Biochemical and Biophysical Research Communications 391 (2010) 467–470

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Hyperthermophilic archaeal prefoldin shows refolding activity at low temperature

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ARTICLE INFO

Article history:
Received 8 November 2009
Available online 15 November 2009

Keywords:
Molecular chaperone
Prefoldin
Refolding activity
Lysozyme
Interaction study
Surface plasmon resonance

ABSTRACT

Prefoldin is a molecular chaperone that captures a protein-folding intermediate and transfers it to a group II chaperonin for correct folding. Previous studies of archaeal prefoldins have shown that prefoldin only possesses holdase activity and is unable to fold unfolded proteins by itself. In this study, we have demonstrated for the first time that a prefoldin from hyperthermophilic archaeon, *Pyrococcus horikoshii* OT3 (PhPFD), exhibits refolding activity for denatured lysozyme at temperatures relatively lower than physiologically active temperatures. The interaction between PhPFD and denatured lysozyme was investigated by use of a surface plasmon resonance sensor at various temperatures. Although PhPFD showed strong affinity for denatured lysozyme at high temperature, it exhibited relatively weak interactions at lower temperature. The protein-folding seems to occur through binding and release from PhPFD by virtue of the weak affinity. Our results also imply that prefoldin might be able to contribute to the folding of some cellular proteins whose affinity with prefoldin is weak.

Introduction

Prefoldin is a heterohexameric chaperone that cooperates with a group II chaperonin in archaea and eukaryotic cytosol [1]. Eukaryotic prefoldin captures substrate proteins such as actin and tubulin in the unfolded state and transfers them to a cytosolic chaperonin, chaperonin containing TCP-1 (CCT) for functional folding [2–4]. To date, archaeal prefoldins from *Methanobacterium thermoautotrophicum* (MtGimC) and *Pyrococcus horikoshii* OT3 (PhPFD) have been functionally characterized [5–11]. These are also capable of capturing non-native proteins and releasing them for subsequent chaperonin-dependent folding *in vitro* (capture-deliver function). The crystal structures of MtGimC and PhPFD, and electron microscopic investigation of eukaryotic (bovine and human) prefoldin have shown that prefoldin has the appearance of a jellyfish consisting of a double beta barrel assembly with six long protruding 'tentacles' comprising coiled coils [12–15]. Biochemical and structural studies indicate that these tentacles bind to substrate proteins in a concerted manner and can move outward for efficient interaction with substrate proteins [6,7]. Previous studies have also shown that archaeal prefoldin only has the ability to capture a denatured protein and subsequently deliver it to a chaperonin. Furthermore, prefoldin has no ATPase activity. Consequently, it has been thought that prefoldin itself is unable to refold denatured proteins.

In this study, we have studied the refolding of denatured lysozyme at various temperatures and demonstrated for the first time that PhPFD possesses refolding activity at temperatures relatively lower than physiologically active temperatures. Interaction analysis at various temperatures using a surface resonance sensor suggests that the weak affinity of PhPFD for denatured lysozyme could assist refolding with high efficiency, while strong affinity prevents refolding and aggregation of denatured lysozyme. These results also suggest that prefoldin might be able to contribute to the folding of some cellular proteins whose affinity with prefoldin is weak, or at low temperature conditions.

Materials and methods

Reagents. Hen egg white lysozyme, Guanidine hydrochloride (GdnHCl), Dithiothreitol (DTT), oxidized glutathione (GSSG) and reduced glutathione (GSH) were purchased from Wako (Osaka, Japan). Dried *Micrococcus lysodeikticus* cells were obtained from Sigma–Aldrich (St. Louis, MO, USA). PhPFD was expressed in *Escherichia coli* BL21 (DE3) and purified as previously described [8].

Abbreviations: PhPFD, prefoldin from *Pyrococcus horikoshii* OT3; TEM, transmission electron microscopy

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doi:10.1016/j.bbrc.2009.11.081

Unfolding and refolding of lysozyme in the presence of prefoldin. Native lysozyme (20 mg/mL) was denatured and reduced for 13 h at room temperature in denaturing-reducing buffer (100 mM Tris–HCl, pH 8.0, 6 M GdnHCl, 50 mM NaCl, 1 mM EDTA and 20 mM DTT) as described [16]. A solution of denatured lysozyme (1.4 mM) was then diluted 100-fold using refolding buffer (100 mM Tris–HCl pH 8.0, 50 mM NaCl, 3 mM GSH, 0.3 mM GSSG and 1 mM EDTA) in the absence or presence of 14 μM PhPFD. Refolding solutions were incubated using Thermomixer Comfort (Eppendorf, Hamburg, Germany) with shaking at 1100 rpm at 20, 25, 30, 40, 50 and 60 °C for 56 h, 12 h, 6 h, 150 min, 90 min and 30 min, respectively, before being assayed for enzymatic activity. The incubation time when the refolding yield was saturated was set as the refolding time.

