to digestion than they were in the presence of ATP alone, suggesting that the chaperonin–ADP–BeFx complex is stabilized in the closed conformation.

When we analyzed the electron micrographs of negatively stained *Thermococcus* chaperonins, we found numerous side-on views of the β16mer–ADP–BeFx complex, but only a few top views (Fig. 6a, b). By contrast, most α16mer and β16mer, with or without nucleotide, were observed as top views (data not shown). Comparison with a projection model of the closed conformation constructed using the atomic coordinates (Fig. 6g) (Shomura et al. 2004), the averaged side view from the electron micrographs indicated that both the

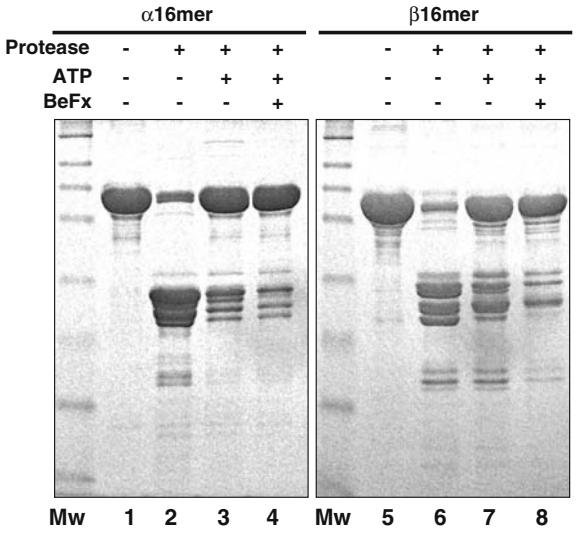

Fig. 5 Thermolysin digestion of *Thermococcus* chaperonins in the presence of ATP and BeFx. Chaperonin–ADP–BeFx complexes were generated by preincubation with 1 mM ATP, 5 mM NaF and 1 mM $BeSO_4$. The thermolysin concentrations were 1 ng/μl for α16mer and 3 ng/μl for β16mer, and digestion was carried out for 10 min at 60°C. Lane Mw contains molecular weight markers (175, 83, 62, 47.5, 32.5, 25, 16.5, 6.5 kDa); *lane 1* α16mer; *lane 2* α16mer without addition of ATP before protease digestion; *lane 3* α16mer incubated with ATP before protease digestion; *lane 4* α16mer incubated with ATP and BeFx before protease digestion; *lane 5* β16mer; *lane 6* β16mer without addition of ATP before protease digestion; *lane 7* β16mer incubated with ATP before protease digestion; *lane 8* β16mer incubated with ATP and BeFx before protease digestion

α16mer and β16mer chaperonin–ADP–BeFx complexes mainly adopt the symmetric closed conformation (Fig. 6c, d). However, one of two lids of the α16mer might be slightly open, as the density of one of the lid regions was obscure and appeared blob-like in structure. A Student's *t* map analysis comparing the original images of *Thermococcus* chaperonins and their 180° rotated images showed overall symmetry (Fig. 6e, f); the differences seen may be due to the titling of the chaperonin particles. In archaeal chaperonins, lid closure is reportedly triggered by ATP binding (Gutsche et al. 2001; Iizuka et al. 2003). Consistent with those findings, our results indicate that two built-in lids of each ADP–BeFx complex of α16mer and β16mer were both in the closed state (Fig. 6h).

Discussion

The protein folding cycle of chaperonins is usually described in terms of the ATPase cycle. ATP binding and hydrolysis serve as switches mediating transition from one step to the next during the folding. The chaperonin–ADP–BeFx complex is thought to mimic an intermediate state in the chaperonin ATPase cycle, between ATP binding and ATP hydrolysis. In this study, chaperonin–ADP–BeFx complexes were generated by incubation with ATP and BeFx. Our electron microscopy and protease digestion experiments showed the ADP–BeFx complex of both α16mer and β16mer to adopt the symmetric closed conformation (Figs. 5, 6). In the presence of ATP and BeFx, β16mer-mediated GFP folding proceeded faster than the folding in the presence of ATP alone; moreover, the yield of folded GFP was approximately twofold higher than that in the presence of AMP–PNP (Fig. 2a). The conformation of AMP–PNP-bound β16mer is asymmetric with one built-in lid opened and the other lid closed (Yoshida et al. 2002c). If the chaperonin–ADP–BeFx complex adopts the symmetric closed conformation, and one molecule of GFP is folded in each of its two cavities, the twofold higher yield would be reasonable. Consistent with this idea, our finding that the ATP + BeFx suppressed the recovery of activity of the dimeric enzyme CS mediated by *Thermococcus* chaperonins (Fig. 4) suggested that BeFx stopped the folding cycle of either α16mer or β16mer in the state of both built-in lids closed. As a result, the folded CS monomers were not released from the cavities of the chaperonin–ADP–BeFx complexes and could not form active dimers.

