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Preheating induced homogeneity of the small heat shock protein from *Methanococcus jannaschii*

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Abstract

Small heat shock proteins usually exhibit increased chaperone-like activity either at high temperatures or after preheating. However, the activation mechanism is still unclear. In the current study, we investigated the preheating-activation process of Mj HSP16.5, using various biophysical methods. Although Mj HSP16.5 was reported to be the most monodispersed sHSPs, we found that the newly purified Mj HSP16.5 was actually heterogeneous. 85 °C-preheating could activate Mj HSP16.5 and turn it into a more compact homogeneous species at the same time. Different cooling rates after preheating did not change the activity of Mj HSP16.5, suggesting that the 85 °C-preheated Mj HSP16.5 is in the most active and also the most stable state. These results demonstrate that the activation process of Mj HSP16.5 might accompany a refolding process.

Keywords: Heat shock protein; Chaperone; Protein folding; Homogeneity; HSP 16.5

1. Introduction

Small heat shock proteins (sHSPs) are ubiquitous among all organisms. As monomers, their sizes range from 12 to 43 kDa, assembling into larger oligomers of 9–40 monomers. All known sHSPs share a conserved sequence of "the α-crystallin domain". Crystal structures of Mj HSP16.5, HSP16.5 from *Methanococcus jannaschii*, and HSP16.9 from wheat, reveal a very similar dimeric organization with a strand of one monomer exchanged with a strand from the other monomer, suggesting that this dimeric unit is a common building block and the most stable subunit of all sHSPs [1,2].

sHSPs possess chaperone-like activity that protects other unfolded proteins from aggregation [3,4]. The unfolded proteins arrested by sHSPs might be recovered later with the help of other chaperones [5,6]. It is suggested that the 'native' assembly form of sHSP is used as a storage state and can quickly dissociate into active small subunits in response to external high temperature [2,7–13]. In the case of HSP 26, a 24-mer, the active species was a dimer [10]. Consistent with this suggestion, many sHSPs exchange a subunit between the native assemblies [2,7,14,15], even between assemblies of different homologies of sHSPs [2,14]. It has also been proposed that the ratio of sHSP to its substrate may depend on the type of substrate investigated [16]. For example, a 1:1 monomeric molar ratio of a single chain monellin (SCM) to Mj HSP16.5 could prevent the thermal aggregation of SCM at 80 °C [16]; the ratio of insulin to Mj HSP16.5 required about 1:7 (molar ratio in monomer) to completely prevent the DTT-induced aggregation of the insulin B chain at 37 °C [14], while a 1:40 monomeric molar ratio of citrate synthase (CS) to Mj HSP16.5 was not able to completely prevent the thermal aggregation of CS at 40 °C [16].

Interestingly, many sHSPs show significantly increased chaperone-like activity either at elevated temperatures or after being preheated and subsequently cooled [10,17–21]. Although the mechanism is not completely understood, the activation of sHSPs

Abbreviations: sHSP, small heat shock protein; Mj HSP16.5, sHSP from *Methanococcus jannaschii*; CS, citrate synthase; DTT, DL-dithiothreitol; SCM, single chain monellin; SEC, size exclusion chromatography; FRET, fluorescence resonance energy transfer; DLS, dynamic light scattering; AUC, analytical ultracentrifugation

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has been attributed to many factors, including the destabilization of sHSP oligomers [10], increase of the subunit-exchange rate [15,22,23] and the size of sHSPs at high temperatures [17,22]. However, the data regarding these factors are somewhat inconsistent. For example, it has been found that the increased activity of sHSPs coincides with the destabilization of the oligomer and an increase in the subunit-exchange rate [10,24]. However, a recent report showed that the activation of HSP26 did not require dissociation of the oligomer [25].

To better understand the activation mechanism of sHSPs, we studied the changes in conformation and activity of Mj HSP16.5 before and after preheating, using several techniques, including circular dichroism (CD), fluorescence resonance energy transfer (FRET), dynamic light scattering (DLS) and analytical ultracentrifugation (AUC). Mj HSP16.5 was selected for this study because: (1) its crystal structure has been solved [1]; (2) Mj HSP16.5 was regarded as the most monodispersed (homogeneous) sHSPs [26]; and (3) the 24-mer of Mj HSP16.5 is extremely thermostable even at temperatures as high as 70 °C [14]. Our results show that the newly purified Mj HSP16.5 is actually heterogeneous, and preheating treatment could not only activate Mj HSP16.5, but also turn it to a homogeneous compact species, suggesting a "refolding" mechanism of activation.