Aliquots (20 μL) of refolding solutions were injected into 1380 μL of an assay buffer (50 mM sodium phosphate, pH 6.2) containing suspended *M. lysodeikticus* cells (0.15 mg/mL). The initial rate of enzymatic lysis of the *M. lysodeikticus* cells at 25 °C was determined by measuring the decrease in absorbance at 450 nm using a CARY 50 spectrophotometer (VARIAN, Palo Alto, CA, USA). The refolding yield was expressed as the percentage of the amount of refolded lysozyme in the refolding solution relative to the initial amount of native lysozyme.

Analysis of interaction between denatured lysozyme and PhPFD by surface plasmon resonance. Surface plasmon resonance experiments were performed using a BIAcore T-100 system (Biacore AB, Uppsala, Sweden) at various sensor temperatures (20, 30 and 40 °C). Analysis at temperatures higher than 40 °C could not be performed due to the temperature limit of this instrument. Denatured lysozyme was coupled to the sensor chip (CM5 research grade) via standard *N*-hydroxysuccinimide and *N*-ethyl-*N*-(dimethylaminopropyl) carbodiimide activation. For immobilizing denatured lysozyme, 100 μL of 17 mg/ml lysozyme, denatured as described above in denaturing-reducing buffer, was injected on the sensor surface with HBS-EP buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.05% surfactant P20) being employed as the mobile phase buffer during the immobilizing process. Tris–HCl buffer (100 mM, pH 8.0) was then injected to quench the unreacted *N*-hydroxysuccinimide groups. TN buffer (100 mM Tris–HCl, pH 8.0, containing 50 mM NaCl) was then used as the mobile phase buffer. PhPFD samples at various concentrations (0.25, 0.5, 0.75 and 1.0 μM) were injected as the analytes and bound analytes were subsequently removed by washing with the mobile phase buffer at 300 s after the injection. Denaturation buffer was pulse-injected (30 s) prior to each analyte injection. Kinetic constants were calculated from the sensorgrams using the BIAcore T-100 evaluation software, version 2.1 (Biacore). The kinetic values of the interaction between denatured lysozyme and PhPFD were calculated using a heterogeneous ligand model. Association constants (K_A) were calculated by the resonance unit at equilibrium using the following equation;

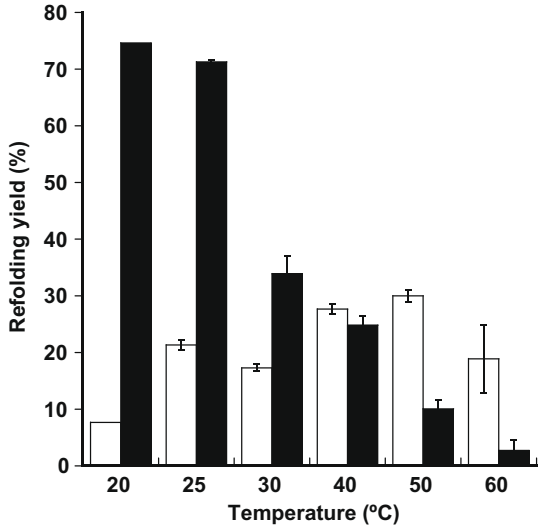

Fig. 1. Refolding of denatured lysozyme in the presence of PhPFD at various temperatures. Native lysozyme (20 mg/mL) was denatured and reduced for 13 h at room temperature in denaturing-reducing buffer (100 mM Tris–HCl, pH 8.0, 6 M GdnHCl, 50 mM NaCl, 1 mM EDTA and 20 mM DTT). A solution of denatured lysozyme (1.4 mM) was then diluted 100-fold in refolding buffer (100 mM Tris–HCl pH 8.0, 50 mM NaCl, 3 mM GSH, 0.3 mM GSSG and 1 mM EDTA) in the absence (white) or presence (black) of 14 μM PhPFD at 20, 25, 30, 40, 50 and 60 °C for 56 h, 12 h, 6 h, 150 min, 90 min and 30 min, respectively, before being assayed for lysozyme activity. The refolding yield was expressed as the percentage of the amount of refolded lysozyme in the refolding solution relative to the initial amount of native lysozyme.

