Nucleotide and Manganese Ion is Required for Chaperonin Function of the Hyperthermostable Group II Chaperonin α from *Aeropyrum pernix* K1

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Prevention of thermal aggregation of the denatured protein by the group II chaperonin from the aerobic hyperthermophilic crenarchaeon *Aeropyrum pernix* K1 (*Ap*cpnA) has been investigated. *Ap*cpnA exists as a homo-oligomer in a ring structure, which protects thermal aggregation of the chemically denatured bovine rhodanese at 50 °C. *Ap*cpnA alone is not sufficient for chaperonin activity, but the chaperonin activity is greatly enhanced in the presence of manganese ion and ATP. Compared to the mesophilic chaperonin GroEL/GroES, *Ap*cpnA is more activated at a higher temperature and protects the aggregation-prone unfolded state of the denatured rhodanese from thermal aggregation. Binding of ATP is sufficient for *Ap*cpnA to perform the chaperonin function *in vitro*, but hydrolysis of ATP is not necessarily required. We propose that utilization of Mn^{2+} and adenosine nucleotide regardless of ATP hydrolysis may be one of peculiar properties of archaeal chaperonins.

Key Words : *Aeropyrum pernix*, Archaea, Group II chaperonin, Nucleotide hydrolysis, Thermal aggregation

Introduction

Chaperonins are ubiquitous and indispensable proteins in bacteria, archaea, and eukarya and assist correct folding and assembling of other proteins in an ATP-dependent manner.[1-4] Chaperonins are classified into two groups based on their evolutionary origin. Group I chaperonins found in bacteria, mitochondria, and chloroplasts form tetradecameric double-toroid oligomer of an approximately 60-kDa subunit (GroEL in bacteria) and require a separate co-chaperonin protein (GroES in bacteria) for substrate folding.[5,6] In contrast, group II chaperonins found in the cytoplasm of eukaryotes and in archaea exist as an eight- or nine-membered rotationally symmetrical double-toroid structure composed of homologous subunits of about 60 kDa without need of GroES like co-chaperone for protein folding.[4,7] The apical domain of group II chaperonin has a built-in lid, which is a protruded helical domain, similar to the co-chaperonin found in group I chaperonins.

The archaeal group II chaperonins have been identified from several thermophilic archaea. Although some archaeal chaperonins possess a single subunit, most have two or three distinct subunits with diverse stoichiometry and rotational symmetry.[8] Chaperonin from *Thermoplasma acidophilum* has two stacked rings of octamer composed of two alternating subunits (α and β) with a stoichiometry of 1:1.[9] On the other hand, the chaperonin from hyperthermophilic methanogen, *Methanopyrus kandleri*, forms a two stacked ring structure composed of only one chaperonin subunit with an eight-fold symmetry.[10] The complete genome sequence of a hyperthermophilic crenarchaeon *Aeropyrum pernix* K1, revealed that this strain has two kind of putative thermosome subunit gene (α and β).[11] Recently, we have cloned and purified a subunit of putative group II chaperonin from the hyperthermophilic crenarchaeon *Aeropyrum pernix* K1 (*Ap*cpnA).[12] Several attempts to express a single subunit type of archael chaperonins in *E. coli* often leads to insoluble or inactive assembled protein.[13,14] However, the homo-oligomeric *Ap*cpnA that was expressed and purified from *E. coli* exhibited a typical chaperonin activity; ATP hydrolysis activity in the presence of manganese ion and prevention of thermal aggregation of rhodanese.[12] Notably, the ATP hydrolysis activity was stimulated not with magnesium ion but with manganese ion, but metal ion dependence and role of ATP hydrolysis for prevention of thermal aggregation of denatured protein by the *Ap*cpnA has not been investigated.

