Interaction of a Small Heat Shock Protein of the Fission Yeast, *Schizosaccharomyces pombe*, with a Denatured Protein at Elevated Temperature

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We have expressed, purified, and characterized one small heat shock protein of the fission yeast *Schizosaccharomyces pombe*, SpHsp16.0. SpHsp16.0 was able to protect citrate synthase from thermal aggregation at 45 °C with high efficiency. It existed as a hexadecameric globular oligomer near the physiological growth temperature. At elevated temperatures, the oligomer dissociated into small species, probably dimers. The dissociation was completely reversible, and the original oligomer reformed immediately after the temperature dropped. Large complexes of SpHsp16.0 and denatured citrate synthase were observed by size exclusion chromatography and electron microscopy following incubation at 45 °C and then cooling. However, such large complexes did not elute from the size exclusion column incubated at 45 °C. The denatured citrate synthase protected from aggregation was trapped by a GroEL trap column at 45 °C. These results suggest that the complex of SpHsp16.0 and denatured citrate synthase at elevated temperatures is in the transient state and has a hydrophobic nature. Analyses of the interaction between SpHsp16.0 and denatured citrate synthase by fluorescence cross-correlation spectrometry have also shown that the characteristics of SpHsp16.0-denatured citrate synthase complex at the elevated temperature are different from those of the large complex obtained after the shift to lowered temperatures.

Small heat shock protein (sHsps) is one of the ubiquitous chaperones, existing in all types of organisms, including archaea, bacteria, and eukaryota (1). sHsps endow thermostolerance to cells in vivo (2, 3) and also protect proteins from thermal aggregation and, in some cases, promote the renaturation of proteins in vitro (4–6). Compared with other chaperones, they are relatively heterogeneous in sequence and size. They are grouped together based on a conserved domain, the α-crystallin domain, which is named after the α-crystallin of the vertebrate eye lens (7). The α-crystallin domain is preceded by a highly variable N-terminal region and is followed by a short, partly conserved C-terminal extension (8). Although all sHsps exist as large oligomeric complexes, their quaternary structures are diverse and are composed of 9–40 subunits (9). To date, the three-dimensional crystal structures of MjHsp16.5, a sHsp from hyperthermophilic archaeon *Methanococcus jannaschii*, and Hsp16.9, a sHsp from wheat, have been determined. MjHsp16.5 forms a hollow spherical complex of 24 subunits (10), whereas Hsp16.9 exists as a dodecameric double disk (11). The crystal structures show that the α-crystallin domain is composed of β-strands, and the two proteins utilize a similar dimer as a higher assembly building block but differ in their quaternary structure. In contrast, some members of the sHsp family like α-crystallin are remarkably polydisperse (12).

It has been revealed that the molecular mechanism of the chaperone function of sHsps resides in the oligomeric structure. The chaperone potential of sHsps is latent when they exist as large oligomeric structures under physiological conditions. At elevated temperatures, the equilibrium shifts to the dissociated state, and the hidden hydrophobic substrate-binding sites are exposed to express chaperone activity (13). In the presence of denatured proteins, small oligomers form a large stable complex to protect against aggregation. Recently, Fu et al. (14) have shown that a mutant of Hsp16.3 of *Mycobacterium tuberculosis* with nine residues missing from the C terminus that cannot form a large oligomeric complex exhibits chaperone activity at low temperatures. The large substrate-sHsp complexes are dissociated to renature by the action of the DnaK system or more efficiently by the cooperation of DnaK and ClpB system (15–19). In the absence of denatured proteins, dissociated small complexes reassemble into a large oligomer after a shift to physiological temperatures.