Three conformations (open, symmetric closed and asymmetric forms) have been described for group II chaperonins (Llorca et al. 1999, 1998; Schoehn et al. 2000a; Schoehn et al. 2000b). Previous studies using small angle X-ray scattering analysis has shown that the nucleotide-free and ADP-bound forms of α16mer adopt the open conformation with opened built-in-lids, and ATP-bound α16mer forms the asymmetric closed conformation (Iizuka et al. 2004). Moreover, α16mer preincubated with ADP and BeFx adopts asymmetric conformation (Iizuka et al. 2005). Based on these earlier observations and the present results, the ATPase cycle in archaeal group II chaperonins is depicted as follows. Nucleotide-free chaperonin forms the open conformation with opened built-in-lids. Upon binding ATP to the one ring of the double ring, the built-in lid is closed, and chaperonin assumes the asymmetric conformation. In this step, the trapped substrate protein within the central cavity of the ring is folded. After ATP hydrolysis formation of the chaperonin–ADP–Pi complex triggers ATP binding to the other ring. This in turn closes the built-in lid of this ring so that the chaperonin adopts a symmetric closed conformation. After hydrolysis of bound ATP is complete, the subsequent release of Pi likely triggers reopening of the built-in lid and release of the folded substrate protein (Fig. 7 in Iizuka et al. 2005).

Table 1 shows the summary of protein folding activity between *Thermococcus* chaperonins. The characteristics of both subunit homooligomers in adding ATP and ADP were same (Table 1). However, comparative analysis on protein folding between subunits revealed that at 60°C protein folding by β16mer was more effective than that by α16mer in the presence of ATP and BeFx (Table 1). It is noteworthy that ATPase activity of the β16mer was lower than the α16mer at this