In the present study, we report that homo-oligomeric *Ap*cpnA contains full chaperonin activity at a high temperature of 50 °C by preventing thermal aggregation of rhodanese, a well-known model substrate protein for chaperonin study.[15] We report that ATP or ADP binding in the presence of manganese ion is sufficient to exert the chaperonin function by showing that thermal aggregation of denatured rhodanese was significantly prevented to a similar extent in the presence of ATP, ADP, or adenosine 5'-[β,γ-imido]triphosphate (AMP-PNP). These results suggest that

[a]equal contributions to this work.
Abbreviations: *Ap*cpnA, *Aeropyrum pernix* K1 chaperonin (α subunit); AMP-PNP, Adenosine 5'-[β,γ-imido] triphosphate

*Ap*cpnA is a suitable model system for studying the chaperonin in protein folding at high temperature.

Materials and Methods

Expression and purification of the *Ap*cpnA and *E. coli* GroEL/GroES. The protein expression plasmid harboring the *Ap*cpnA (pJLA503-*Ap*cpnA) gene, which was constructed in the previous study,[12] was transformed into *E. coli* BL21(DE3) codon plus cells (Novagen, Inc., San Diego, CA). Transformants were grown in 2 × YTA medium (tryptone 16 g/L, yeast extract 10 g/L, NaCl 5 g/L, and ampicillin 100 μg/mL) overnight at 30 °C for small scale culture. The overnight culture was then inoculated into the fresh medium at 30 °C until the optical density (A_{600}) reached 0.6-0.8, and protein expression was induced by shifting the temperature to 40 °C. After induction for 4 h, cells were collected by centrifugation at 8,000 g for 10 min at 4 °C. The harvested cells were disrupted by sonication in buffer A (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mM dithiothreitol, 20% glycerol) containing DNase/RNase. The suspension of disrupted cells was centrifuged at 27,000 g for 30 min and the supernatant fraction was heat-treated at 85 °C for 30 min followed by recentrifugation. The supernatant was loaded on a DEAE-cellulose (Sigma) column (3.5 × 25 cm) equilibrated in buffer A and the bound protein was eluted with a linear gradient of NaCl (0 to 1.0 M in the same buffer). The protein solution was concentrated using a centricon 10 filter from Amicon (Millipore, Bedford, MA, USA) and dialyzed against buffer B (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, 20% glycerol). The purity of recombinant protein was confirmed by SDS-PAGE. Protein concentration was determined using a Bio-Rad protein assay system (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard. Hereafter, molar concentration of *Ap*cpnA refers to hexadecamer with a monomeric subunit of 60 kDa.

GroEL/GroES chaperone proteins were prepared as described previously.[16] *E. coli* DH5α and BL21 (DE3) codon plus cells were used as the cloning and expression host cells, respectively. Protein concentration was determined using the following extinction coefficient: $E^{0.1\%}$ = 0.142 (at 276 nm) for GroES and $E^{0.1\%}$ = 0.173 (at 280 nm) for GroEL.[16] Hereafter, GroEL and GroES concentrations refer to 14-mer and 7-mer, respectively.

Unfolding-folding of rhodanese. Recombinant bovine rhodanese (thiosulfate:cyanide sulfur transferase, EC2.8.1.1) was purchased (from Sigma) and further purified as described previously[17] to remove ammonium sulfate in which the protein was suspended. Rhodanese concentrations were determined using a value of $E^{0.1\%}$ = 1.75 (at 280 nm).[18] For chemical denaturation of the protein, 1.67 mg/mL (equivalent to 50 μM) rhodanese was denatured for at least 2 hrs at 25 °C in the denaturation buffer (50 mM Tris-HCl, pH 7.2, plus 6 M Guanidinium chloride). For spontaneous folding, unfolded rhodaneses was diluted to 16.7 μg/mL (0.5 μM) in 20 mM MOPS-NaOH buffer (pH 7.2) containing 50 mM sodium thiosulfate, 100 mM KCl, 1 mM dithiothreitol (rhodanese folding buffer), which was preincubated at the desired temperature. For chaperonin-assisted refolding of the chemically denatured rhodanese, the refolding mixture containing the denatured rhodanese (0.5 μM) was incubated in the presence of either *Ap*cpnA (0.5 μM) or GroEL (0.5 μM)/GroES (1.0 μM) with ATP and divalent metal ion at the designated temperature. Chemically denatured rhodanese was also refolded in the presence of *Ap*cpnA with ATP, ADP, or nonhydrolysable ATP analogue, AMP-PNP, as indicated in figure legend. Thermal aggregation of the rhodanese was monitored by incubating the native rhodaese (0.5 μM in the rhodanese folding buffer) in the presence or absence of *Ap*cpnA at 50 °C. The time course of thermal aggregation of rhodanese and aggregation during refolding of the chemically denatured rhodanese was monitored by the increase of light scattering at 320 nm, using an UV-visible spectrophotometer (Ultrospec 2100, Amersham Biosciences).