2. Experimental

2.1. Materials

Cy3 and Cy5 mono NHS ester dyes were obtained from GE Amersham. Porcine heart citrate synthase (CS), bovine insulin, thyroglobulin (669 kDa), catalase (232 kDa), bovine serum albumin (66 kDa) and mineral oil were purchased from Sigma. The pET21a plasmid containing the Mj HSP16.5 gene was a generous gift from Dr. Sung-Hou Kim at the University of California at Berkeley. The pET21a plasmid containing the Mj HSP16.5 gene was transformed into the *E. coli* Strain, Rossetta (DE). Expression and purification of Mj HSP16.5 was performed according to the reported procedures [27] with minor modifications. Briefly, *E. coli* cells were cultured at 37 °C, and harvested at 4 °C by centrifugation. The harvested cell pellet was then resuspended in PBS buffer and sonicated. After centrifugation at 4 °C, the supernatant was collected and mixed with solid ammonium sulfate to a final concentration of $(NH_4)_2SO_4$ of 260 g/L, and incubated at 4 °C for 15 min. The sample was then centrifuged, and the supernatant was mixed with solid ammonium sulfate again to a final concentration of $(NH_4)_2SO_4$ of 460 g/L, and incubated at 4 °C for 15 min and then centrifuged. The pellet was dissolved in 50 mM PBS buffer (pH 6.9), and applied onto a 5-mL HiTrap Q column (GE Amersham), washed with a linear gradient from 0 to 0.5 M NaCl. The fraction corresponding to Mj HSP16.5 was pooled and concentrated by ultrafiltration (Amicon, YM30), then applied onto a Sephacryl S-200 HR column (GE Amersham), eluting with 40 mM PBS, 100 mM NaCl buffer (pH 6.9). Newly Purified Mj HSP 16.5 was concentrated by ultrafiltration (Amicon, YM30), and used immediately or stored at 4 °C for no more than 72 h.

2.2. Preparation of dye-labeled protein

Mj HSP16.5 was labeled with either Cy3 or Cy5 mono NHS ester dye according to the protocol provided by the manufacturer. The labeled protein was separated from free dye by separation on a Sephadex G-25 desalting column (GE Amersham). The final dye:Mj HSP16.5 (24-mer) molar ratios were estimated to be 3.5 and 8.5 for the Cy3-labeled and Cy5-labeled Mj HSP16.5, respectively.

2.3. Preheating treatment

The preheating of the newly purified and dye-labeled Mj HSP16.5 was performed by incubating proteins in a water bath, and the subsequent cooling was performed in an ice-water bath, or air, or at a controlled cooling rate. Preheated Mj HSP16.5 was used immediately or stored at 4 °C for no more than 72 h.

2.4. Native-pore gradient PAGE

Mj HSP 16.5 was loaded with 100 mM Tris, 10% Glycerol, 0.2% bromphenol blue buffer (pH 8.6), and native-pore gradient PAGE was performed in 25 mM Tris, 192 mM Glycine running buffer (pH 8.3) at 4 °C for 12 h on pore gradient gels with a gradient from 4–30%, then stained with Coomassie brilliant blue. Thyroglobulin (669 kDa), catalase (232 kDa), and bovine serum albumin (66 kDa) were used as molecular weight markers.

2.5. Aggregation assay

The aggregations of CS at 45 °C and reduced insulin at 37 °C were monitored at one-minute time intervals by light scattering at 360 nm using Molecular Devices-SpectraMax 190 (Molecular Devices). CS was used at a concentration of 1.69 μM (monomer) in 50 mM PBS and 1 mM EDTA (pH 6.9). Insulin was first prepared as a high concentration stock solution in 20 mM Glycine–HCl (pH 3.0) and stored at 4 °C, before its subsequent use at a final concentration of 53.6 μM. The aggregation of reduced insulin was initiated by the addition of DTT at a final concentration of 2 mM with different concentrations of Mj HSP16.5 in 50 mM PBS and 1 mM EDTA (pH 6.9). Five microliters of mineral oil was added to each plate to prevent evaporation, and plates were gently shaken (automatically controlled by SpectraMax 190) before each measurement to prevent the sedimentation of aggregated proteins. The concentrations of Mj HSP16.5, CS, and insulin were determined by absorbance at 280 nm using a Lambda 45 UV/Vis spectrometer (Perkin Elmer) with extinction coefficients of 0.565 mg^{-1} cm^2 [28], 1.0 mg^{-1} cm^2, and 1.78 mg^{-1} cm^2 [29], respectively.

2.6. Circular dichroism

CD spectra were recorded on a Jobin Yvon CD6 at 25 °C with 1-cm and 0.1-mm pathlength cylinder quartz cuvettes for near-UV and far-UV, respectively. Mj HSP16.5 was used at concentrations of 1.4 mg/mL and 1.0 mg/mL in 10 mM PBS (pH 6.9) for near-UV and far-UV CD, respectively.

2.7. Fluorescence spectra

Fluorescence spectra were obtained from a Jobin Yvon Fluorolog-312 fluorometer at 25 °C. Samples were excited at 280 nm with an excitation slit of 2 nm and the emission spectra were recorded between 300–500 nm with an emission slit of 3 nm.

2.8. Subunit exchange

0.423 μM of newly purified (or preheated) Cy3-labeled Mj HSP16.5 in 10 mM PBS (pH 7.0) was mixed with 0.135 μM of newly purified (or preheated) Cy5-labeled Mj HSP16.5 in 10 mM PBS (pH 7.0) at 4 °C to obtain an equal molar ratio of Cy3-and Cy5-labeled Mj HSP16.5 monomers. The mixture was incubated at different temperatures as indicated in the figures. At certain time intervals, samples were taken out and cooled to 25 °C in a water bath. FRET was monitored on a Jobin Yvon Fluorolog-312 fluorometer at 25 °C. Samples were excited at 520 nm with an excitation slit of 2 nm and the emission spectra were recorded between 550–750 nm with an emission slit of 3 nm. The time constants (τ) for the subunit exchange were estimated by fitting the acceptor fluorescence increase at 668 nm using Origin 6.0 (Microcal Software, Inc.) to the Eq.: $y=y_0+Ae^{-t/\tau}$.

