Expression and biochemical characterization of two small heat shock proteins from the thermoacidophilic crenarchaeon *Sulfolobus tokodaii* strain 7

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Abstract

We expressed and characterized two sHsps, StHsp19.7 and StHsp14.0, from a thermoacidophilic crenarchaeon, *Sulfolobus tokodaii* strain 7. StHsp19.7 forms a filamentous structure consisting of spherical particles and lacks molecular chaperone activity. Fractionation of *Sulfolobus* extracts by size exclusion chromatography with immunoblotting indicates that StHsp19.7 exists as a filamentous structure in vivo. On the other hand, StHsp14.0 exists as a spherical oligomer like other sHsps. It showed molecular chaperone activity to protect thermophilic 3-isopropylmalate dehydrogenase (IPMDH) from thermal aggregation at 87°C. StHsp14.0 formed variable-sized complexes with denatured IPMDH at 90°C. Using StHsp14.0 labeled with fluorescence or biotin probe and magnetic separation, subunit exchanges between complexes were demonstrated. This is the first report on the filament formation of sHsp and also the high molecular chaperone activity of thermophilic archaeal sHsps.

Keywords: small heat shock protein; archaea; thermoacidophile; chaperone activity; oligomerization

All organisms express heat shock proteins (Hsps) to protect themselves from harm during times of stress. The major conserved families of these proteins have been shown to be involved in protein folding as molecular chaperones (Gething and Sambrook 1992; Hendrick and Hartl 1993; Parsell and Lindquist 1994). Five major families of Hsps have been identified (Buchner 1996). Among them, small heat shock proteins (sHsps) are ubiquitous in all types of organisms, including archaea, bacteria, and eukarya (Narberhaus 2002), and their expression is up-regulated significantly under conditions of cellular stress like heat shock (Horwitz 2000; Laksanalamai et al. 2001). In spite of a relatively low overall sequence homology among them, they were grouped together based on a conserved domain, the α-crystallin domain, which is named after the α-crystallin of the vertebrate eye lens (Caspers et al. 1995). The α-crystallin domain is preceded by a highly variable N-terminal region and is followed by a short, poorly conserved C-terminal extension (Leroux et al. 1997).

sHsps form large multimeric complexes of 9–40 subunits (12–42 kD in monomer size). To date, the three-dimensional crystal structures of MjHsp16.5, an sHsp from a hyperthermophilic archaeon, *Methanococcus jannaschii*, and Hsp16.9, an sHsp from wheat, have been determined. MjHsp16.5 forms a hollow spherical complex of 24 subunits (K.K. Kim et al. 1998), whereas Hsp16.9 exists as a dodecameric double disk (van Montfort et al. 2001). The

crystal structures show that the α-crystallin domain is composed of β-strands and the two proteins use 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 polydispersed (Haley et al. 2000). The quaternary structure of sHsps is highly dynamic, which is often reflected by pronounced size heterogeneity or rapid subunit exchange (Bova et al. 2000, 2002). It was reported that oligomers of Hsp16.9 and Hsp26, an sHsp of *Saccharomyces cerevisiae*, underwent a reversible temperature-dependent dissociation into smaller species, and can also form high-molecular-weight complexes with a model substrate as a function of increased temperature (Haslbeck et al. 1999; van Montfort et al. 2001).

The sHsps endow thermotolerance to cells in vivo (van den IJssel et al. 1994; Plesofsky-Vig and Brambl 1995), and also protect some proteins from thermal aggregation and in some cases promote renaturation in vitro (Horwitz 1992; Jakob et al. 1993; Muchowski and Clark 1998). It has been suggested that sHsps bind to their substrate proteins at denaturing temperatures and form a stable complex with folding intermediates (Lee et al. 1997). As described above, the sHsp chaperone activity required the dissociation of the complex at heat shock temperatures (Haslbeck et al. 1999).

