Nucleotide specificity of an archaeal group II chaperonin from *Thermococcus* strain KS-1 with reference to the ATP-dependent protein folding cycle

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Received 22 November 2001; revised 28 January 2002; accepted 28 January 2002

First published online 18 February 2002

Edited by Jesus Avila

Abstract The archaeal chaperonin-mediated folding of green fluorescent protein (GFP) was examined in the presence of various nucleotides. The recombinant α- and β-subunit homo-oligomers and natural chaperonin oligomer from *Thermococcus* strain KS-1 exhibited folding activity with not only ATP but also with CTP, GTP, or UTP. The ADP-bound form of both recombinant and natural chaperonin had the ability to capture non-native GFP, but could not refold it in the presence of CTP, GTP or UTP until ATP was supplied. The archaeal chaperonin thus utilized ATP, but could not use other nucleoside triphosphates in the cytoplasm where ADP was present.

Key words: Archaea; ATPase cycle; Group II chaperonin; Molecular chaperone; Nucleoside specificity; Protein folding

1. Introduction

In vivo protein folding is assisted by molecular chaperones. Chaperonins are a type of the molecular chaperone with a molecular weight of 60 kDa and form a double-ring composed of two back-to-back stacked oligomeric rings [1]. They are able to bind the denatured protein or intermediate of the folding protein and refold it in an ATP-dependent manner. Based on their amino acid sequence and their structural homology, the chaperonins are divided into two groups [2,3]. Group I chaperonins from bacteria and organelles, typified by *Escherichia coli* GroEL, are composed of a 60-kDa subunit, and cooperate with the co-chaperonin, GroES.

Group II chaperonins are found in archaea and in the cytosol of eukaryotes. The major difference from group I chaperonins is that the group II chaperonins have no co-chaperonin, but a built-in lid in their apical domain [4]. The archaeal group II chaperonins exist as an eight or nine rotationally symmetric double-ring toroidal structure composed of one to three kinds of homologous subunits of about 60 kDa [5,6]. In the hyperthermophilic archaeum, *Thermococcus* strain KS-1, the natural chaperonin forms a hetero-oligomer with variable subunit composition according to the growth temperature [7]. The recombinant α- and β-chaperonin homo-oligomers and natural chaperonin isolated from *Thermococcus* strain KS-1 have shown a protein-folding activity in an ATP-dependent manner [8,9]. The protein folding has been shown to occur in the *cis*-cavity of *Thermococcus* chaperonin [10]. However, details of protein folding in the ATP-dependent cycle of archaeal chaperonins remain unclear.

The GroEL–GroES complex has exhibited effective protein-refolding activities with not only ATP but also with CTP, UTP or ADP [11,12]. GroEL was capable of hydrolyzing ATP, CTP, and UTP but not ADP [11]. In the currently accepted model, binding of the nucleotide to GroEL is sufficient to drive productive folding in the *cis*-cavity of GroEL-GroES complex, and ATP hydrolysis provides a timer for the substrate to fold and primes GroEL to release GroES, allowing the folded substrate to exit to the medium [13]. The archaeal group II chaperonin from *Methanococcus thermolithotrophicus*, which is composed of only one subunit, promoted refolding of the thermophilic citrate synthase in the presence of ATP, CTP, and UTP, but not with GTP [14]. In contrast to the GroEL–GroES complex, refolding of the citrate synthase by *Methanococcus* chaperonin was not promoted by ADP [14]. This result suggests that the nucleotide dependence of archaeal chaperonin-mediated protein folding is different from that of group I chaperonins. However, it is not known whether protein folding by hyperthermophilic archaeal chaperonins is facilitated by nucleotides other than ATP. To elucidate archaeal chaperonin-mediated protein folding, we examined the nucleotide specificity of protein folding mediated by *Thermococcus* strain KS-1 chaperonin by using green fluorescent protein (GFP) as the substrate.

2. Materials and methods

2.1. Materials

ATP and ADP were purchased from Sigma, the other nucleotides (AMP, CTP, GTP, and UTP) and reagents being obtained from Wako Pure Chemicals. The high-performance liquid chromatography (HPLC) nucleotide analysis showed that there was no contamination by ATP in these ADP, AMP, CTP, GTP, and UTP.