2.9. Dynamic light scattering

DLS experiments were performed at 25 °C with 1 mg/mL Mj HSP16.5 in 50 mM PBS and 100 mM NaCl buffer (pH 6.9) using ALV/DLS/CGS-5022F (ALV) with He–Ne laser (632.8 nm).

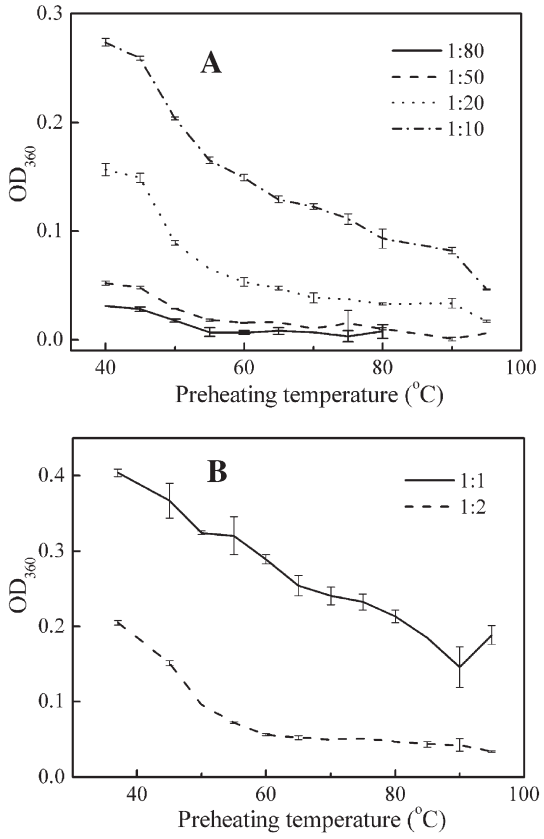

Fig. 1. Preheating enhances the chaperone-like activity of Mj HSP16.5. (A) Suppression of the thermal aggregation of 1.69 μM CS was monitored at 45 °C by absorbance at 360 nm in the presence of different monomeric molar ratios of CS to Mj HSP16.5. (B) Suppression of the reductive aggregation of 53.6 μM insulin in 2 mM DTT was monitored at 37 °C by absorbance at 360 nm in the presence of different monomeric molar ratios of insulin to Mj HSP16.5.

2.10. Analytical ultracentrifugation

Sedimentation velocity experiments were carried out using a Beckman Optima XL-A analytical ultracentrifuge with an An-60Ti rotor. To find out if there is any concentration-dependent behavior, three concentrations (0.071 mg/mL, 0.88 mg/mL, and 3.5 mg/mL) of Mj HSP16.5 in 50 mM PBS and 100 mM NaCl buffer (pH 6.9) were investigated, and the absorbance was monitored at 220 nm, 280 nm, and 290 nm for the concentrations of 0.071 mg/mL, 0.88 mg/mL, and 3.5 mg/mL, respectively. Three different samples, i.e. the newly purified, 65 °C-preheated, and 85 °C-preheated Mj HSP16.5, with the same concentration were centrifuged at 24,000 rpm at 20 °C concurrently in the same rotor to ensure an identical experimental condition. Data analyses were performed using the Enhanced van Holde–Weischet [28,29] and Time Derivative (dC/dt) [30] methods in the UltraScan software package (http://www.ultrascan.uthscsa.edu/;).

3. Results

3.1. The chaperone-like activity of Mj HSP16.5 significantly increased after preheating

Every datum point in Fig. 1 represents a final OD of the thermal aggregation of CS (Fig. 1A) or reductive aggregation of insulin (Fig. 1B), and a lower value means a higher chaperone-like activity of Mj HSP 16.5. Fig. 1A shows that the newly purified Mj HSP16.5 (37 °C data) has a low chaperone-like activity, and that even a monomeric molar ratio of 1:80 of CS to Mj HSP16.5 was insufficient to completely prevent the thermal aggregation of CS at 45 °C. After Mj HSP16.5 was preheated and cooled, its chaperone-like activity increased significantly, and higher preheating temperatures resulted in greater chaperone-like activities. After being preheated at 85 °C and then cooled down, a molar ratio of 1:20 of CS to Mj HSP16.5 was sufficient to prevent the thermal aggregation of CS at 45 °C. Similarly, to completely prevent the DTT-induced aggregation of the insulin B chain at 37 °C, the ratio of insulin/newly purified Mj HSP16.5 has to be as high as about 1:7 (molar ratio in monomer) [14]; however, after Mj HSP16.5 has been preheated at 65 °C or at higher temperatures and cooled down, a molar ratio of about 1:2 can efficiently prevent the DTT-induced aggregation of the insulin B chain at the same condition (Fig. 1B).