Electron microscopy experiment. Purified *Ap*cpnA (1.2 mg/mL) that was diluted ten-fold in 50 mM Tris-HCl buffer (pH 7.3) containing 1 mM dithiothreitol and 100 mM NaCl were cross-linked with 2.5% (v/v) glutaraldehyde and 1% (w/v) osmium tetroxide for 1 hr and washed to remove excess fixative. Samples were then mounted on carbon-coated copper grids, and negatively stained with 1% (w/v) uranyl acetate for 30 s. Transmission electron microscopy (JEM 1200EX-II, JEOL, Japan) was performed with an applied voltage of 80 kV. Images were recorded at a magnification of ×40,000 onto Kodak electron image films.

Results and Discussion

Expression and purification of homo-oligomeric *Ap*cpnA. Previously, we have identified an ORF (accession No. APE0907) encoding the thermosome α subunit in the *A. pernix* K1 genome database, and cloned into the protein expression plasmid DNA.[12] The gene encoding the thermosome α was introduced and expressed in *E. coli*, and the recombinant protein (*Ap*cpnA) was purified as described in Materials and Methods. During the purification process, anion exchange on a DEAE-cellulose column was critical to separate *Ap*cpnA from endogenous *E. coli* GroEL, which ensures absence of contaminating bacterial chaperonin in any chaperonin activity that was observed subsequently. The molecular mass of the purified *Ap*cpnA (60 kDa) that was calculated from the amino acid sequence was in agreement with the value obtained by SDS-PAGE (Fig. 1A). To estimate the molecular weight of the homo-oligomeric *Ap*cpnA, the purified protein was applied to Superdex S300 gel-filtration column and eluted as a ~950 kDa complex (data not shown). Electron microscopy observation of the negatively stained *Ap*cpnA revealed that it exists as a typical ring-like structure with a diameter of about 30 nm (Fig. 1B). Thus homo-oligomeric *Ap*cpnA is believed to exist as hexadecameric double-ring structure, as observed in several hyperthermophilic group II chaperonin.[9,10,19]

Chaperonin function of *Ap*cpnA in the presence of Mn^{2+} and ATP. The chaperonin activity of *Ap*cpnA was

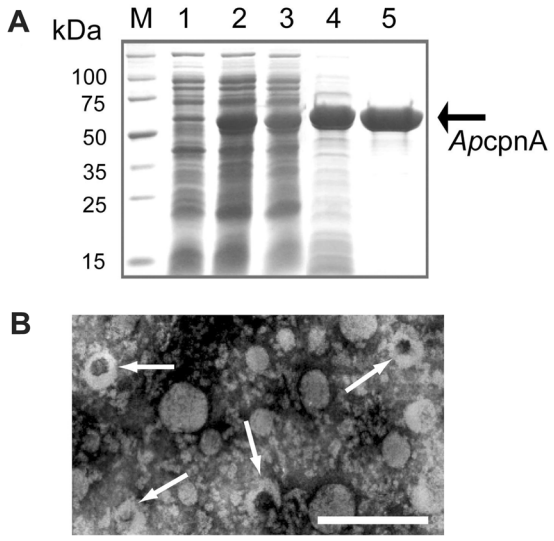

Figure 1. Purification and electron micrograph of homo-oligomeric *Ap*cpnA. (A) SDS-PAGE (10% gel) of *Ap*cpnA during purification. Lane 1, crude extract without heat-induction at 40 °C; lane 2, crude extract after heat-induction; lane 3, supernatant of crude extract after DNase/RNase treatment; lane 4, supernatant after heat treatment at 85 °C for 30 min; lane 5, purified protein after DEAE-cellulose column. (B) Transmission electron micrograph of purified *Ap*cpnA (1.25 μM) fixed with 2.5% (v/v) glutaraldehyde, and negatively stained with 1% (w/v) uranyl acetate. Scale bar, 100 nm, and arrows indicate ring-like structure of *Ap*cpnA.