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Abbreviations: α16mer, α-subunit homo-oligomer; β16mer, β-subunit homo-oligomer; GFP, green fluorescent protein; HPLC, high-performance liquid chromatography; nαβ16mer, natural chaperonin oligomer

0014-5793/02/$22.00
PII: S0014-5793(02)02377-3

2.2. Purification of the chaperonin subunit homo-oligomers

The *Thermococcus* strain KS-1 chaperonin α- and β-subunit homo-oligomers (α16mer and β16mer) were respectively expressed in *E. coli* strain BL21 (DE3) cells by expression vectors pK1Eα2 and pK1Eβ [8,9]. They were grown aerobically overnight at 37°C in a 2×YT medium supplemented with 100 μg/ml of ampicillin or 75 μg/ml of kanamycin. The recombinant chaperonin subunit homo-oligomers (α16mer and β16mer) were purified as described previously [7], and were shown to be nucleotide-free by the method described later in the section on the nucleotide hydrolysis and binding assay. Proteins were analyzed by polyacrylamide gel electrophoresis on 12% polyacrylamide gel in the presence of sodium dodecyl sulfate after being denatured by incubating at boiling temperature in the sample buffer [15]. The gel was stained with Coomassie brilliant blue R-250. The protein concentration was measured by the Bradford method with a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), using bovine serum albumin as the standard [16].

2.3. Purification of the natural chaperonin from Thermococcus strain KS-1 cell

Thermococcus strain KS-1 was grown anaerobically as previously described [7]. The cells grown at 90°C were subjected to heat shock at 93°C for 20 min prior to being harvested. This temperature change took approximately 2 min. After cultivation, the culture was immediately cooled to room temperature by circulating water for approximately 10 min and then pooled in plastic containers in an ice-chilled water bath, before the cells were harvested with a continuous centrifuge (Kokusan Co., Tokyo, Japan) at 10 000×*g*. The natural chaperonin oligomer (nαβ16mer) was purified from *Thermococcus* strain KS-1 cell as previously described [7]. The ratio of β/α in the purified nαβ16mer from the cells grown at 90°C was determined to be approximately 8.5 by an enzyme-linked immunosorbent assay [7]. The purified nαβ16mer was stored at 4°C.

2.4. Purification and folding of GFP

A heat-stable mutant of GFP was used [10,17]. GFP was purified from recombinant *E. coli* strain BL21 (DE3) cells harboring the expression vector, pET21C-GFP (His) as described previously [10,17].

The folding reaction of GFP was performed at 60°C. GFP (10 μM) was denatured by incubation in 0.0125 N HCl and 5 mM dithiothreitol (DTT) at room temperature for 30 min. At 0 min, the denatured GFP solution was diluted (0.05 μM final dilution) 200-fold with the folding buffer (50 mM Tris–HCl, pH 7.5, 100 mM KCl, 25 mM $MgCl_2$, and 5 mM DTT) containing 0.05 μM α16mer or β16mer. For nαβ16mer, denatured GFP was diluted (0.03 μM final dilution) 300-fold with the folding buffer containing 0.03 μM nαβ16mer. The folding mixture was preincubated at 60°C for 10 min before denatured GFP was diluted. A nucleotide (1 mM final concentration) was added to the refolding mixture at the appropriate time. The fluorescence at 510 nm by excitation light at 396 nm was continuously monitored with a fluorometer (FP-777, Jasco, Tokyo, Japan), the reaction mixture being continuously stirred throughout the folding reaction. As a control, native GFP was diluted with the folding buffer that did not contain a chaperonin oligomer. The fluorescence intensity of native GFP was taken as 100%.