In the fission yeast *Schizosaccharomyces pombe*, two small heat shock protein genes (SPCC338.06c and SPBC3E7.02c) have been identified. Expression of SpHsp16.0 (SPBC3E7.02c) is induced by a number of experimental stimuli including heat shock. In addition, its expression is also responsive to depletion of deoxyribonucleotides or DNA damage, and this response is dependent on the *spc1* MAPK pathway and the transcription factor *atf1* (20). SPCC338.06c encodes sHsp with the molecular mass of 15,806 Da, which shares 34.8% amino acid sequence
identity with SpHsp16.0. It is also induced by heat shock. However, their biochemical characterizations have not been performed previously.

In this study, we have expressed and characterized SpHsp16.0. Similar to other sHsps, it existed as a large oligomeric complex and dissociated into small oligomers at elevated temperatures to function as a molecular chaperone. It formed a large complex with a denatured protein after heat incubation like other sHsps. We found that the complex of SpHsp16.0 and denatured protein was in a different state at elevated temperatures. It seems to be in the transient state and have a hydrophobic nature. By fluorescence cross-correlation spectroscopy, the interactions of SpHsp16.0 and a denatured protein at different conditions were compared.

**MATERIALS AND METHODS**

**Proteins, Reagents, and Strains—**Ex Taq DNA polymerase, restriction enzymes, and other reagents for gene manipulation were obtained from TAKARA Bio Inc. (Shiga, Japan). Citrate synthase (CS) from porcine heart was purchased from Sigma-Aldrich. GFP was purified as described previously (21). A bacterial chaperonin GroEL trap mutant (D398A-GroEL) (GroELtrap) was used to trap an unfolded protein (22).

Fluorescent labeling of SpHsp16.0 and CS was performed using BODIPY-FL (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid) succinimidyl ester (Molecular Probes, Eugene, OR) and Cy5 Mono-reactive Dye (Amersham Biosciences). Other reagents were the products of Wako Pure Chemical Industries (Osaka, Japan). S. pombe ARC032 (972h+) was used for preparation of the genomic DNA. Escherichia coli strains DH5α and BL21(DE3) were used for plasmid construction and protein expression, respectively.

**Expression and Purification of SpHsp16.0—**Because the gene is not split by introns, a full-length gene for SpHsp16.0 was amplified from the total genomic DNA of *S. pombe* ARC032 by PCR using as primers 5’-CCC-ATA-TGT-CTT-TGC-AAC-CTT-TTT-T-3’ and 5’-GGG-AAT-TCT-TAC-TTA-ATA-GCA-ATT-TGT-T-3’ and then subcloned into the pT7Blue T vector. After confirmation of the sequence, the gene was excised with Ndel and EcoRI and then introduced into the Ndel/EcoRI site of pET23b to construct pSpHsp16.0E.

*E. coli* BL21(DE3) transformed with pSpHsp16.0E was grown at 37 °C in Luria-Bertani medium containing 100 µg/ml of ampicillin. The cells were harvested and centrifuged at 4,600 × g for 20 min at 4 °C. The harvested cells were resuspended in buffer A (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, and 1 mM dithiothreitol) and disrupted by sonication. The suspension was clarified by centrifugation at 20,000 g for 30 min at 4 °C. The supernatant was applied to a DEAE-Toyopearl anion exchange column (Tosoh, Tokyo, Japan) equilibrated with buffer A. The proteins were eluted with a linear gradient of 0–50 mM NaCl in buffer A. Fractions containing SpHsp16.0 were pooled, concentrated by ultrafiltration (Amicon Ultra, Millipore, Billerica, MA), and then applied to a UnosQ6 column (Bio-Rad) equilibrated with buffer A. The proteins were eluted with a linear gradient of 0–500 mM NaCl in buffer A. Fractions containing SpHsp16.0 were pooled, concentrated by ultrafiltration (Amicon Ultra), and then applied to a HiLoad 26/60 Superdex 200-µg size exclusion column (Amersham Biosciences) equilibrated with buffer B (50 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, and 150 mM NaCl). Fractions containing SpHsp16.0 were collected and then concentrated by ultrafiltration.