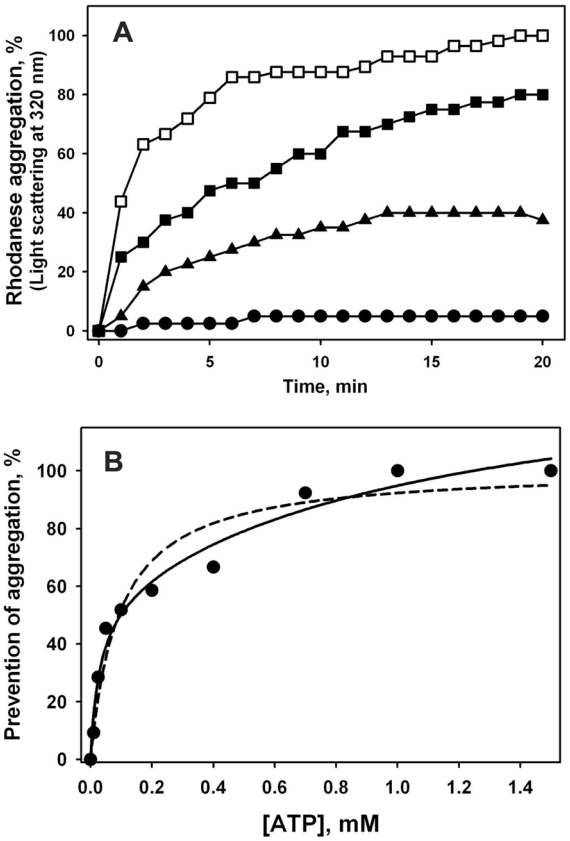

Figure 2. Prevention of the thermal aggregation of rhodanese by *Ap*cpnA. (A) Chemically denatured rhodanese (50 μM) was diluted 100-fold into the rhodanese folding buffer and incubated at 50 °C in the absence (□) or presence of 0.5 mM *Ap*cpnA and other components (●, 5 mM $MnCl_2$ and 2 mM ATP; ▲, 5 mM $MgCl_2$ and 2 mM ATP; ■, 5 mM $MnCl_2$). Time courses for the formation of aggregation were monitored by measuring apparent light scattering at 320 nm using an UV-visible spectrophotometer. (B) Dependence of ATP concentrations on the prevention of rhodanese aggregation by *Ap*cpnA. The extent of thermal aggregation was measured 20 min after incubation of the chemically denatured rhodanese (0.5 μM) in the rhodanese folding buffer containing various concentrations of ATP and 5 mM $MnCl_2$ in the presence of 0.5 μM *Ap*cpnA at 50 °C. The extent of prevention of rhodanese aggregation monitored at 2 mM ATP was set to 100%.

examined by the ability of purified *Ap*cpnA to prevent aggregation of unfolded rhodanese, which is widely used in protein folding studies. Rhodanese has a pronounced tendency to aggregate upon dilution from the denaturant guanidinium-Cl (GdmCl) into a physiological buffer solution. This is easily monitored by the increase in absorbance of the reaction due to light scattering. *Ap*cpnA was capable of preventing this aggregation to a degree dependent on the concentration of chaperonin in the reaction.[12] In order to address ATP and divalent metal ion requirement of the *Ap*cpnA in prevention of protein aggregation, chemically denatured rhodanese was refolded by *Ap*cpnA in the absence or presence of ATP and divalent metal ions, Mg^{2+} or Mn^{2+} (Fig. 2A). When the denatured rhodanese was diluted in 20 mM MOPS-NaOH buffer preincubated at 50 °C, it aggregated extensively within 5 min. Adding *Ap*cpnA into the refolding mixture at a 1:1 molar ratio significantly reduced the extent of protein aggregation by 20%. This chaperonin effect was more enhanced in the presence of ATP and divalent metal ions, such as Mg^{2+} and Mn^{2+} (Fig. 2A). Most of chaperonins including *E. coli* GroEL undergo global conformational changes upon binding and hydrolysis of ATP in the presence of Mg^{2+} during its function as a chaperonin. However, *Ap*cpnA belongs to thermosomes that display very little ATPase activity in the presence of Mg^{2+}, and *Ap*cpnA showed the most enhanced ATPase activity in the presence of Mn^{2+}.[12] Consistent with this observation, *Ap*cpnA with Mn^{2+} and ATP in the refolding mixture almost completely prevented progress of thermal aggregation of the denatured rhodanese up to 20 min at 50 °C. Interestingly, even without ATP hydrolysis activity in the presence of Mn^{2+}, *Ap*cpnA significantly prevented thermal aggregation of the denatured rhodanese (Fig. 2A).