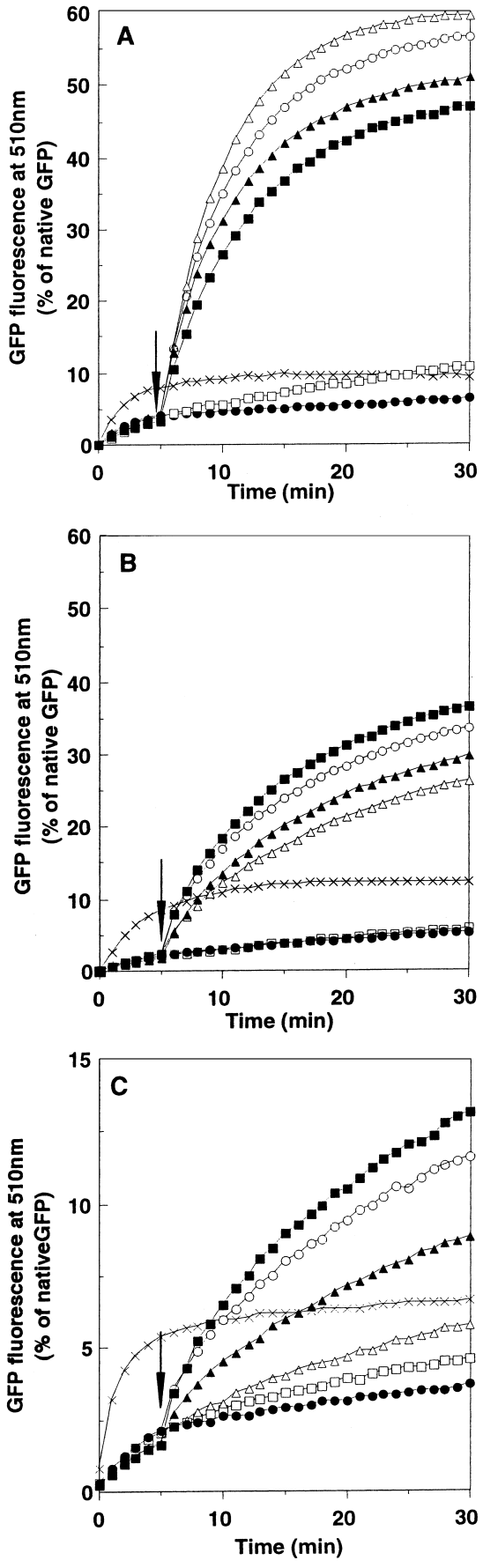

Fig. 1. Effect of various nucleotides on protein folding mediated by the *Thermococcus* chaperonin oligomer. The folding mixture was incubated at 60°C. At 0 min, denatured GFP was diluted into a folding mixture containing the nucleotide-free α16mer, β16mer or nαβ16mer. At 5 min (arrow), a nucleotide (1 mM final concentration) was added to the folding mixture. The refolding of GFP was continuously monitored by the fluorescence at 510 nm with excitation at 396 nm. The fluorescence intensity of native GFP at the same concentration is taken as 100%. A: Nucleotide specificity of α16mer-mediated GFP folding. B: Nucleotide specificity of β16mer-mediated GFP folding. C: Nucleotide specificity of nαβ16mer-mediated GFP folding. ×, spontaneous refolding; ○, folding in the presence of ATP; ●, folding in the presence of ADP; □, folding in the presence of AMP; ■, folding in the presence of GTP; △, folding in the presence of UTP; ▲, folding in the presence of CTP.

2.5. Nucleotide hydrolysis and binding assay

The nucleotide hydrolyzing activity of the recombinant chaperonin homo-oligomers and the natural chaperonin oligomer was measured as described previously [8]. The chaperonin oligomers were incubated in the folding buffer with or without ADP at 60°C for 5 min. The reaction was started by adding the nucleotide at a final concentration of 1 mM, and was terminated with 2% (w/v) perchloric acid. Liberated inorganic phosphate was measured by the malachite green method [18,19]. The rate of the spontaneous nucleotide hydrolysis at each temperature was measured and taken into account for determining the nucleotide hydrolyzing activity.