The molecular chaperone system of thermophilic archaea is very simple compared with that of other organisms. Among major chaperone families, only genes encoding Hsp70 and its cochaperones, group II chaperonin and sHsp, have been found in the genome sequences, and genes for Hsp104/ClpB and Hsp90 are absent (Bult et al. 1996; Kawarabayasi et al. 1998, 2001). Moreover, genes for the Hsp70/DnaK system were not found in the total genomic sequences of hyperthermophilic archaea. Therefore, it is believed that the sHsp plays the main role in the molecular chaperone system in hyperthermophilic archaea. To date, sHsps from two hyperthermophilic archaea, *M. jannashii* and *Thermococcus* sp. strain KS-1, have been characterized (R. Kim et al. 1998; Usui et al. 2001; Bova et al. 2002). Although they prevented thermal aggregation of some proteins in vitro, they were not very effective compared with other sHsps. Heat shock induction of sHsps in hyperthermophilic archaea was demonstrated in *Thermococcus* sp. strain KS-1 and *Pyrococcus furiosus* (Laksanalamai et al. 2001; Usui et al. 2001).

In this study, we characterized two sHsps, StHsp19.7 and StHsp14.0, from a thermoacidophilic crenarchaeon, *Sulfolobus tokodaii* strain 7, which grows best in an acidic and aerobic conditions at ~80°C (Kawarabayasi et al. 2001; Suzuki et al. 2002). In studies of the molecular chaperone systems of *S. tokodaii* strain 7, the chaperonin subunits α and β were found to assemble into homo-oligomers, respectively, and arrest the spontaneous refolding of chemically denatured proteins in the same way as the purified *Sulfolo-*

bus chaperonin (Nakamura et al. 1997; Yoshida et al. 1998). However, neither wild-type chaperonin nor recombinant homo-oligomers exhibited any ATP-dependent refolding activity. Recently, another chaperonin gene was found in the complete genomic sequence (Kawarabayasi et al. 2001).

We have found two open reading frames encoding sHsp, and expressed them in *Escherichia coli*. One of them, named StHsp19.7, formed a filamentous structure, and the other, StHsp14.0, exhibited efficient molecular chaperone activity at 87°C.

Results

Two sHsps exist in S. tokodaii *strain 7*

The deduced amino acid sequences of ST0555 and ST1653 of *S. tokodaii* strain 7 exhibited significant homology in the α-crystallin domain with MjHsp16.5 and some eukaryotic sHsps (Fig. 1). Among five residues (corresponding to G62, L111, A122, G127, and L129 of MjHSP16.5) highly conserved in sHsps (Narberhaus 2002), four exist in the amino acid sequences of ST0555 and ST1653. In addition, the secondary structure prediction showed that β-strands were also conserved in their putative α-crystallin domains (data not shown). Thus, it was concluded that they encode small heat shock proteins, which were named StHsp19.7 and StHsp14.0 by their deduced molecular masses of 19,717 D and 14,045 D, respectively.

The StHsp19.7 and StHsp14.0 genes were cloned in pET23a vectors and used for the expression of StHsp19.7 and StHsp14.0 in *E. coli*, respectively. The expressed proteins were confirmed to be StHsp19.7 and StHsp14.0 by N-terminal amino acid sequencing. StHsp19.7 has lost the N-terminal methionine.

StHsp19.7 exists as a filament and StHsp14.0 as a spherical complex

Size exclusion chromatography of the purified StHsp19.7 and StHsp14.0 revealed that they existed as oligomers (Fig. 2A). The molecular mass of StHsp19.7 was larger than expected. Surprisingly, the electron microscopic image of negatively stained StHsp19.7 showed that it forms a filamentous structure (Fig. 2B). The width of the filaments was measured to be 13–14 nm. Size exclusion chromatography and electron microscopic images showed that the StHsp19.7 filament was dissociated into spherical particles of 13–17 nm and shorter filaments by heat shock at 80°C, the optimal growth temperature of *S. tokodaii* strain 7 (Fig. 2C).