When the chaperonin activity was measured with varying ATP concentrations in the presence of 5 mM Mn^{2+} and 0.5 μM *Ap*cpnA, prevention of thermal aggregation of the denatured rhodanese gradually increased with increasing ATP concentrations and saturated at around 1 mM as shown in Fig. 2B. Double hyperbolic equation with two different binding constants was used for a better fit, because fitting with a single hyperbolic equation shows a significant deviation (dashed line in Fig. 2A). Two half-saturation concentrations ($K_{1/2}$) for ATP that reflect strong and weak binding affinity was determined to be 0.027 (±0.01) and 1.1 (±0.5) mM, respectively. $K_{1/2}$ value for tight ATP binding is

close to the K_M value for ATP hydrolysis (10 μM) that was previously obtained.[12] At present it is not clear why the ATP-dependence on the chaperonin activity shows biphasic behavior with two different $K_{1/2}$ values. One possible explanation would be that in the refolding mixture there exist two forms of oligomeric *Ap*cpnA with two distinct ATP binding affinities.

Comparison of chaperonin activity with *E. coli* GroEL at two different temperatures. The chaperonin activity of *Ap*cpnA was compared with *E. coli* GroEL in prevention of thermal aggregation of rhodanese at two different temperatures. Chemically denatured rhodanese was diluted (0.5 μM) and mixed with the respective chaperonin at 1:1 molar ratio. At the low temperature of 20 °C the GroEL (0.5 μM) together with GroES (1.0 μM) efficiently prevented the progress of thermal aggregation in the presence of 5 mM Mg^{2+} and 2 mM ATP, whereas thermophilic *Ap*cpnA did not show much of chaperonin activity in the presence of 5 mM Mn^{2+} and 2 mM ATP (Fig. 3A). As observed in many archaeal chaperonins that come from thermophilic sources, *Ap*cpnA exhibits limited chaperonin activity *in vitro* at temperatures below its elevated growth optima.[13,14,20,21] Same experiment was performed at the temperature of 50 °C. At the elevated temperature *Ap*cpnA completely prevented thermal aggregation of the denatured rhodanese, but the GroEL/GroES was not able to do so (Fig. 3B). These results indicate that *Ap*cpnA is fully activated at the high temperature and protects the aggregation-prone unfolded state of the chemically denatured rhodanese from thermal denaturation. We have also tested whether *Ap*cpnA is capable of suppressing thermal aggregation of the native rhodanese at the high temperature. Native rhodanese (0.5 μM) was mixed with the respective chaperonin at 1:1 molar ratio and was incubated at 50 °C. As shown in Fig. 3C, the elevated temperature results in gradual increase of light scattering due to extensive aggregation of the native rhodanese. Rate of thermal aggregation process depends on initial status of the substrate protein; thermal aggregation of the native rhodanese showed a slower kinetics than that of the fully denatured rhodanese. In the presence of *Ap*cpnA together with 5 mM Mn^{2+} and 2 mM ATP, progress of the thermal aggregation was lessened at 50 °C, which is more efficient than that with the complete GroEL system (Fig. 3C). The results indicate that *Ap*cpnA interacts with the thermally unfolded substrate protein under ATP-hydrolysis condition, preventing its denaturation even at the elevated temperature. Thus *Ap*cpnA is appropriate as a model chaperonin system that can promote refolding of substrates at high temperatures, using the traditional mesophilic model substrate proteins for folding assay.