In addition, when the 65 °C-preheated Mj HSP16.5 was heated again at 45 °C, and subsequently cooled to room temperature, its chaperone-like activity remained the same as the sample that was only preheated once at 65 °C. Meanwhile when the 45 °C-preheated Mj HSP16.5 was heated again at 65 °C and subsequently cooled to room temperature, its chaperone-like activity was also the same as the sample that was only preheated once at 65 °C (data not shown). Our experiments also showed that the preheating time (in the range from 1 h to 6 h) and subsequent cooling rate (either cooled at 0.1 °C/min or cooled by ice-water bath) had no obvious influence on the chaperone-like activity of the preheated Mj HSP 16.5 (data not shown).

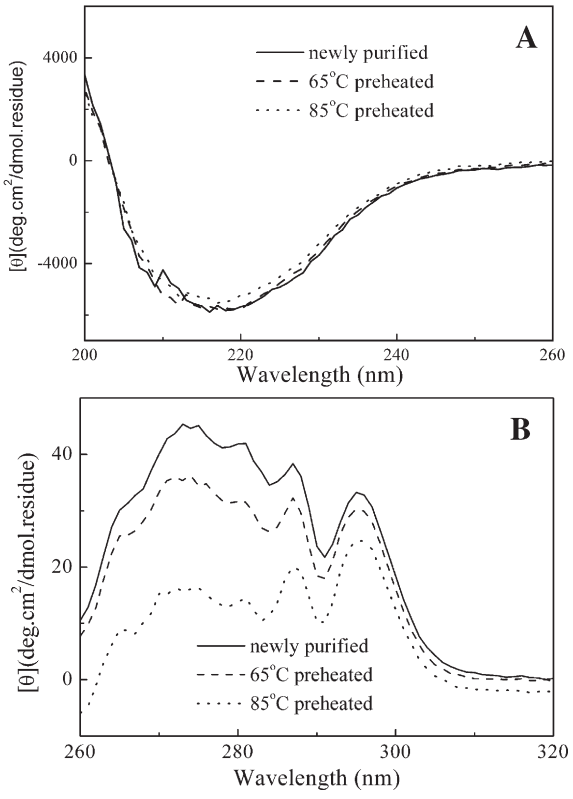

Fig. 2. CD spectra of the newly purified and preheated Mj HSP16.5. Concentrations of Mj HSP16.5 were 1.4 mg/mL and 1.0 mg/mL in 10 mM PBS (pH 6.9) for near-UV and far-UV CD, respectively.

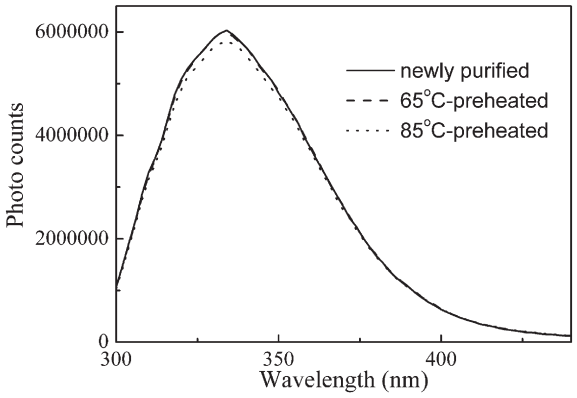

Fig. 3. Fluorescence spectra of the newly purified and preheated Mj HSP16.5. Concentration of Mj HSP16.5 was 0.18 mg/mL in 10 mM PBS pH 6.9 buffer.

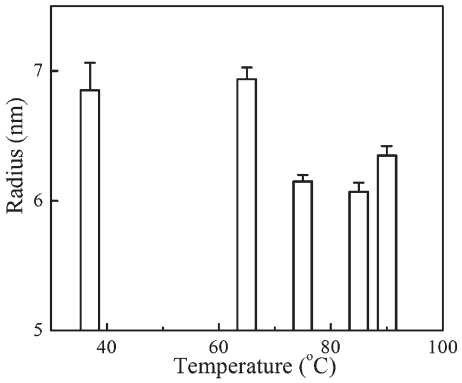

Fig. 5. Mass-based hydrodynamic radius of Mj HSP16.5 calculated from DLS experiments.

3.2. No secondary structure change but significant aromatic packing change after preheating

Far-UV spectra of Mj HSP16.5 both before and after preheating showed no observable differences (Fig. 2A). These results indicate that only a very small change, if any, in the secondary structure of Mj HSP16.5 occurred after preheating. Fluorescence spectra (Fig. 3) show little change between the newly purified and the preheated Mj HSP16.5, indicating little changes in the hydrophobic environment of both tryptophan and tyrosine residues after preheating. However, significant differences between the newly purified and the preheated Mj HSP16.5were observed for near-UV CD spectra (Fig. 2B), indicating significant changes in the local (chiral) packing of the aromatic side chains of Mj HSP16.5 after preheating.

3.3. Mj HSP16.5 became more homogeneous after preheating

Consistent with previously published data [14], Mj HSP16.5 maintained its oligomeric state after preheating as indicated by the size exclusion chromatographic data (data not shown). Native-pore gradient PAGE also showed that the majority of the newly purified and preheated Mj HSP16.5 migrated as a 24-mer (Fig. 4). However, several very weak bands migrated between the 24-mer band and the 669 kDa molecular weight marker for the newly purified and 65 °C-preheated Mj HSP16.5 samples, but not for the 85 °C-preheated Mj HSP16.5 sample. These weak bands may be larger oligomers and/or significantly distorted 24-mers.