StHsp14.0 eluted as a single peak, and the molecular mass was estimated to be ~220 kD (Fig. 2A). This result implies that StHsp14.0 should exist as a uniform hexadecameric oligomer because the deduced molecular mass of the StHsp14.0 monomer was ~14.0 kD. However, because of
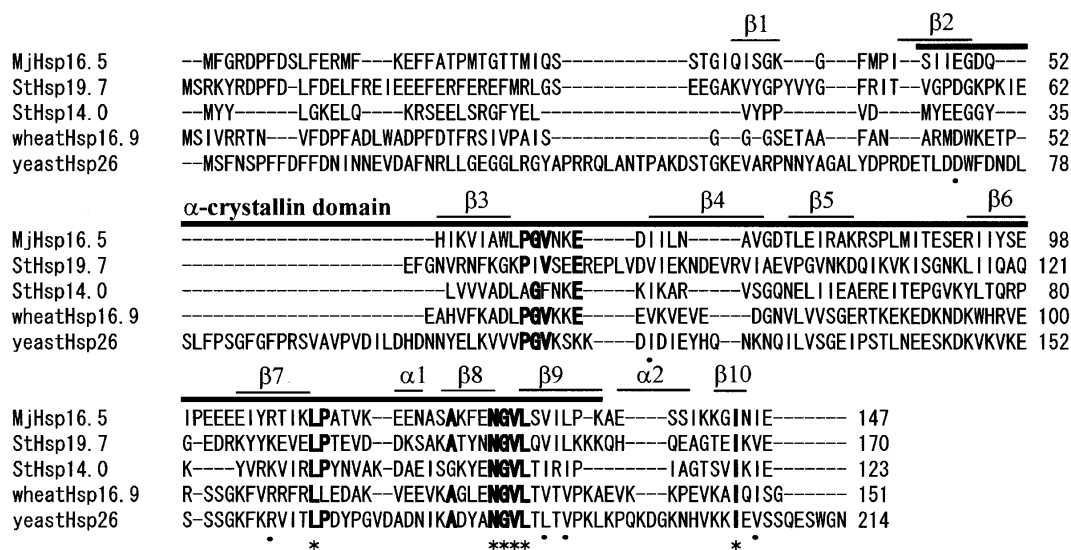
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                                                          β1              β2
MjHsp16.5    --MFGRDPFDSLFERMF---KEFFATPMTGTTMIQS------------STGIQISGK---G---FMPI--SIIEGDQ---   52
StHsp19.7    MSRKYRDPFD-LFDELFREIEEEFERFEREFMRLGS------------EEGAKVYGPYVYG---FRIT--VGPDGKPKIE   62
StHsp14.0    --MYY------LGKELQ----KRSEELSRGFYEL-------------------VYPP-------VD-----MYEEGGY---   35
wheatHsp16.9 MSIVRRTN---VFDPFADLWADPFDTFRSIVPAIS-------------G--G-GSETAA---FAN---ARMDWKETP-   52
yeastHsp26   --MSFNSPFFDFFDNINNEVDAFNRLLGEGGLRGYAPRRQLANTPAKDSTGKEVARPNNYAGALYDPRDETLDDWFDNDL   78

α-crystallin domain      β3                    β4        β5              β6
MjHsp16.5    ---------------------HIKVIAWLPGVNKE-----DIILN-----AVGDTLEIRAKRSPLMITESERIIYSE   98
StHsp19.7    ---------------------EFGNVRNFKGKPIVSEEREPLVDVIEKNDEVRVIAEVPGVNKDQIKVKISGNKLIIQAQ  121
StHsp14.0    ---------------------LVVVADLAGFNKE-----KIKAR-----VSGQNELIIEAEREITEPGVKYLTQRP   80
wheatHsp16.9 ---------------------EAHVFKADLPGVKKE-----EVKVEVE----DGNVLVVSGERTKEKEDKNDKWHRVE  100
yeastHsp26   SLFPSGFGFPRSVAVPVDILDHDNNYELKVVVPGVKSKK----DIDIEYHQ--NKNQILVSGEIPSTLNEESKDKVKVKE  152