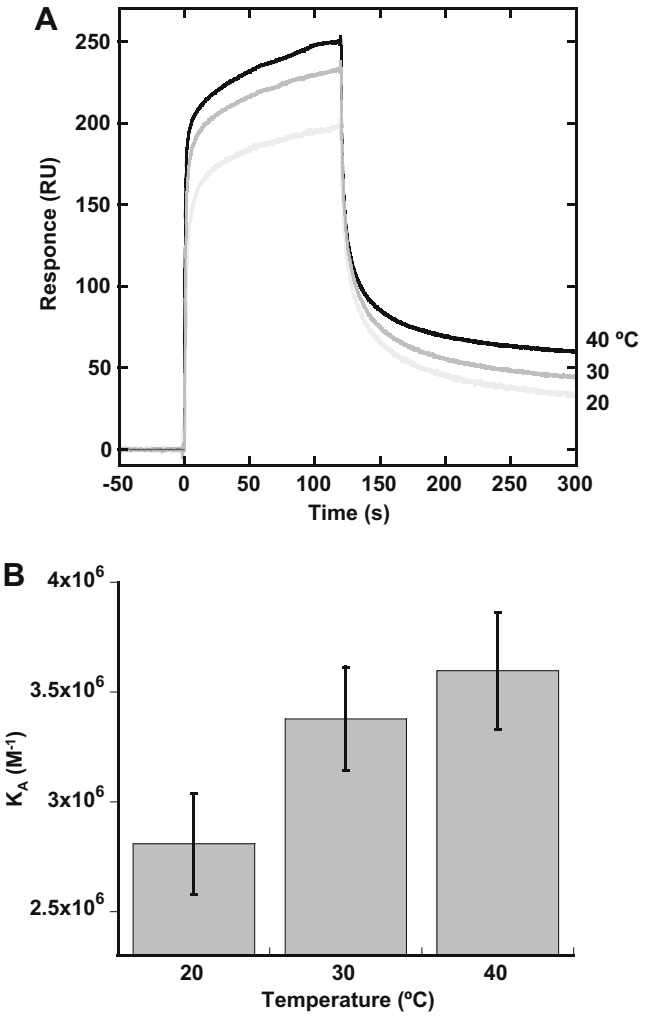

Fig. 2. Examination of the interaction between PhPFD and denatured lysozyme using a surface plasmon resonance sensor. (A) Surface plasmon resonance experiments were performed using a BIAcore T-100 system (Biacore AB, Uppsala, Sweden) at various sensor temperatures (20, 30 and 40 °C). Denatured lysozyme was immobilized onto the BIAcore sensor chip to 10,000 RU. PhPFD was injected at 0.25 μM as the analytes at 20 (gray), 30 (dark gray) and 40 °C (black) are shown for comparison. (B) Kinetic constants were calculated from the sensorgrams using the BIAcore T-100 evaluation software, version 2.1 (Biacore). The kinetic values of the interaction between denatured lysozyme and PhPFD were calculated using a heterogeneous ligand model. Association constants (K_A) were calculated by the resonance unit at equilibrium using the following equation; $R_{eq} = R_{max} \cdot C/(C + 1/K_A)$ where R_{eq} represents the equilibrium resonance units, R_{max} is the resonance signal at saturation, and C is the concentration of free analyte.

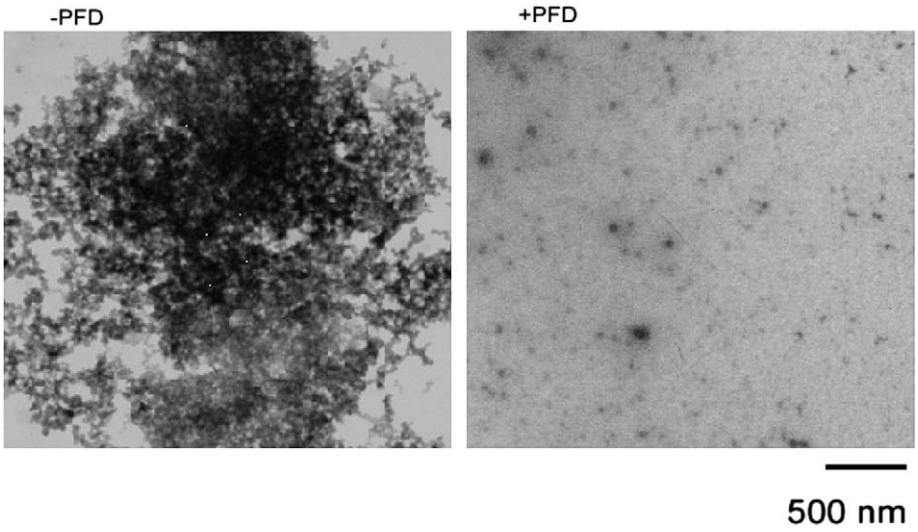

Fig. 3. TEM analysis of lysozyme samples incubated with PhPFD at 25 °C. Denatured lysozyme was incubated at 25 °C for 12 h in the absence or presence of PhPFD. Samples were examined with an excitation voltage of 100 kV using a JEM-1011 TEM (JEOL, Tokyo, Japan). The scale bar represents 500 nm.