**Thermal Aggregation Measurements—**Thermal aggregation of CS from porcine heart was monitored by measuring light scattering at 500 nm with a spectrofluorometer (FP-6500; Jasco, Tokyo, Japan) at 45 °C. Native CS (150 nM, monomer) was incubated in the assay buffer (5 mM Tris-HCl, pH 8.0) with or without SpHsp16.0. The assay buffer was preincubated at 45 °C and continuously stirred throughout the measurement.

**Effect of SpHsp16.0 on Spontaneous Refolding of GFP—**GFP was denatured by incubation in acidic buffer (100 mM HCl, 50 mM Tris, 100 mM KCl, and 5 mM dithiothreitol) for 30 min at room temperature. Refolding was started by diluting the denatured GFP to be 50 nM in the dilution buffer (50 mM Tris-HCl, pH 8.0, 100 mM KCl, and 5 mM dithiothreitol) without or with SpHsp16.0 (100 nM or 10 µM). The fluorescence of the refolded GFP was monitored at 510 nm with excitation at 400 nm using a spectrofluorophotometer (FP-6500) with continuous stirring at 45 °C.

**Electron Microscopy—**An aliquot of SpHsp16.0 or SpHsp16.0-CS complex solution was applied on specimen grids covered with a thin carbon support film, which had been made hydrophilic by the ion sputtering device (HDT-400; JEOL, Tokyo, Japan) and then negatively stained with 1% uranyl acetate for 30 s. The images were recorded by making use of a slow scan CCD camera (Gatan Retractable Multiscan camera) under low electron dose conditions at a magnification of 50,000× in an electron microscope (Tecnai F20; Philips Electron Optics, Hillsboro, OR) operated at 120 kV. The images were analyzed on computers using digital micrograph.

**Size Exclusion Chromatography-Multi-angle Light Scattering (SEC-MALS)—**The purified SpHsp16.0 complex was analyzed by SEC-MALS on a TSKgel G3000SW column (Tosoh) connected with a multi-angle light scattering detector (Mini DAWN; Wyatt Technology, Santa Barbara, CA) and a differential refractive index detector (Shodex RI-101; Showa Denko, Tokyo, Japan) by a HPLC system, PU-980i (JASCO). A 100-µl aliquot of sample was injected into the column and eluted with buffer C (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, and 1 mM dithiothreitol) at 1.0 ml/min. The molecular mass and protein concentration were determined according to the instructional manual (23).

**Size Exclusion Chromatography at Elevated Temperatures—**Size exclusion chromatography was performed with a column, SB-804HQ (Showa Denko), using a HPLC system, PU-1580i, connected with a multi-wavelength detector MD1515 (JASCO). The purified SpHsp16.0 was diluted to 4 µM in buffer B. A 100-µl aliquot of diluted SpHsp16.0 was heated at the specified temperature for 60 min and then loaded on the column heated at the same temperature. Elution was performed with buffer C at a flow rate of 0.5 ml/min. To examine the reversibility of the dissociation, SpHsp16.0 heated at 45 °C was analyzed by size exclusion chromatography at room temperature after cooling at 4 °C for 30 min.

**Labeling SpHsp16.0 and CS with Fluorescent Dyes—**Before labeling, both SpHsp16.0 and CS were gel-filtrated on a NAP5 column (Sephadex G-25; Amersham Biosciences) equilibrated with 50 mM sodium phosphate, pH 7.5. Then 200 µl of SpHsp16.0 (3 mg/ml) was mixed with 5 µl of 1 M K2CO3 to pH 8.5. Subsequently, BODIPY-FL succinimidyl ester was added to the mixture to 121 µM and incubated for 3 h at room temperature. After the reaction, unreacted BODIPY-FL succinimidyl ester was removed by gel filtration with a NAP5 column. After mixing 200 µl of CS (22.4 µM) with 20 µl of 1 M Na2HPO4 to pH 9.0, Cy5 monoreactive dye (Amersham Biosciences) was added to 83 µM, and the mixture was incubated for 3 h at room temperature. Then the unreacted Cy5 monoreactive dye was removed by gel filtration with a NAP5 column. The fluorescence-labeled SpHsp16.0 and CS were designated BODIPY-SPHsp16.0 and Cy5-CS, respectively. BODIPY-SPHsp16.0 exhibited almost same chaperone activity as SpHsp16.0 without modification.