            β7          α1  β8    β9      α2   β10
MjHsp16.5    IPEEEIYRTIKLPATVK--EENASAKFENGVLSVILP-KAE----SSIKKGINIE------- 147
StHsp19.7    G-EDRKYYKEVELPTEVD--DKSAKATYNNGVLQVILKKKQH----QEAGTEIKVE------- 170
StHsp14.0    K----YVRKVIRLPYNVAK-DAEISGKYENGVLTIRIP--------IAGTSVIKIE------- 123
wheatHsp16.9 R-SSGKFVRRFRLLEDAK--VEEVKAGLENGVLTVTVPKAEVK---KPEVKAIQISG------ 151
yeastHsp26   S-SSGKFKRVITLPDYPGVDADNIKADYANGVLTLTVPKLKPQKDGKNHVKKIEVSSQESWGN  214
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Figure 1. Amino acid sequence alignment of StHsp19.7 and StHsp14.0 with other sHsps. The sequences (with accession numbers) aligned are Hsp16.5 (AAB98273) of *M. jannaschii*, StHsp19.7 (BAB65551), StHsp14.0 (BAB66734), Hsp16.9 (S21600) of wheat, and Hsp26 (P15992) of *S. cerevisiae*. The alignment was performed by using CLUSTAL W. Asterisks indicate identical amino acids, and dots represent similar amino acids among all five sHsps compared. Boldface letters indicate amino acids that are conserved among at least four of the five sequences. The α-crystallin domain and the secondary structure assignment of MjHSP16.5 are shown on *top* of the alignment according to the crystal structure (K.K. Kim 1998).

the uncertainty of molecular weight predictions by size exclusion chromatography, more detailed studies would be necessary to determine the correct stoichiometry of the complex. Electron microscopic images of negatively stained StHsp14.0 showed that it exists as a spherical particle of 11–13 nm (Fig. 2D). These results showed that StHsp14.0 has almost the same oligomeric structure as MjHsp16.5. Thus, we concluded that the StHsp14.0 complex also consists of 24 subunits based on analogy with other sHsps from hyperthermophilic archaea (K.K. Kim et al. 1998; Usui et al. 2001).

StHsp14.0 prevents the thermally induced aggregation of IPMDH

To examine if StHsp19.7 or StHsp14.0 possesses chaperone activity, their effects on the thermal aggregation of IPMDH from the thermophilic bacterium *Thermus thermophilus* HB8 were examined. IPMDH is a homodimer of the subunit with the molecular mass of ~37 kD (Imada et al. 1991). Although it is much more stable against thermal denaturation than mesophilic enzymes, it denatures to aggregate at 87°C as detected by light scattering at 360 nm. As shown in Figure 3A, StHsp14.0 inhibited completely the thermal aggregation of IPMDH at 87°C at a 24 : 1 ratio of StHsp14.0 monomer to IPMDH monomer. Thus, the StHsp14.0 24-mer prevented the aggregation of IPMDH monomer at an almost equimolar ratio.

In previous reports, about 40–80-fold excess mole of MjHsp16.5 or *T* KS-1 sHsp 24-mer was required to prevent the thermal aggregation of the CS monomer at 40°–45°C (R. Kim et al. 1998; Usui et al. 2001). StHsp14.0 was also ineffective in suppressing the aggregation of the citrate synthase at 45°C, and a 32-fold excess of the StHsp14.0 24-mer over the CS monomer was required for complete suppression (Fig. 3B). Therefore, sHsps of hyperthermophilic archaea are active around or over their optimal growth temperatures.

On the other hand, StHsp19.7 could not protect the thermal aggregation of IPMDH (Fig. 3C). Because StHsp19.7 itself doesn't aggregate at the temperature (data not shown), the aggregation of IPMDH should be enhanced in the presence of StHsp19.7.