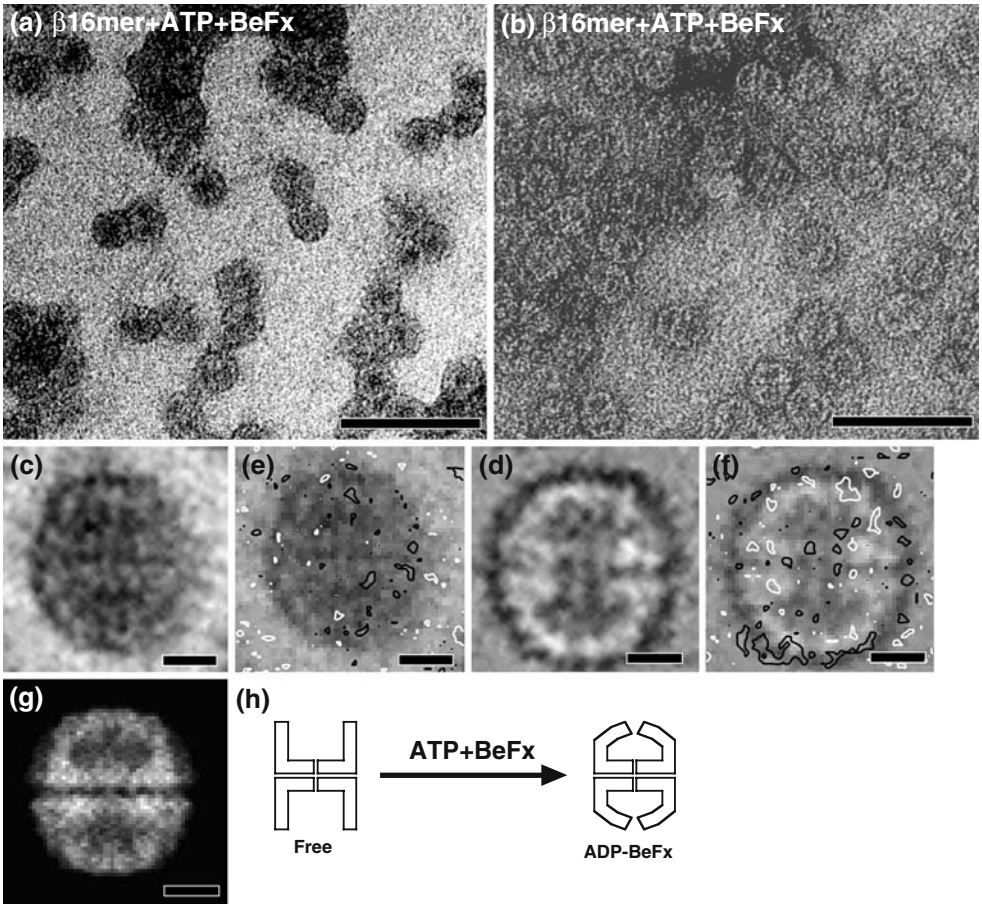

Fig. 6 Electron microscopic analysis of the conformation of *Thermococcus* chaperonins in the presence of both ATP and BeFx. Electron micrographs of negatively stained α16mer (**a**) and β16mer (**b**) in the presence of ATP and BeFx. Averaged images of α16mer (**c**) and β16mer (**d**). Student's *t* maps of the difference between the original and 180° rotated images (95% confidence limit) of α16mer (**e**) and β16mer (**f**) are shown to examine their symmetry. *White areas* (high density) and *black areas* (low density) indicate differences between the original and rotated images. (**g**) Projection model of the closed conformation created from the atomic coordinates of α16mer. (**h**) Schematic drawing illustrating a model of the *Thermococcus* chaperonin–ADP–BeFx complex. Nucleotide free chaperonins adopt an open conformation; upon binding ATP and BeFx, the built-in lids are closed to form the symmetric closed conformation. Scale bars: 50 nm (**a**, **b**) or 5 nm (**c**, **d**, **e**, **f**, **g**)

temperature. ATP–BeFx-dependent folding mediated by β16mer proceeded more rapidly than that mediated by α16mer and it reached a higher plateau. Moreover, the ATP–BeFx-dependent folding mediated by β16mer was unaffected by trapα, whereas that mediated by α16mer was markedly suppressed by trapα. ATP-dependent folding by group II chaperonins is proceeded by multiple rounds of binding and release of nonnative forms of the substrate protein (Farr et al. 1997; Yoshida et al. 2002c). During ATP dependent refolding, the refolding intermediate is in a cyclic reaction of binding to and releasing from the chaperonin. However, when β16mer formed a complex with ADP–BeFx, the ATPase cycle was stopped and the complex formed the symmetric closed conformation. The substrate protein captured by β16mer–ADP–BeFx complex was kept in the central cavity and was completely folded (Figs. 2 and 3). On the contrary, our previous study has indicated that α16mer preincubated with ADP and BeFx which forms the asymmetric closed conformation encapsulates the unfolded protein in the closed-ring but does not fold it (Iizuka et al. 2005). These differences between α16mer and β16mer may indicate that their nucleotide binding affinities are different. The optimal temperature for the ATPase activity of β16mer (>90°C) is higher than that for α16mer (approx. 60°C). Although at 60°C the ATPase activity of β16mer is lower than that of α16mer (Yoshida et al. 2001), binding affinities of β16mer to nucleotide and a substrate protein may be higher than those of α16mer. The closing rate of the built-in lid of β16mer may also be faster than that of α16mer.

Expressions of the α and β subunits are regulated differentially and only the β subunit is thermally inducible (Yoshida et al. 2001). Thus, the proportion of the β subunit in natural chaperonins in vivo increases with temperature, and β subunit-rich chaperonin is more thermostable than α subunit-rich chaperonin (Yoshida et al. 2002a). At higher temperatures, the chaperonin oligomer dissociates to ATPase-inactive monomers. The encapsulation of denatured protein by chaperonin is critical for the protein folding. The β16mer is probably more suitable for protein folding than that of α16mer at higher temperatures. Although the basic protein folding mechanisms of α16mer (Fig. 7 in Iizuka et al. 2005) and β16mer are similar, these subunits differ in particular activities including those observed in the presence of BeFx in the present study. As these subunits show approximately 20% difference in amino acid sequence including the highly diverged C-terminal sequences, these altered residues may be involved in the changed activities. Understanding the cause of these differences may lead to better understanding of the mechanisms underlying the protein folding cycles of *Thermococcus* chaperonin subunits.

Table 1 Summary of protein folding activity between *Thermococcus* chaperonins

The nucleotide added in the reaction mixture	Characteristics of the subunit homooligomer	α16mer	β16mer
ATP	Protein folding	+	+
	Retention of the protein in the cavity	−	−
	Sensitivity to the trapα	+	+
	Conformation	Asymmetric[a]	ND
ADP	Protein folding	−	−
	Retention of the protein in the cavity	−	−
	Sensitivity to the trapα	nd	nd
	Conformation	Open[a]	Open[e]
ADP + BeFx	Protein folding	−	nd
	Retention of the protein in the cavity	+[b]	nd
	Sensitivity to the trapα	nd	nd
	Conformation	Asymmetric[c]	nd
AMP–PNP	Protein folding	−	+
	Retention of the protein in the cavity	ND	+
	Sensitivity to the trapα	ND	−
	Conformation	Asymmetric[a]	Asymmetric[e]
ATP + BeFx	Protein folding	+	+[f]
	Retention of the protein in the cavity	+	+
	Sensitivity to the trapα	+	−
	Conformation	Symmetric closed[c,d]	Symmetric closed[d]

+ Folding of the substrate protein; − no folding of the substrate protein; *ND* not detected; *nd* not determined
[a]Data from Iizuka et al. 2004
[b]Encapsulate the substrate protein in the cavity without folding of the substrate protein
[c]Data from Iizuka et al. 2005
[d]Data from this work. Temperature, 60°C
[e]Data from Yoshida et al. 2002c
[f]Folding yield is higher than that with AMP–PNP

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