Fig. 5 shows the mass-weighted radius of Mj HSP16.5 that was calculated from DLS experiments both before and after preheating at different temperatures. There is almost no change

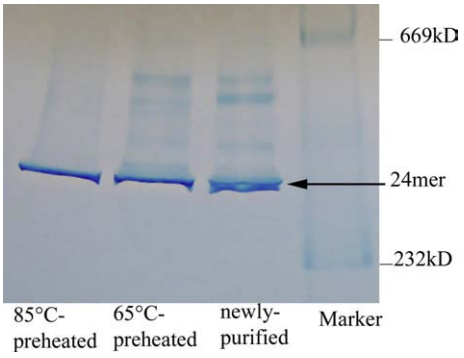

Fig. 4. Effects of Mj HSP16.5 preheating on native-pore gradient PAGE. Thyroglobulin (669 kDa) and catalase (232 kDa) were used as molecular weight markers.

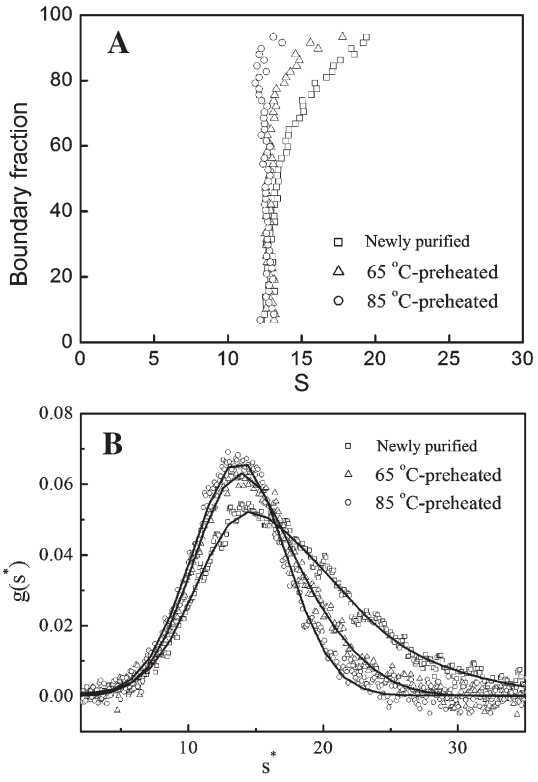

Fig. 6. Analysis of sedimentation velocity of Mj HSP 16.5. (A) Van Holde–Weischet distribution plot of the newly purified (open squares), 65 °C-preheated (open triangles), and 85 °C-preheated (open circles) Mj HSP16.5; the concentration of Mj HSP16.5 was 3.5 mg/mL. (B) The g(s*) profiles by Time Derivative (dC/dt) data analysis of the newly purified (open squares), 65 °C-preheated (open triangles), and 85 °C-preheated (open circles) Mj HSP16.5. Solid lines are the Gaussian-fitted curves, where the data of the newly purified, 65 °C-preheated, and 85 °C-preheated Mj HSP 16.5 were fitted to three, two, and one component(s), respectively.

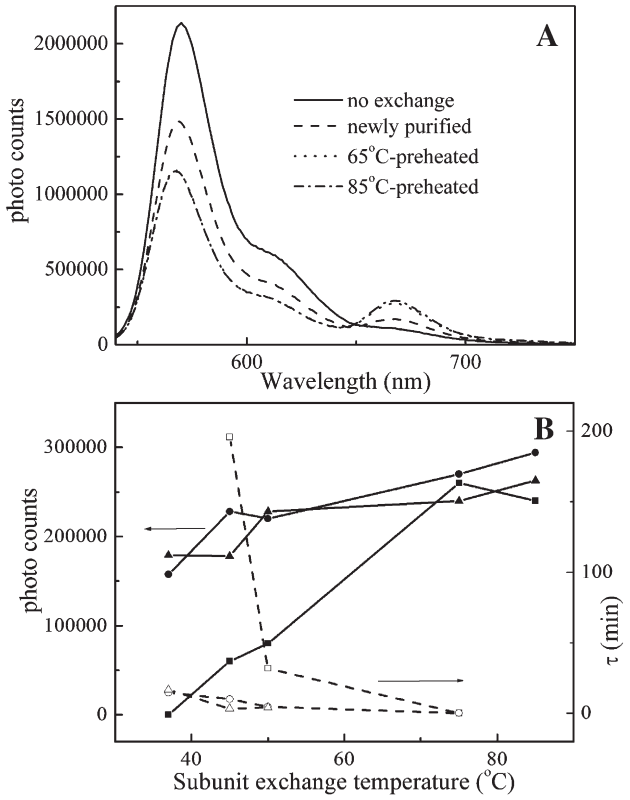

Fig. 7. Subunit exchange of Mj HSP 16.5. (A) The FRET spectra at 25 °C of the mixtures of the Cy3-labeled and Cy5-labeled Mj HSP16.5 after being incubated at 50 °C for 2 h, except for the bold line, which is the spectrum of the initial state of the mixture without incubation. Prior to being incubated at 50 °C for 2 h, the Cy3-labeled and Cy5-labeled Mj HSP16.5 had also been individually preheated at 65 °C (dotted-line) and 85 °C (dashed-dotted-line) (Dotted-line overlapped with dotted-dashed-line). (B) Sold lines show the peak value of the FRET spectra at 668 nm (left y-axis) when the subunit exchange had been finished at each subunit exchange (incubation) temperature (horizontal axis), and dashed-line represents the calculated time constants (right y-axis) of the subunit exchange. The square symbols (both open and closed) represent the newly purified Mj HSP 16.5, the circle symbols represent the individually 65°C-preheated, and triangles represent the individually 85 °C-preheated.