StHsp14.0 binds to nonnative IPMDH and forms a large complex

To examine whether *Sulfolobus* sHsps directly bind to IPMDH in a native or denatured state, immunoprecipitation from the mixture of StHsp19.7 or StHsp14.0 and IPMDH incubated at 65°C and 90°C was performed using anti-StHsp19.7 and anti-StHsp14.0 antibodies. StHsp19.7 bound to neither native IPMDH at 65°C, nor denatured IPMDH at 90°C, reflecting its lack of molecular chaperone activity (Fig. 4A). IPMDH was precipitated with StHsp14.0 when preincubated at 90°C, but only StHsp14.0 was detected after incu-
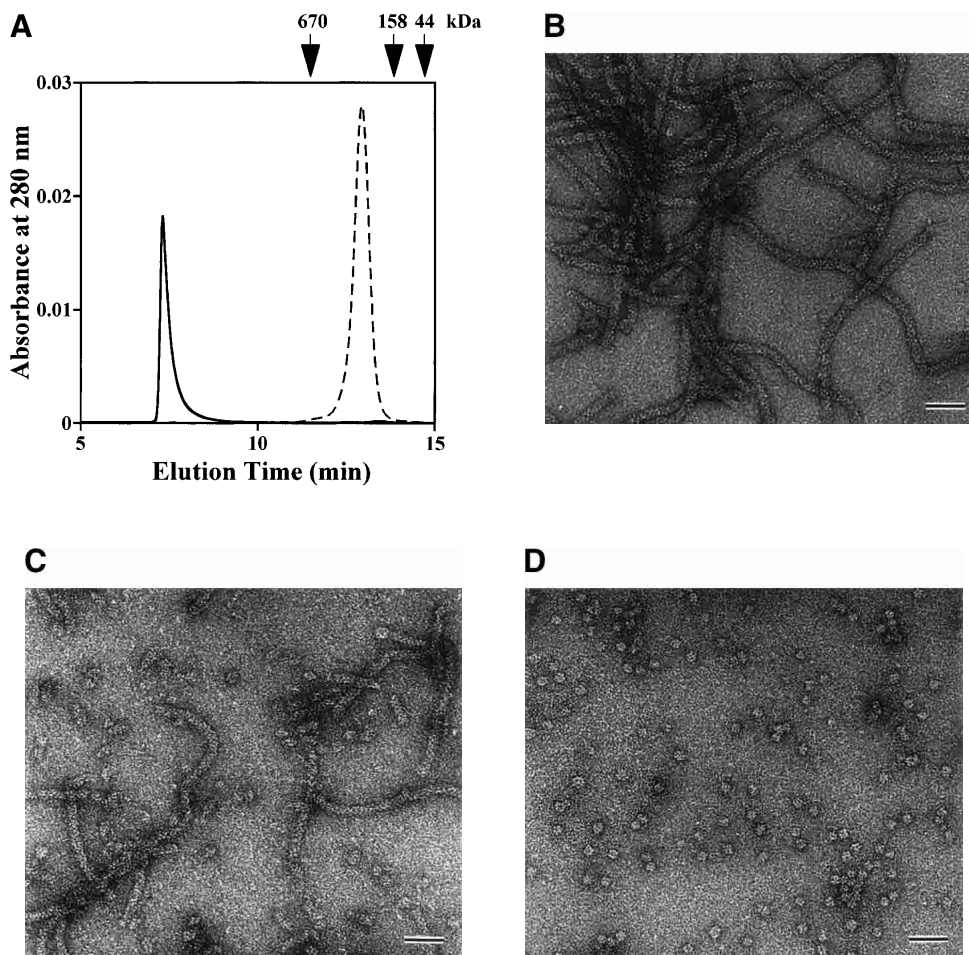
Figure 2. Quaternary structures of StHsp19.7 and StHsp14.0. (*A*) Size exclusion chromatography was performed using a TSKgel G4000SW$_{XL}$ column (Tosoh) as described in Materials and Methods. Purified StHsp19.7 (43.2 μM as monomer; solid line) and StHsp14.0 (43.2 μM as monomer; dotted line) elute as single peaks. The column was standardized with the following markers as indicated *above* the figure: thyroglobulin (670 kD); γ-globulin (158 kD); and ovalbumin (44 kD). (*B*) Negative-stain electron microscopic image of StHsp19.7 (7.1 μM). (*C*) Negative-stain electron microscopic image of StHsp19.7 (50.8 μM) kept at 80°C for 20 min, cooled on ice for 10 min, and centrifuged at 15,600*g* at 4°C for 10 min. The supernatant was diluted to 5.1 μM and then applied to grids as described in Materials and Methods. (*D*) Negative-stain electron microscopic image of StHsp14.0 (7.2 μM). Scale bar, 50 nm.

bation at 65°C (Fig. 4A). Thus, it is confirmed that StHsp14.0 directly bound to denatured IPMDH to suppress thermal aggregation.