The chaperonin-bound nucleotide was analyzed by HPLC. The chaperonin oligomers were incubated in a buffer of 50 mM Tris–HCl, pH 7.5, 100 mM KCl, and 25 mM $MgCl_2$ with or without the nucleotide to a final concentration of 1 mM at 60°C for 35 min. After the incubation, the reaction mixture was loaded onto a gel filtration spin column (Bio-Spin 30, Bio-Rad Laboratories, Hercules, CA, USA), which had been equilibrated with 50 mM Tris–HCl, pH 7.5, 100 mM KCl, 25 mM $MgCl_2$ at 60°C, and then subjected to centrifugation. Perchloric acid (2% final concentration) was added into the eluted solution, and the mixture incubated at 0°C for 20 min. After removing the precipitated protein by centrifugation, the supernatant was neutralized and then loaded onto a reverse-phase column (OSD-Tm80, Tosoh) which had been equilibrated with a 100 mM sodium phosphate buffer at pH 6.9. The eluted nucleotide was detected by its UV absorbance.

3. Results

3.1. Nucleotide specificity of GFP refolding mediated by the chaperonin oligomers

The triphosphate nucleotides, GTP, ATP, CTP, and UTP, induced refolding of GFP mediated by α16mer, β16mer or nαβ16mer (Fig. 1A–C). Neither ADP nor AMP induced folding (Fig. 1A–C). Although the former four nucleoside triphosphates were hydrolyzed at about the same rate as ATP by α16mer, β16mer, and nαβ16mer, no hydrolysis of ADP by α16mer, β16mer, or nαβ16mer was detectable (Table 1). At 60°C, plateaus (final yields) of the fluorescence of GFP mediated by α16mer were higher than those by β16mer. These results indicate that *Thermococcus* chaperonins were both able to use not only ATP but also other triphosphate nucleotides for promoting protein refolding at similar efficiency.