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

where R_{eq} represents the equilibrium resonance units, R_{max} is the resonance signal at saturation, and C is the concentration of free analyte.

Transmission electron microscopy (TEM). Denatured lysozyme was incubated at 25 °C for 12 h in the absence or presence of PhPFD as described above. The samples were then diluted 10-fold with distilled water and soon placed onto a carbon-coated copper grid and allowed to absorb. The grid was air dried prior to negative staining with uranyl acetate. Excess stain was then removed from the grid by air drying. Samples were examined with an excitation voltage of 100 kV using a JEM-1011 TEM (JEOL, Tokyo, Japan).

Results and discussion

Fig. 1 shows the refolding yields of denatured lysozyme in the absence or presence of PhPFD at various temperatures. At higher temperatures, which are closer to the physiological conditions of PhPFD, refolding of lysozyme was suppressed in the presence of PhPFD. This result agrees well with previous observations that PhPFD is a holdase which captures a folding intermediate and then subsequently transfer it to a group II chaperonin for folding [6–10]. The spontaneous refolding of lysozyme in the absence of PhPFD was relatively similar at all temperatures examined. Surprisingly, however, the refolding yields at lower temperatures were much higher in the presence of PhPFD than in the absence of PhPFD (Fig. 1). The refolding yield in the presence of PhPFD increased with decreasing incubation temperature, and exceeded 70% at room temperature. This result indicates that PhPFD possesses refolding activity at room temperature, which is lower than the physiological temperature.

In an effort to elucidate the mechanism of the refolding activity of PhPFD at lower temperatures, the interaction between PhPFD and denatured lysozyme at various temperatures (20, 30 and 40 °C) was examined by surface plasmon resonance using the BIAcore T-100 system. Higher temperatures could not be employed due to limitation of the apparatus. Fig. 2A and B clearly show that the affinity was temperature-dependent and became weaker with decreasing temperature. The association constant (K_A) values were estimated as 2.8, 3.4 and 3.6 ($\times 10^6\ M^{-1}$) for 20, 30 and 40 °C, respectively. This result implies that the refolding activity of PhPFD might emerge at lower temperatures due to weak interactions between PhPFD and lysozyme. It is important to note that this weak interaction should be strong enough to prevent aggregation of lysozyme molecules, considering high refolding efficiency. On the other hand, the interaction at higher temperatures is so strong that refolding of denatured lysozyme is prevented. These results support a novel notion that the interaction between molecular chaperone proteins and their substrates can determine the fate of substrate proteins, such as whether the substrates undergo refolding or are maintained in their folding intermediate states.

In order to further confirm that aggregation of lysozyme was prevented by PhPFD at lower temperatures, samples incubated at 25 °C were examined by TEM. As shown in Fig. 3, denatured lysozyme formed large aggregates when incubated in the absence of PhPFD, which is consistent with the low refolding yield (Fig. 1). On the other hand, aggregation of denatured lysozyme during the refolding process was efficiently prevented in the presence of PhPFD at 25 °C (Fig. 3). This result also supports our idea that the relatively weaker affinity between PhPFD and denatured proteins at lower temperatures could prevent aggregation during the refolding process and, more importantly, could facilitate refolding. At higher temperatures, while stronger affinity could prevent aggregation of the denatured proteins, it also precluded refolding of the substrate protein.

In conclusion, we demonstrated for the first time that PhPFD possesses refolding activity of denatured lysozyme at relatively lower temperatures than physiological temperatures. It was also suggested that the weak affinity prevents aggregation and may assist the refolding of denatured lysozyme. On the other hand, refolding of lysozyme was prevented at higher temperatures due to the higher affinity with PhPFD. Thus, it is plausible that the affinity of the interaction between molecular chaperone proteins and denatured substrate proteins could modulate the refolding process of the substrates. Our results also suggest that prefoldin might be able to contribute to the folding of some cellular proteins whose affinity with prefoldin is weak, or at relatively lower temperature conditions than physiological temperatures. Further investigations are required to confirm these possibilities.

Acknowledgments

This work is financially supported by RIKEN (Nano-scale Science & Technology Research) and the Ministry of Education, Sci-

ence, Sports, Culture, and Technology (MEXT) of Japan. We thank Mrs. Yuriko Sakamaki (RIKEN Research Resource Center) for TEM analysis.

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