Using size exclusion chromatography and negative-stain electron microscopy, we examined the formation of complex between StHsp14.0 and nonnative IPMDH. When the mixture of StHsp14.0 and IPMDH was applied to the gel filtration column without heat treatment, two clearly separated peaks appeared (Fig. 4B). The high-molecular-weight peak corresponds to the StHsp14.0 oligomer, and the low-molecular-weight peak corresponds to the IPMDH (dotted line). When StHsp14.0 was incubated with IPMDH at 90°C before loading, new broad and sharp peaks appeared at shorter elution times (Fig. 4B, solid line).

Complex formation between StHsp14.0 and IPMDH at 90°C was also analyzed by electron microscopy. As shown in Figure 4C, various sized particles, a spherical particle of

17–18 nm or a spread particle of 17–39 nm in diameter, larger than the spherical particles of the StHsp14.0 oligomer, were observed. Thus, StHsp14.0 likely binds to nonnative IPMDH to form complexes of variable size and shape. Eukaryotic sHsp also forms such complexes when incubated with substrate proteins under heat shock conditions (Ehrnsperger et al. 1997; Lee et al. 1997; Haslbeck et al. 1999).

Subunit exchange of StHsp14.0

sHsp oligomers show a dynamic exchange of subunits at physiological temperatures, which is thought to be related to chaperone activity (Bova et al. 2000, 2002). To elucidate whether the StHsp14.0 oligomers also exchange subunits, we labeled StHsp14.0 with a fluorescent dye, Alexa Fluor
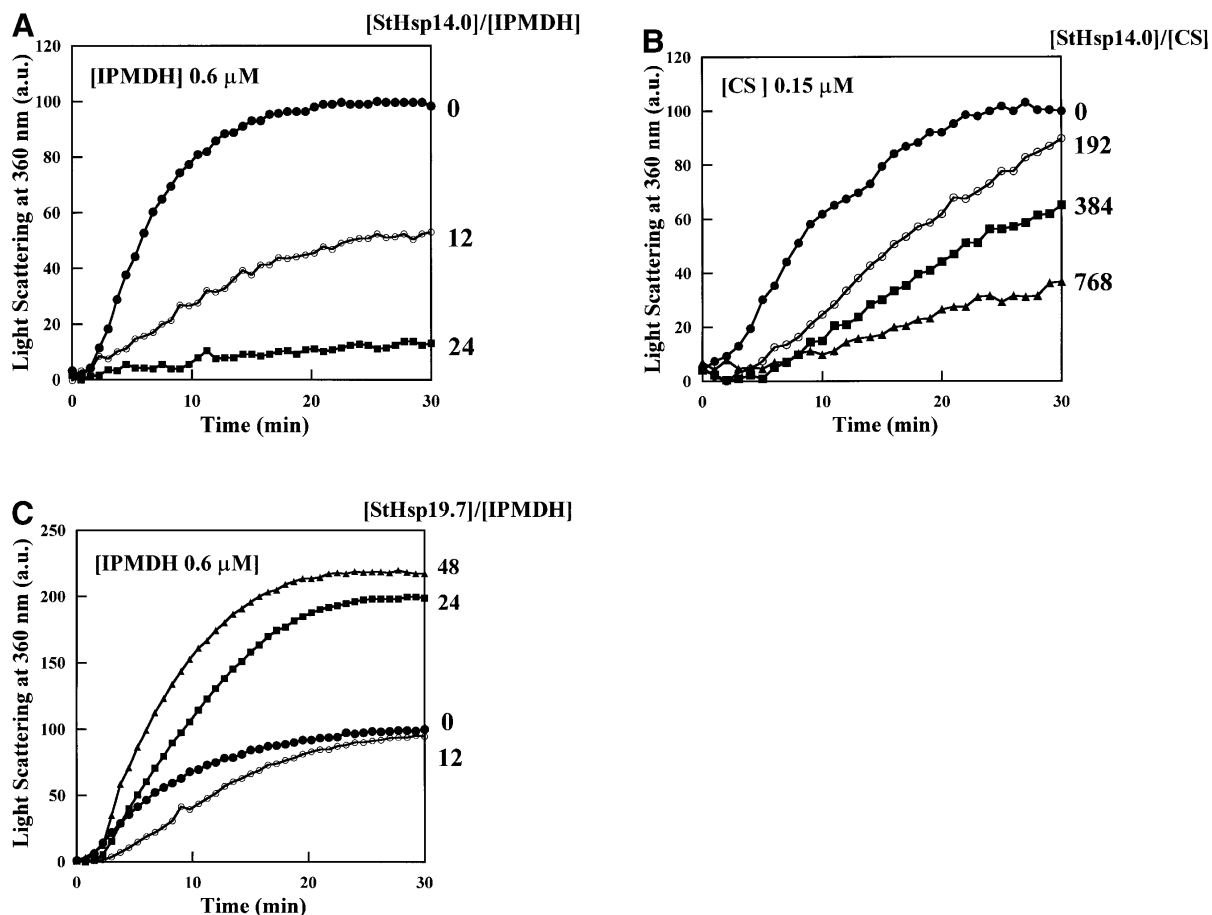
Figure 3. Thermal aggregation of IPMDH and CS was prevented by StHsp14.0. (*A*) IPMDH (0.6 μM as monomer) was incubated at 87°C in 50 mM Tris-HCl (pH 8.0) buffer, without or with StHsp14.0. (*B*) CS (0.15 μM as monomer) was incubated at 45°C in 50 mM Tris-HCl (pH 8.0) buffer, with or without StHsp14.0. (*C*) IPMDH (0.6 μM as monomer) was incubated at 87°C in 50 mM Tris-HCl (pH 8.0) buffer, without or with StHsp19.7. The aggregation of IPMDH or CS was monitored by measuring apparent light scattering at 360 nm with a spectrophotometer.