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**Interaction of SpHsp16.0 with a Denatured Protein**
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Analysis of SpHsp16.0-CS Complex by Size Exclusion Chromatography—The interaction in the native structure was examined by size exclusion chromatography at room temperature. A mixture of 1 μM SpHsp16.0 and 0.2 μM Cy5-CS in buffer B was analyzed with a size exclusion column, SB-804HQ, using a HPLC system PU-1580i with buffer B delivered at the flow rate of 0.5 ml/min. Proteins and Cy5-CS were monitored at the absorbance at 220 and 650 nm, respectively. SpHsp16.0-CS complex was analyzed as follows. SpHsp16.0 solution (1 μM) was heated at 45 °C from 5 min, and the Cy5-CS was added to 0.2 μM. After further incubation at 45 °C, the mixture was analyzed on the column at 45 °C or at room temperature after cooling on ice for 30 min. To examine whether other chaperone can rob denatured CS from SpHsp16.0, GroEL, and SpHsp16.0-CS to 1 μM (as tetradecamer) after 30 min of incubation at 45 °C. The mixture was analyzed on the column at 45 °C.

FCS and FCCS Measurement—FCS and FCCS measurements were carried out with a ConfoCor2 (Carl Zeiss, Oberkochen, Germany), which consisted of a CW Ar+ laser and helium-neon laser, a water immersion objective (C-Apochromat, 40×, 1.2 NA, Carl Zeiss), and two channels of avalanche photodiodes (SPCM-200-PQ; EG&G). BODIPY-FL was excited at 488 nm, and Cy5 was excited at 633 nm. The confocal pinhole diameter was adjusted to 90 μm for 633 nm and 50 μm for 488 nm. The emission signals were split by a dichroic mirror (635-nm beam splitter) and detected at 505–550 nm by the green channel for BODIPY-FL and through a 650-nm-long path filter by the red channel for Cy5.

Measurement was performed using 0.2 μM BODIPY-SpHsp16.0 and 0.05 μM Cy5-CS solutions in 50 mM phosphate buffer, pH 7.5. To measure FCS, 100 μl of each labeled protein solution was used for the measurements separately at room temperature or at 42 °C after 5 min of incubation at 45 °C. FCCS at room temperature was measured using 100 μl of the mixture of both labeled proteins. To examine the interaction at 42 °C, 0.2 μM BODIPY-SpHsp16.0 was incubated at 45 °C for 5 min, and then Cy5-CS was added to 0.05 μM. After a further 5-min incubation at 45 °C, 100 μl of the mixture was subjected to FCCS at 42 °C. The large complex of BODIPY-SpHsp16.0 and Cy5-CS was formed by cooling the mixture at 4 °C for 4 min after 5 min of incubation at 45 °C, and then the FCCS measurement was performed at room temperature.

Data Analysis of FCS and FCCS—The fluorescence autocorrelation functions of the red and green channels, Gx(τ) and Gy(τ), and the fluorescence cross-correlation function, Gs(τ), are calculated using Equation 1,

\[ G_s(\tau) = \frac{\langle \delta I_s(t) \cdot \delta I_s(t + \tau) \rangle}{\langle I_s(t) \rangle \langle I_s(t + \tau) \rangle} \]

(Eq. 1)

where \( \tau \) denotes the time delay, \( I_s \) is the fluorescence intensity of the red channel (\( i=r \)) or green channel (\( i=g \)), and \( G_s(\tau) \) denotes the auto correlation function of red (\( i=j=x=r \)), green (\( i=j=x=g \)), and cross-correlation (\( i=r, j=g, x=c \)), respectively. Acquired \( G_s(\tau) \) were fitted by a one-, two-, or three-component model as shown in Equation 2,

\[ G_s(\tau) = 1 + \frac{1}{N} \sum_i F_i \left( 1 + \frac{\tau}{\tau_i} \right)^{-1} \left( 1 + \frac{\tau}{\tau_i^2} \right)^{-1/2} \]