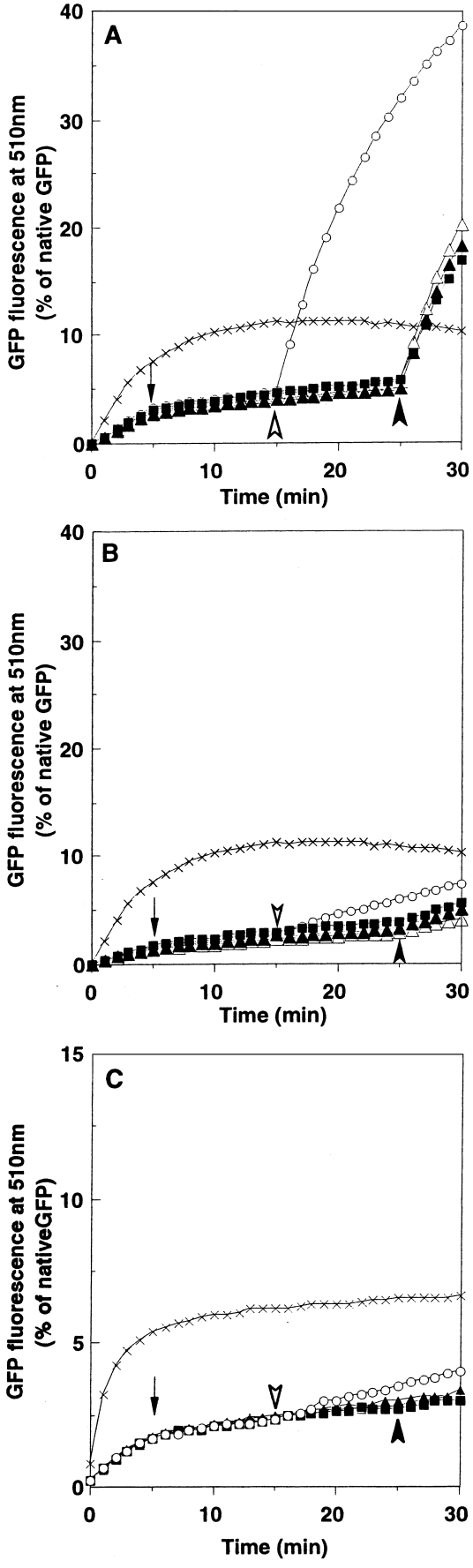

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Fig. 2. Effect of nucleotides on the folding of protein which had been captured by the ADP-bound *Thermococcus* chaperonins. The folding mixture was incubated at 60°C. At 0 min, denatured GFP was diluted into a folding mixture containing the nucleotide-free α16mer, β16mer or nαβ16mer. At 5 min, ADP (1 mM final concentration) was added to the folding mixture (arrow). The refolding of GFP was continuously monitored by the fluorescence at 510 nm with excitation at 396 nm. The fluorescence intensity of native GFP at the same concentration is taken as 100%. A: Effect of triphosphate nucleotides after adding ADP on GFP captured by the α16mer. B: Effect of triphosphate nucleotides on GFP captured by β16mer after adding ADP. C: Effect of triphosphate nucleotides on GFP captured by nαβ16mer after adding ADP. ×, spontaneous GFP refolding; ○, refolding of GFP mediated by α16mer, β16mer, or nαβ16mer with the addition of ATP at 15 min (open arrowhead); ■, refolding of GFP mediated by α16mer, β16mer, or nαβ16mer with the addition of GTP at 15 min (open arrowhead), and further addition of ATP at 25 min (closed arrowhead); ▲, refolding of GFP mediated by α16mer, β16mer, or nαβ16mer with the addition of CTP at 15 min (open arrowhead), and further addition of ATP at 25 min (closed arrowhead); △, refolding of GFP mediated by α16mer, β16mer, or nαβ16mer with the addition of CTP at 15 min (open arrowhead), and further addition of ATP at 25 min (closed arrowhead).

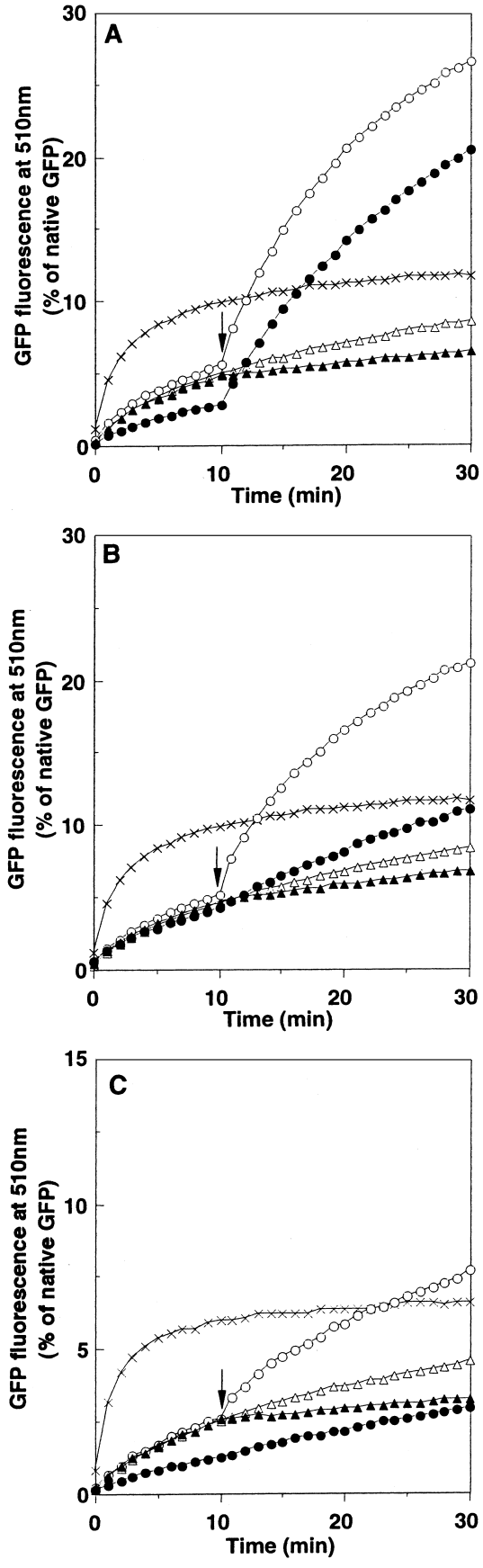

Table 1
Nucleotide hydrolyzing activities of the *Thermococcus* chaperonin oligomer

Nucleoside	α16mer	β16mer	nαβ16mer
ATP	21	10	20
ADP	0	0	0
CTP	23	10	10
GTP	19	10	10
UTP	26	10	10

The α16mer, β16mer and nαβ16mer were incubated at 60°C. The concentration of each chaperonin oligomer was 0.25 μg/μl. The rate of hydrolysis is presented as the released phosphate concentration (nmol/min/mg of protein).