in the radius of Mj HSP16.5 when preheated below 65 °C. However, if Mj HSP16.5 is preheated to a temperature higher than 75 °C and then cooled, its radius decreases significantly from approximately 6.9 nm to 6.2 nm. Further increases in the preheating temperature had no effect on the radius of the protein. It must be noted that the decrease in the radius of Mj HSP16.5 after preheating does not contradict the increase in size of Mj HSP16.5 at high temperatures [17]. In this study, we compared the radii of different samples at the same temperature (25 °C) with different preheating temperatures, while prior studies compared the radii measured at different temperatures [17].

AUC is a powerful technique to determine the homogeneity/heterogeneity of macromolecular solutions. It can detect changes in both the shape and size of macromolecules that many other techniques cannot [31,32]. Although previous work by others suggested that Mj HSP16.5 could be homogeneous, the AUC experiments described here suggest that this suggestion may not be entirely true. Fig. 6A shows the van Holde–Weischet distribution plots of the AUC results of Mj HSP16.5. The vertical lines for the Mj HSP16.5 sample preheated to 85 °C suggest that it is homogeneous, whereas the tilted lines suggest that the newly purified Mj HSP16.5 is heterogeneous. This observation held true when concentrations between 0.071–3.5 mg/mL were tested, suggesting that it is not a concentration-dependent feature (for clarity, Fig. 6 shows only one concentration). Fig. 6B shows the g (s*) profiles of the newly purified, the 65 °C-preheated, and the 85 °C-preheated Mj HSP16.5 samples by Time Derivative (dC/dt) data analysis. The average sedimentation coefficients ($s_{20,w}$) were calculated to be 18.0 S, 14.5S, and 14.0S for the newly purified, the 65 °C-preheated, and the 85 °C-preheated Mj HSP16.5 samples, respectively. The newly purified Mj HSP16.5 showed a wide distribution of sedimentation coefficients, which could only be fitted to at least three Gaussian components. The 65 °C-preheated sample had a narrower distribution of sedimentation coefficients that could be fitted to two components, and the 85 °C-preheated sample had the narrowest distribution of sedimentation coefficients that could be fitted to a single Gaussian component, also indicating homogeneity.

3.4. Subunit-exchange rate increased after preheating

Cy3 and Cy5 labeling did not change the 24-mer assembly of Mj HSP16.5 (data not shown). Fig. 7 shows the FRET results of the subunit exchange of Mj HSP16.5 at different temperatures. At 37 °C, there was almost no subunit exchange occurred for the newly purified Mj HSP16.5 sample, while significant subunit exchange was observed for the 65 °C-preheated and the 85 °C-preheated Mj HSP16.5 samples. At 45 °C and above, subunit exchange of the newly purified Mj HSP16.5 sample was also observed. Large differences in both the subunit-exchange rate and the magnitude between the newly purified and preheated Mj HSP16.5 samples were observed at 75 °C and above. Unexpectedly, there was no significant difference in either the subunit-exchange rate or the magnitude between the 65 °C-preheated and the 85 °C-preheated Mj HSP16.5, though the chaperone-like activity difference is obvious. At 85 °C, subunit-exchange time constants were too fast comparing to the manual mixing time, therefore the time constants were not calculated.

4. Discussion

4.1. Enhanced chaperone-like activity of Mj HSP16.5 after preheating

Mj HSP16.5 is usually regarded as an inefficient chaperone with broad substrate specificity [14,16]. Previous reports indicate that at a monomeric molar ratio of 1:1, Mj HSP16.5 could prevent the thermal aggregation of SCM at 80 °C [16]. To completely prevent DTT-induced aggregation of the insulin B chain at 37 °C, the required monomeric molar ratio of insulin to the newly purified Mj HSP16.5 was 1:7. However, our results showed that after Mj HSP16.5 was preheated at temperatures 65 °C or higher and subsequently cooled down, a molar ratio of approximately 1:2 could efficiently prevent DTT-induced aggregation of the insulin B chain under the same conditions.

This observation indicates that the preheated protein is 3.5-fold more active than the newly purified form. These data suggest that preheated Mj HSP16.5, which is much similar to its native state in the hyperthermophilic archaeon than the newly purified, is likely an efficient chaperone.