(Eq. 2)

where \( F_i \) and \( \tau_i \) are the fraction and diffusion time of component \( i \), respectively. \( N \) is the average number of fluorescent particles in the excitation-detection volume defined by radius \( w_r \) and length \( 2s \), and \( s \) is the structure parameter representing the ratio \( s = z_s/w_r \). The pinhole adjustment of the FCS setup, structural parameter, and detection volume were calibrated for 488- and 633-nm excitation using FCS measurements of rhodamin 6G and Cy5 solution, respectively, with a concentration of 10−7 M. Molecular masses of labeled protein could be evaluated with the Stokes-Einstein equation relation for a spherical molecule and with the molecular masses of rhodamin 6G or Cy5 as references (24). The average number of red fluorescent particles (\( N_r \)) and green fluorescent particles (\( N_g \)), and particles that have both red and green fluorescence (\( N_rg \)) can be calculated with \( N_r = 1/G_r(0) \), \( N_g = 1/G_g(0) \), and \( N_rg = G_r(0)/G_r(0)G_g(0) \), respectively. When \( N_r \) and \( N_g \) are constant, \( G_r(0) \) is directly proportional to \( N_r \). For a quantitative evaluation of the cross-correlation among various samples, \( G_s(0) \) is normalized by \( G_r(0) \) (cross-ratio; \( G_r(0)/G_g(0) \)) (25).

RESULTS

SpHsp16.0 Efficiently Protects Porcine Heart Citrate Synthase from Thermal Aggregation but Cannot Arrest Spontaneous Refolding of Acid-denatured GFP. A thermal aggregation of CS from porcine heart was monitored by measuring light scattering at 500 nm using a spectrophotometer at 45 °C with continuous stirring. Monitoring started with the addition of CS (150 mM as monomer) to 50 mM Tris-HCl buffer, pH 8.0, preincubated at 45 °C without (closed circle) or with 150 (open triangle), 300 (closed triangle), 450 (open square), 600 (closed square) M SpHsp16.0. GFP folding was monitored by measuring the fluorescence at 510 nm with excitation at 400 nm using a spectrophotofluorometer. The fluorescence measurement was started by the addition of acid-denatured GFP (final concentration, 50 nM) into the dilution buffer (50 mM Tris-HCl, pH 8.0, 100 mM KCl, 5 mM dithiothreitol) without (closed circle) or with 100 nM (open circle) or 10 μM (closed square) SpHsp16.0 at 45 °C.
In *S. pombe*, two small heat shock protein genes have been identified. Among them, expression of SPBC3E7.02c is highly induced by a number of experimental stimuli including heat shock. It is named SpHsp16.0 from its deduced molecular mass of 15,967 Da. To examine whether SpHsp16.0 possesses molecular chaperone activity, its effect on the thermal aggregation of CS from porcine heart were examined. As shown in Fig. 1A, SpHsp16.0 efficiently prevented the thermal aggregation of CS. An excess of SpHsp16.0 monomer of ~2 mol was enough to suppress the increase in light scattering, which coincides well with previous observations obtained with sHsps from *Saccharomyces cerevisiae* (13, 17). These results support the notion that sHsps function as dimers under heat-stressed conditions.

Then we examined the effects of SpHsp16.0 on the spontaneous refolding of GFP. Chaperonin or prefoldin can capture the folding intermediate of acid denatured GFP and arrest its spontaneous refolding (26, 27). SpHsp16.0 could not suppress the increase of fluorescence even in the presence of a 200-fold excess (Fig. 1B). This result suggests that the complex of SpHsp16.0 and denatured protein is not stable as observed for other chaperones.