3.2. *Effect of ADP on chaperonin-mediated protein folding*

When denatured GFP was diluted with the folding buffer containing a chaperonin, it was captured by the chaperonins and the increase in fluorescence caused by refolding was suppressed (Fig. 1A–C). When ADP was added to the folding mixture in which the folding intermediate of GFP had been captured by α16mer, β16mer or nαβ16mer, the gradual increase in fluorescence was more significantly suppressed than that in its absence (Fig. 2A–C). ADP was found to bind to nucleotide-free α16mer or β16mer without GFP (data not shown). These results suggest that *Thermococcus* chaperonins, which captured non-native GFP, bound ADP, but did not induce the folding of GFP. The folding of captured GFP was initiated by adding ATP (Fig. 2A–C). However, the fluorescence was not increased by the addition of GTP, CTP or UTP, unlike ATP (Fig. 2A–C). ADP inhibited the hydrolysis of these nucleotides except ATP by α16mer or β16mer (data not shown). When ATP was added to the refolding mixture 10 min after adding GTP, CTP or UTP, the fluorescence increased, indicating that refolding of GFP had started. In the presence of ADP, no other triphosphate nucleotide except ATP promoted this increase in fluorescence. These results suggest that ADP bound to *Thermococcus* chaperonins with higher affinity than GTP, CTP and UTP, but with lower affinity than ATP.

The ADP-bound forms of α16mer, β16mer and nαβ16mer, which had been preincubated with ADP for 10 min, captured unfolded GFP, and then refolded it when ATP was added (Fig. 3A–C). The efficiency of ATP-dependent folding by

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Fig. 3. Ability of the ADP-bound *Thermococcus* chaperonin oligomer to bind denatured GFP. The folding mixture was incubated at 60°C. At 0 min, denatured GFP was diluted with the folding buffer. The refolding of GFP was continuously monitored by the fluorescence at 510 nm with excitation at 396 nm. The final concentration of each added nucleotide was 1 mM, and the fluorescence intensity of native GFP at the same concentration is taken as 100%. GFP refolding mediated by the α16mer, β16mer or nαβ16mer is shown in A, B and C, respectively. ×, spontaneous GFP refolding; △, refolding of GFP mediated by α16mer, β16mer, or nαβ16mer in the absence of nucleotide; ▲, refolding of GFP mediated by α16mer, β16mer, or nαβ16mer with the addition of ADP at 10 min (arrow); ○, refolding of GFP mediated by α16mer, β16mer, or nαβ16mer with the addition of ATP at 10 min (arrow); ●, GFP refolding mediated by α16mer, β16mer, or nαβ16mer which had been preincubated with ADP for 10 min before dilution, ATP being supplemented at 10 min (arrow).