488, and biotin, independently. The labeling efficiencies were estimated to be 0.3 mole of Alexa Fluor 488 and 1.7 mole of biotin per StHsp14.0 subunit. The subunit exchange reaction was started by mixing equimolar amounts of fluorescence-labeled StHsp14.0 and biotin-labeled StHsp14.0. After incubation, streptavidin-linked magnetic beads were added to the reaction solution, and then StHsp14.0 complexes containing biotin-labeled sHsp subunits were recovered by magnetic separation. The recovered magnetic beads were suspended in buffer and used for fluorescence measurement. If StHsp14.0 oligomers exchange subunits, fluorescence would be detected because fluorescence-labeled subunits would be incorporated into complexes containing biotin-labeled complexes, which are captured by the streptavidin-linked magnetic beads. Although very little fluorescence was observed after the incubation at room temperature, the fluorescence of the captured fraction increased with the reaction temperature (Fig. 5, stippled bars). When the reaction temperature was increased to 90°C, a marked increase in fluorescent intensity was observed. The intensity

was ~43% of the total fluorescent intensity of fluorescence-labeled StHsp14.0. At 45°C, the fluorescent intensity was ~20% of that at 90°C. This result corresponds with the low molecular chaperone activity of StHsp14.0 at 45°C (Fig. 3B).

To unequivocally show that the florescence of the magnetic beads fraction is caused only by an exchange of subunits between oligomers, we performed an additional control experiment. We mixed excess unreacted free fluorescence label with biotin-labeled StHsp14.0 or excess unreacted free biotin label with fluorescence-labeled StHsp14.0, and performed the same experiment as described above. We did not observe any remarkable fluorescence increase in either experiment (Fig. 5).