Nucleotide dependence on *Ap*cpnA chaperonin activity. We examined which nucleotide-bound state of *Ap*cpnA is active in preventing thermal aggregation of the denatured rhodanese, and whether or not ATP hydrolysis is necessary (Fig. 4). GroEL chaperonin strictly requires GroES and nucleotide for folding of the chemically denatured rhodanese.[15,22] However, GroEL does not strictly require nucleotide hydrolysis for chaperonin activity, because GroEL/GroES readily folded denatured substrate protein in the presence of the non-hydrolysable ATP analogue (AMP-

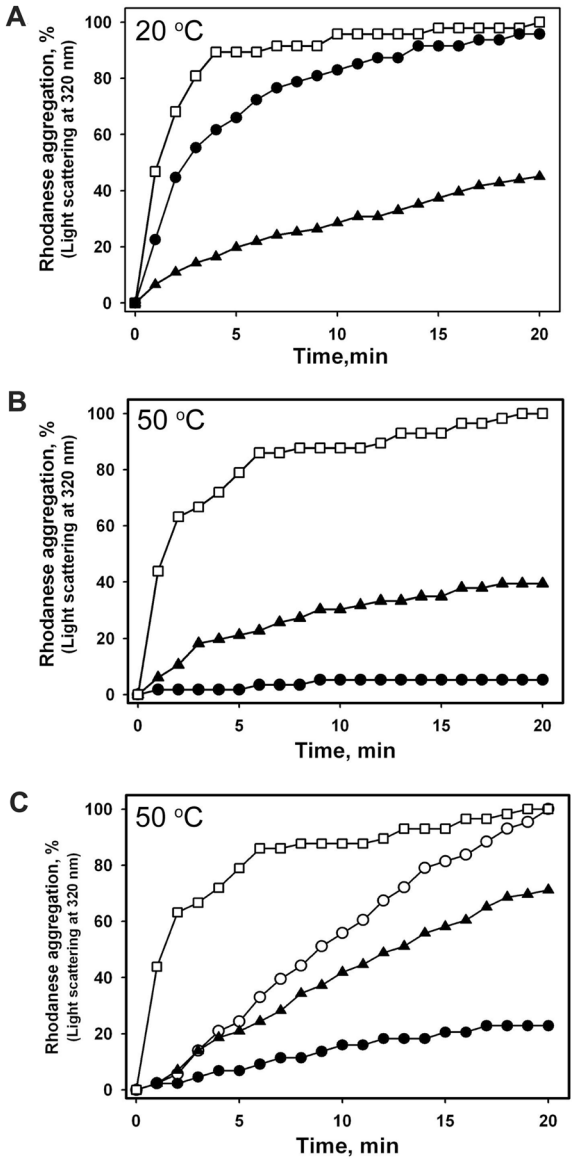

Figure 3. Comparison of chaperonin activity of *Ap*cpnA and GroEL/GroES. Chemically denatured rhodanese (50 μM) was diluted 100-fold into the rhodanese folding buffer and incubated in the presence or absence of chaperonin proteins at two different temperatures of (A) 20 °C and (B) 50 °C. Open squares (□), rhodanese aggregation in the absence of chaperonin; closed triangles (▲), in the presence of GroEL(0.5 μM)/GroES(1.0 μM) plus 5 mM $MgCl_2$/2 mM ATP; closed circles (●), *Ap*cpnA (0.5 μM) plus 5 mM $MnCl_2$/2 mM ATP. (C) Time courses of thermal aggregation of the native rhodanese was monitored by incubating rhodaese (0.5 μM in the rhodanese folding buffer) in the absence or presence of chaperonin at 50 °C. Open circles (○), rhodanese aggregation in the absence of chaperonin; closed triangles (▲), in the presence of GroEL(0.5 μM)/GroES(1.0 μM) plus 5 mM $MgCl_2$/2 mM ATP; closed circles (●), *Ap*cpnA (0.5 μM) plus 5 mM $MnCl_2$/2 mM ATP. Aggregation of the chemically denatured rhodanese (open squares, □) was overlaid for comparison with the thermal aggregation of the native rhodanese.