4.2. Activation mechanism for the chaperone activity of Mj HSP16.5

Subunit exchange is a unifying feature of all sHSPs. Since an increase in the subunit-exchange rate correlates with an increase in its chaperone-like activity in some studies, it was proposed that subunit exchange was important for the chaperone-like activity of sHSPs [15,23,25]. However, we showed that with a monomeric molar ratio of 1:80 of CS to Mj HSP16.5, the newly purified Mj HSP16.5 exhibited obvious chaperone-like activity at 37 °C, but no subunit exchange was observed at the same temperature (Fig. 7). No subunit exchange at 37 °C for Mj HSP16.5 is consistent with a previous report that there was no subunit exchange for Mj HSP16.5 at 50 °C with different labeling groups [14]. Newly purified Mj HSP16.5 without labeling groups would be expected to be more stable and more difficult to exchange a subunit than the labeled ones. This result demonstrates that subunit exchange is not a prerequisite for the chaperone-like activity of sHSPs, which is consistent with a recent report that the activation of HSP26 did not require dissociation of the oligomer [25].

Our results showed that Mj HSP16.5 underwent little secondary structural and hydrophobic environment change after preheating. However, significant changes in the local packing (chiral environment) of the aromatic side chains were observed in near-UV CD spectra. Since tryptophan and tyrosine residues are the dominant contributors to near-UV CD spectra, and the only one tryptophan and one of the two tyrosine residues in the Mj HSP16.5 monomer was found near its dimeric interface, the changes in the near-UV CD spectrum should mainly arise from local monomeric and/or dimeric conformational change. And this local conformational change between the newly purified and the 85 °C-preheated Mj HSP 16.5 indicates that one of these two species must be trapped in misfolded local minima. The cooling rate after preheating and the repeated preheating of Mj HSP16.5 at temperatures lower than the first preheating temperature did not affect the chaperone activity of the preheated Mj HSP16.5, indicating that the 85 °C-preheated Mj HSP16.5 was in its most stable state. Therefore, the preheating-activation process is likely a refolding process.

It must be noted that the stable state described here refers to the folding/unfolding process, and does not refer to the dissociation/reassembly process of the oligomer. In fact, the locally misfolded monomers/dimers might have a “self-sticky” area and are tightly stuck to each other in the latent distorted oligomers, making it more difficult for both subunit exchange and dissociation. As a result, the activation of sHSPs coincides with the destabilization, or dissociation, of the oligomer and increase in the rate of subunit exchange.

In summary, our results demonstrate that the newly purified Mj HSP16.5 expressed in *E. coli* at 37 °C was heterogeneous and less active, which is possibly locally misfolded. Preheating or high temperature can activate the newly purified Mj HSP16.5 likely by refolding it into the most stable and compact state. It was proposed that HSPs emerged from the hazardous environment near hydrothermal systems [33], so it is possible that the activation of other sHSPs is similar to that of Mj HSP16.5.

Acknowledgements

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References

[1] K.K. Kim, R. Kim, S. Kim, Crystal structure of a small heat-shock protein, Nature 394 (1998) 595–599.
[2] R.L. van Montfort, E. Basha, K.L. Friedrich, C. Slingsby, E. Vierling, Crystal structure and assembly of a eukaryotic small heat shock protein, Nat. Struct. Biol. 8 (2001) 1025–1030.
[3] M. Haslbeck, sHsps and their role in the chaperone network, Cell. Mol. Life Sci. 59 (2002) 1649–1657.
[4] P. van den IJssel, D.G. Norman, R.A. Quinlan, Molecular chaperones: small heat shock proteins in the limelight, Curr. Biol. 9 (1999) R103–R105.
[5] M. Ehrnsperger, S. Graber, M. Gaestel, J. Buchner, Binding of non-native protein to hsp25 during heat shock creates a reservoir of folding intermediates for reactivation, EMBO J. 16 (1997) 221–229.
[6] M. Haslbeck, T. Franzmann, D. Weinfurtner, J. Buchner, Some like it hot: the structure and function of small heat-shock proteins, Nat. Struct. Mol. Biol. 12 (2005) 842–846.
[7] M.P. Bova, H.S. Mchaourab, Y. Han, B.K. Fung, Subunit exchange of small heat shock proteins. Analysis of oligomer formation of αA-crystallin and Hsp27 by fluorescence resonance energy transfer and site-directed truncations, J. Biol. Chem. 275 (2000) 1035–1042.
[8] K.C. Giese, E. Vierling, Changes in oligomerization are essential for the chaperone activity of a small heat shock protein *in vivo* and *in vitro*, J. Biol. Chem. 277 (2002) 46310–46318.
[9] L. Gu, A. Abulimiti, W. Li, Z. Chang, Monodisperse hsp 16.3 nonamer exhibits dynamic dissociation and reassociation, with the nonamer dissociation prerequisite for chaperone-like activity, J. Mol. Biol. 319 (2002) 517–526.
[10] M. Haslbeck, S. Walke, T. Stromer, M. Ehrnsperger, H.E. White, S. Chen, H.R. Saibil, J. Buchner, Hsp26: a temperature-regulated chaperone, EMBO J. 18 (1999) 6744–6751.
[11] R. Shashidharamurthy, H.A. Koteiche, J. Dong, H.S. Mchaourab, Mechanism of chaperone function in small heat shock proteins: dissociation of the HSP27 oligomer is required for recognition and binding of destabilized T4 lysozyme, J. Biol. Chem. 280 (2005) 5281–5289.
[12] T. Stromer, M. Ehrnsperger, M. Gaestel, J. Buchner, Analysis of the interaction of small heat shock proteins with unfolding proteins, J. Biol. Chem. 278 (2003) 18015–18021.
[13] T. Stromer, E. Fischer, K. Richter, M. Haslbeck, J. Buchner, Analysis of the regulation of the molecular chaperone Hsp26 by temperature-induced dissociation: the N-terminal domain is important for oligomer assembly and the binding of unfolding proteins, J. Biol. Chem. 279 (2004) 11222–11228.
[14] M.P. Bova, Q. Huang, L. Ding, J. Horwitz, Subunit exchange, conformational stability, and chaperone-like function of the small heat shock protein 16.5 from *Methanococcus jannaschii*, J. Biol. Chem 277 (2002) 38468–38475.
[15] X. Fu, Z. Chang, Temperature-dependent subunit exchange and chaperone-like activities of Hsp16.3, a small heat shock protein from *Mycobacterium tuberculosis*, Biochem. Biophys. Res. Commun. 316 (2004) 291–299.