**Oligomeric Structure of SpHsp16.0**—Size exclusion chromatography of the purified SpHsp16.0 revealed that it existed as an oligomer. Electron microscopic image of negatively stained SpHsp16.0 showed that it existed as a spherical particle (Fig. 2A). The diameter was estimated to be less than 15 nm. The size is almost the same as that of Hsp26 from *S. cerevisiae* (13). Then we determined the molecular mass of the oligomer by SEC-MALS. The molecular mass of the oligomer was calculated to be 250 kDa (Fig. 2B). Thus, the SpHsp16.0 oligomer is likely to be composed of 16 subunits.

**Temperature-dependent Oligomeric Structural Change of SpHsp16.0**—It is postulated that the oligomeric dissociation of Hsp is prerequisite for exposure of the substrate-binding site and then molecular chaperone functions (11, 13, 28–30). We have examined the dissociation of the complex by size exclusion chromatography on HPLC at elevated temperatures. Because we could not observe any structural change when the samples were applied to a column kept at room temperature, analyses were performed with the column heated to the temperature of the sample. As shown in Fig. 3A, we observed a temperature-dependent dissociation of the oligomer. At 45 °C, all of the large oligomers disappeared and small complexes appeared.

The dissociation is reversible as shown in Fig. 3B. The SpHsp16 oligomer was completely dissociated into small oligomers at 45 °C.
small oligomers immediately disappeared, and the original oligomeric complex reappeared when the sample was cooled. The dissociation equilibrium is also affected by the concentration as observed for Hsp27 (31) (data not shown). Therefore, we were unable to determine the molecular mass of the small dissociated oligomers by SEC-MALS because of the limit of sensitivity.

Interaction between SpHsp16.0 and Heat-denatured CS—Then interaction between SpHsp16.0 and CS was examined by size exclusion chromatography and electron microscopy. When the mixture of SpHsp16.0 and CS labeled with Cy5 (Cy5-CS) was applied to the size exclusion column without heat treatment, two clearly separated peaks appeared (Fig. 4A). The high molecular mass peak corresponds to the SpHsp16.0 oligomer, and the low molecular mass peak corresponds to Cy5-CS (Fig. 4A, dotted line). Then SpHsp16.0 was incubated with Cy5-CS at 45 °C for 30 min and cooled at 4 °C for 30 min. This mixture was analyzed by size exclusion chromatography at room temperature. Broad and sharp peaks composed of both proteins appeared at shorter elution times (Fig. 4B). The large complexes were analyzed by electron microscopy. Various sized elliptical particles of 25–37.5 nm in the major axis, larger than the spherical particles of the SpHsp16.0 oligomer, were observed (Fig. 5). SpHsp16.0 likely binds to non-native CS to form complexes of variable size and shape like other sHsps (13, 17, 29, 32).

Then we examined the complex of SpHsp16.0 and Cy5-CS by the column heated at 45 °C. Unexpectedly, we could not observe any large complexes of SpHsp16.0 and Cy5-CS (Fig. 4C). Moreover, most of SpHsp16.0 and Cy5-CS were lost, and only small peaks were observed. The proteins were eluted from the column afterward by washing with ethylene glycol, which is thought to weaken hydrophobic interaction (data not shown). The result suggests that the complex of SpHsp16.0 and Cy5-CS is in the transient state and has hydrophobic nature at elevated temperatures. The observed stable large complex of SpHsp16.0 and CS is likely to be formed after the shift to the nonstress temperature.