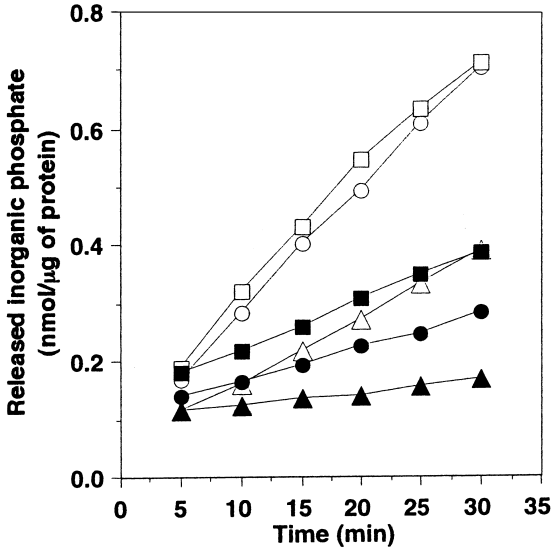

Fig. 4. ATPase activity of the *Thermococcus* chaperonin oligomers in the presence of ADP. ATP hydrolysis by the *Thermococcus* chaperonin oligomers was conducted at 60°C. The amount of inorganic phosphate is shown that was produced in an assay mixture containing α16mer and 1 mM ATP (○); α16mer, 1 mM ADP and 1 mM ATP (●); β16mer and 1 mM ATP (△); β16mer, 1 mM ADP and 1 mM ATP (▲); nαβ16mer and 1 mM ATP (□); nαβ16mer, 1 mM ADP and 1 mM ATP (■).

α16mer in the refolding mixture with or without ADP was almost the same. However, in the presence of ADP, the ATP-dependent folding efficiency of GFP by β16mer and nαβ16mer was significantly lower than that in the refolding mixture without ADP. The ATPase activity of the chaperonin oligomers was partially inhibited by ADP (Fig. 4), indicating that ADP competed with ATP for the binding site of the chaperonin. These results suggest that the difference between the binding affinity of β16mer or nαβ16mer; to ADP and to ATP was less than the case of α16mer.

4. Discussion

The GroEL–GroES complex in the group I chaperonins has been reported to promote the folding of captured protein in the presence of ATP, CTP, and UTP [11,12], GroEL having the hydrolyzing activity of these nucleotides. *Methanococcus* chaperonin has also exhibited protein-folding activity in the presence of ATP, CTP, and UTP, but not GTP [14]. In contrast, the recombinant α16mer, β16mer and nαβ16mer from *Thermococcus* refolded the monomeric protein, GFP, with ATP, CTP, UTP, and GTP. All of these nucleoside triphosphates were also hydrolyzed by α16mer, β16mer and nαβ16mer. These results suggest that the *Thermococcus* chaperonin was able to utilize these nucleoside triphosphates for protein folding.

The GroEL–GroES complex and GroEL without GroES have exhibited ADP-dependent protein-folding activity [11,12]. However, α16mer, β16mer and nαβ16mer did not mediate protein folding in an ADP-dependent manner. ADP also did not promote protein folding mediated by the *Methanococcus* chaperonin [14]. The ADP-bound form of α16mer, β16mer and nαβ16mer had the ability to capture non-native GFP, but was not able to refold it even with an excess of CTP, GTP or UTP being present until ATP had been supplied. In the presence of ADP, ATP was only one nucleotide promoting protein folding by α16mer, β16mer and nαβ16mer among those tested. The binding affinity of the nucleotides was probably in the order ATP > ADP > GTP = CTP = UTP. These results suggest that ATP is the natural nucleotide substrate for chaperonin in the cell of *Thermococcus* strain KS-1, and the ADP, which bound to the *Thermococcus* chaperonin, was exchanged only with free ATP. In the presence of ADP, the yield of ATP-dependent folding by α16mer was higher than that by β16mer and nαβ16mer. The exchange rate of ADP to ATP by α16mer was probably faster than that by β16mer and nαβ16mer.

GroEL and the eukaryotic group II chaperonins mediate protein folding in an ATP-dependent manner which proceeds by multiple rounds of binding and release of the non-native form of the substrate protein [20,21], the folded protein finally being released from the chaperonin. This multiple cycle proceeds until all of the molecules to reach the native form. ATP hydrolysis is the driving force for the cyclic reaction of chaperonin-mediated folding. Our results suggest that *Thermococcus* chaperonin would utilize ATP for protein folding, but would not use other trinucleoside phosphates in the cytoplasm where ADP is present. Chaperonin-mediated protein folding in *Thermococcus* cell probably depends on the cycle of ATP binding and hydrolysis.

Acknowledgements: We thank Ms. C. Ohkami and Ms. H. Iwabuchi for their technical assistance. We acknowledge Dr. H. Taguchi of Tokyo Institute of Technology for the kind gift of the GFP expression vector. This study was conducted at the Marine Biotechnology Institute of Japan as part of the Basic Knowledge Creation and Development Program supported by the New Energy and Industrial Technology Development Organization of Japan.

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