[16] R. Kim, K.K. Kim, H. Yokota, S.H. Kim, Small heat shock protein of *Methanococcus jannaschii*, a hyperthermophile, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 9129–9133.
[17] D.R. Kim, I. Lee, S.C. Ha, K.K. Kim, Activation mechanism of HSP16.5 from *Methanococcus jannaschii*, Biochem. Biophys. Res. Commun. 307 (2003) 991–998.
[18] R. Kim, L. Lai, H.H. Lee, G.W. Cheong, K.K. Kim, Z. Wu, H. Yokota, S. Marqusee, S.H. Kim, On the mechanism of chaperone activity of the small heat-shock protein of *Methanococcus jannaschii*, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 8151–8155.
[19] Q. Mao, D. Ke, X. Feng, Z. Chang, Preheat treatment for *Mycobacterium tuberculosis* Hsp16.3: correlation between a structural phase change at 60 °C and a dramatic increase in chaperone-like activity, Biochem. Biophys. Res. Commun. 284 (2001) 942–947.
[20] B. Raman, CH, M. Rao, Chaperone-like activity and quaternary structure of a-crystallin, J. Biol. Chem. 269 (1994) 27264–27268.
[21] B. Raman, CH, M. Rao, Chaperone-like activity and temperature-induced structural changes of a-crystallin, J. Biol. Chem 272 (1997) 23559–23564.
[22] M.R. Burgio, C.J. Kim, C.C. Dow, J.F. Koretz, Correlation between the chaperone-like activity and aggregate size of a-crystallin with increasing temperature, Biochem. Biophys. Res. Commun. 268 (2000) 426–432.
[23] K. Usui, O.F. Hatipoglu, N. Ishii, M. Yohda, Role of the N-terminal region of the crenarchaeal sHsp, StHsp14.0, in thermal-induced disassembly of the complex and molecular chaperone activity, Biochem. Biophys. Res. Commun. 315 (2004) 113–118.
[24] X. Fu, C. Liu, Y. Liu, X. Feng, L. Gu, X. Chen, Z. Chang, Small heat shock protein Hsp16.3 modulates its chaperone activity by adjusting the rate of oligomeric dissociation, Biochem. Biophys. Res. Commun. 310 (2003) 412–420.
[25] T.M. Franzmann, M. Wuhr, K. Richter, S. Walter, J. Buchner, The activation mechanism of Hsp26 does not require dissociation of the oligomer, J. Mol. Biol. 350 (2005) 1083–1093.
[26] D.A. Haley, M.P. Bova, Q.L. Huang, H.S. Mchaourab, P.L. Stewart, Small heat-shock protein structures reveal a continuum from symmetric to variable assemblies, J. Mol. Biol. 298 (2000) 261–272.
[27] K.K. Kim, H. Yokota, S. Santoso, D. Lerner, R. Kim, S.H. Kim, Purification, crystallization, and preliminary X-Ray crystallographic data analysis of small heat shock protein homolog from *Methanococcus jannaschii*, a hyperthermophile, J. Struct. Biol. 121 (1998) 76–80.
[28] B. Demeler, H. Saber, J.C. Hansen, Identification and interpretation of complexity in sedimentation velocity boundaries, Biophys. J. 72 (1997) 397–407.
[29] K.E. van Holde, W.O. Weischet, Boundary analysis of sedimentation velocity experiments with monodisperse and paucidisperse solutes, Biopolymers 17 (1978) 1387–1403.
[30] W.F. Stafford, Boundary analysis in sedimentation transport experiments: a procedure for obtaining sedimentation coefficient distributions using the time derivative of the concentration profile, Anal. Biochem. 203 (1992) 295–301.
[31] J.L. Cole, J.C. Hansen, Analytical ultracentrifugation as a contemporary biomolecular research tool, J. Biomol. Tech. 10 (1999) 163–176.
[32] J. Lebowitz, M.S. Lewis, P. Schuck, Modern analytical ultracentrifugation in protein science: A tutorial review, Protein Sci. 11 (2002) 2067–2079.
[33] E. Nisbet, The realms of archaean life, Nature 405 (2000) 625–626.