FIGURE 4. Interaction between SpHsp16.0 and denatured CS. Interaction of SpHsp16.0 and Cy5-CS was analyzed on a size exclusion column, SB-804HQ, by monitoring the absorbance at 220 and 650 nm. A, gel filtration analysis of the mixture of SpHsp16.0 and Cy5-CS at room temperature. B and C, SpHsp16.0 solution was heated at 45 °C from 5 min, and the Cy5-CS was added. After further incubation at 45 °C, the mixture was analyzed with the column at 45 °C (C) or at room temperature after cooling on ice for 30 min (B). D, GroELtrap was added to the mixture of SpHsp16.0 and Cy5-CS after 30 min incubation at 45 °C, and the mixture was analyzed at 45 °C. As a control, the chromatogram obtained without addition of GroELtrap is also shown. The retention time for GroELtrap is marked.
To examine the state of the complex at the elevated temperatures, we examined whether GroEL trap mutant (GroELtrap, D398A-GroEL) can rob the denatured Cy5-CS from SpHsp16.0. GroELtrap can capture an unfolded protein but is unable to renature it in an ATP-dependent manner (22). As shown in Fig. 4D, most of the fluorescence intensity of Cy5-CS was observed at the position where GroELtrap appeared, indicating that the complex of SpHsp16.0 and denatured Cy5-CS is not stable in the elevated temperature, and binding of denatured protein is likely to be in the dynamic equilibrium state.

**Detection of Interaction between SpHsp16.0 and CS at Elevated Temperature by FCS and FCCS**—We did not observe a complex of SpHsp16.0 and CS at elevated temperatures by size exclusion chromatography or other methods probably because the complex is in the transient state and also because of the highly hydrophobic nature of the complex. FCS is used to analyze the interaction of proteins in solution (33, 34). It provides information about the diffusion properties of fluorescently tagged target molecules at a very low concentration (pM). FCS measurements are based on single photon counting of fluorescence in a defined detection volume (~0.25 fl) generated by a laser beam and fine detection optics. The diffusion constant and the concentration of target molecule can be determined from the autocorrelation function (G(t)), allowing us to monitor the interaction in situ.

Fig. 6 (A and B) shows the results of FCS analyses of BODIPY-SpHsp16.0 and Cy5-CS at room temperature and 42 °C. Molecular masses were estimated from the calculated diffusion time using the Einstein-Stokes equation with rhodamin 6G or Cy5 as a standard. At room temperature, the diffusion times of rhodamin 6G, Cy5, BODIPY-SpHsp16.0, and Cy5-CS were 26, 42, 241, and 224 μs, respectively. So the molecular masses of BODIPY-SpHsp16.0 and Cy5-CS were calculated to be 384 and 119 kDa, respectively. Although these values do not exactly match the molecular masses of the SpHsp16.0 hexadecamer (250 kDa) and CS dimer (98 kDa) determined by SEC-MALS or calculated from the amino acid sequences, they reflect well the quaternary structures. When the temperature was increased to 42 °C, both proteins exhibited a dramatic change in diffusion time. The diffusion time of
BODIPY-SpHsp16.0 was 103 μs (corrected for room temperature). Cy5-CS was composed of three components whose diffusion times were 589, 137, and 80 μs. Their distribution ratios were estimated to be 70, 20, and 10%, respectively. The estimated molecular mass of BODIPY-SpHsp16.0 decreased to 30.4 kDa, almost the same as the molecular mass of the dimer, 32 kDa. In contrast, the quadruplet structure of CS was not uniform. The molecular mass of the main component is calculated to be ~2200 kDa, which is consistent with the formation of an aggregate.

An extended technique of FCS, FCCS, can detect the coincidence of two spectrally distinct fluorescent probes in a small detection area at very low concentrations. FCCS has been used to detect the association-dissociation reaction and interaction between two molecular species. When 100 μl of a mixture containing BODIPY-SpHsp16 (0.2 μM as monomer) and Cy5-CS (0.05 μM as monomer) was observed at room temperature, almost no cross-correlation between them was observed at room temperature (G(0)/G(0) = 0.002) (Fig. 6C). No specific cross-correlation between BODIPY-SpHsp16.0 and only Cy5 dye was observed at room temperature, at 45 °C with cooling to 4 °C, and at 42 °C, respectively (data not shown). By contrast, a significant cross-correlation (G(0)/G(0) = 0.15) was observed when they were incubated at 45 °C and cooled to 4 °C (Fig. 4C). The result coincides with the formation of a large complex. Then we performed FCCS at 42 °C, the upper temperature limit of the thermostatted stage of the system. Although the efficiency was relatively low, SpHsp16.0 could suppress the thermal aggregation of CS at that temperature (data not shown). As shown in Fig. 6C, a cross-correlation between them was clearly detected (G(0)/G(0) = 0.08). The interaction was not as tight as that observed for the large oligomeric complexes obtained after the temperature shift. Thus, we concluded that the complex of SpHsp16.0 and denatured CS should be different from that observed after the temperature drop.