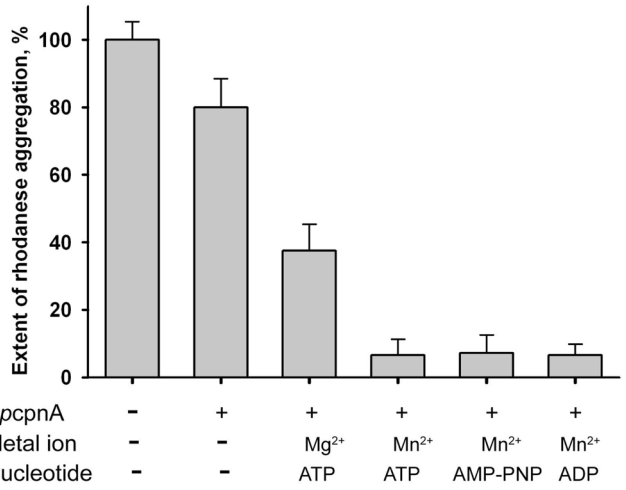

Figure 4. Nucleotide- and metal ion-dependent chaperonin activity of the *Ap*cpnA. Chemically denatured rhodanese (50 μM) was diluted 100-fold into the rhodanese folding buffer and incubated in the presence or absence of *Ap*cpnA at 50 °C. The extent of thermal aggregation was measured 20 min after incubation of the chemically denatured rhodanese (0.5 μM) in the rhodanese folding buffer containing various adenosine nucleotides and 5 mM of $MnCl_2$ or $MgCl_2$ in the presence of 0.5 μM *Ap*cpnA at 50 °C. The extent of rhodanese aggregation measured in the buffer alone was set to 100%.

PNP) or ADP.[23] Previously, we detected a strong ATP hydrolysis activity in *Ap*cpnA in the presence of Mn^{2+}.[12] However, the *Ap*cpnA did not exhibit any ATP hydrolysis activity in the presence of Mg^{2+}. It has been recently reported that chaperonins from *P. furiosus*, *P. horikoshii*, *and M. jannashii* utilize Co^{2+} and Mn^{2+} as divalent cations for stimulation of the ATPase activity.[24] Thus, these results prompted us to evaluate effects of various nucleotides in the presence of Mn^{2+} or Mg^{2+} on the chaperonin activity of the *Ap*cpnA. The chaperonin activity of *Ap*cpnA was examined in the presence or absence of Mn^{2+} (or Mg^{2+})-adenosine nucleotides. Experiments of the prevention of chemically denatured rhodanese at 50 °C in the presence of equi-molar concentration of *Ap*cpnA, 5 mM Mn^{2+} (or Mg^{2+}), and 2 mM adenosine nucleotide were performed. As shown in Figure 4, in the absence of nucleotide, *Ap*cpnA was unable to prevent the substrate protein aggregation at 50 °C. However, *Ap*cpnA prevented aggregation of denatured rhodanese by ~60 % and ~90% in the presence of Mg^{2+}-ATP and Mn^{2+}-ATP, respectively. These results indicate that *Ap*cpnA requires nucleotide and divalent metal ion for efficient chaperonin function *in vitro*. Notably, nucleotide hydrolysis is not necessarily required for the chaperonin activity of *Ap*cpnA, because the protein aggregation was significantly reduced by *Ap*cpnA in the presence of Mg^{2+}-ATP, a condition that *Ap*cpnA is unable to hydrolyze ATP. We found that both ADP and the non-hydrolysable ATP analogue (AMP-PNP) together with Mn^{2+} efficiently supported *Ap*cpnA for prevention of the rhodanese aggregation to a similar extent that was obtained with Mn^{2+}-ATP. This result suggests that binding of ATP or ADP is sufficient for *Ap*cpnA to perform chaperonin function by preventing the rhodanese aggregation under these experimental conditions, and that hydrolysis of ATP is not absolutely required.

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