**DISCUSSION**

SpHsp16.0 shares almost the same characteristics as other sHsps studied so far. It exists as a large oligomeric complex and dissociated into small oligomers at elevated temperatures. The dissociation is completely reversible in the absence of denatured protein. At the medium temperatures, SpHsp16.0 eluted as broad peaks between the original complex and dissociated species, suggesting that SpHsp16.0 was in the equilibrium between complex and dissociation states. The dissociation equilibrium was also affected by the concentration. We could not determine the molecular mass of the dissociated species by SEC-MALS because they were observed only the concentration is low. Molecular mass estimated from the diffusion constant observed by FCS suggests that the small species is dimer. However, further study would be required to determine the structure of sHsp in the dissociated state.

SpHsp16.0 forms a large complex with CS after the heat incubation and cooling. The complex was stable and did not dissociate spontaneously (data not shown). We could not observe such large complexes of SpHsp16.0 and denatured CS by size exclusion chromatography at 45 °C in spite of the fact that the stable large complex was separated by the column. The result seems to contradict with the previous result of Hsp26 that large complexes with denatured proteins were observed by native PAGE at the high temperature (13). The difference is likely to be caused by the hydrophobic nature of the complex at the high temperature. In our experiment, large portions of SpHsp16.0 and CS were lost during size exclusion chromatography, probably by the hydrophobic interaction. Stable large complexes obtained after the temperature drop did not exhibit such hydrophobic nature, and they eluted quantitatively in size exclusion chromatography.

The difference in the characteristics of the complexes before and after cooling was also observed by FCCS. Compared with the stable complex, the interaction between SpHsp16.0 and Cy5-CS of the complex at the elevated temperature is relatively weak. The hydrophobic nature and the relatively weak interaction suggest that the complex is in the transient state. Binding between SpHsp16.0 and denatured proteins should be in the dynamic equilibrium, resulting in that the surface of the complex is hydrophobic. It is also confirmed by the fact that denatured CS that is protected from aggregation is captured by GroELtrap. Incompetence of capturing folding intermediate of GFP is also likely to be due to the same reason because acid denatured GFP folds relatively rapidly compared with de novo folding.

We monitored the decrease of CS activity at 45 °C in the absence or presence of 4-fold molar excess SpHsp16.0 (data not shown). CS activity decreased with the incubation time and was almost completely lost after 5 min of incubation. Similar to the result obtained with Hsp25 (17), the presence of SpHsp16.0 had almost no effect on thermal inactivation of CS, even though it can prevent their thermal aggregation. Thus, we think that SpHsp16.0 binds with CS in the irreversibly unfolded state.

Recently, Franzmann et al. (35) reported that dissociation of the oligomer is not required for activation of Hsp26. They propose existence of two alternative conformations for the Hsp26 oligomer with different affinities for unfolded proteins. Even though we could not observe such conformational changes of SpHsp16.0 oligomers at elevated temperatures, the existence of SpHsp16.0-substrate complex in the transient state with high affinity implies the presence of the conformation with high affinity.

To reveal the molecular mechanism of chaperone activity of small heat shock proteins, structural and functional characterization of the complex in the transient state would be necessary. Because it is difficult to characterize the transient complex by conventional methods because of high hydrophobicity, we believe that FCS and FCCS are important tools for further study.

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