Native StHsp19.7 exists as filaments or spherical particles

To investigate whether StHsp19.7 forms filamentous complexes in vivo, the cellular lysate of *S. tokodaii* strain 7 was
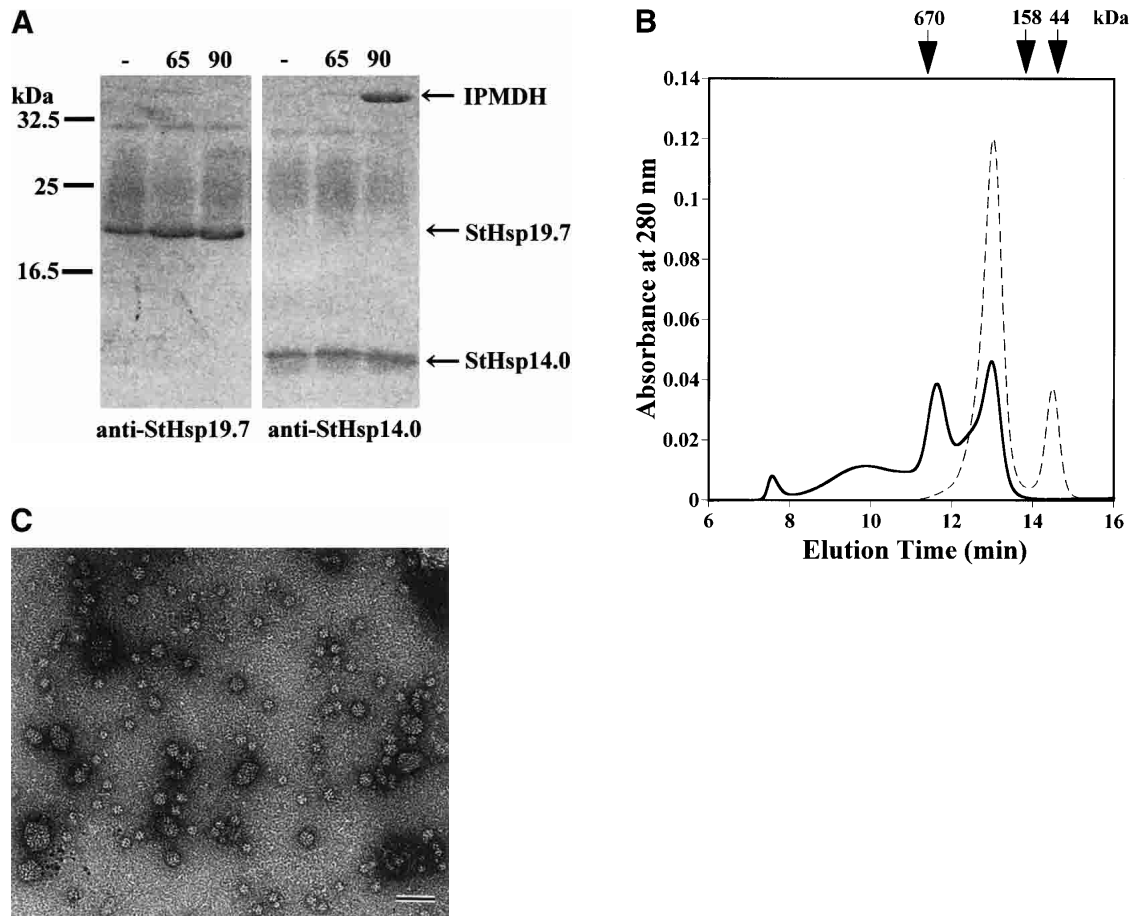
Figure 4. Complex formation between StHsp14.0 and IPMDH. (*A*) Immunoprecipitation of StHsp14.0 or StHsp19.7 and IPMDH. StHsp19.7 or StHsp14.0 and IPMDH were treated at 65°C or 90°C for 20 min, cooled on ice for 10 min, and then centrifuged at 15,600g at 4°C for 10 min. The supernatant was used for immunoprecipitation with IMMUNOchatcher, as described in Materials and Methods. Immunoprecipitation was performed with anti-StHsp19.7 or anti-StHsp14.0. Proteins were detected by SDS-PAGE using Coomassie brilliant blue. (*B*) Size exclusion chromatography was performed using a TSKgel G4000SW$_{XL}$ column (Tosoh) as described in Materials and Methods. StHsp14.0 and IPMDH were incubated at 90°C for 20 min, cooled on ice for 10 min (solid line), or not heat-treated (dotted line) and then centrifuged at 15,600g at 4°C for 10 min. The supernatant was applied to the column. The column was standardized with the markers as indicated in Figure 2A. (*C*) Negative-stain electron microscopic images of complex between StHsp14.0 and IPMDH. StHsp14.0 (216.0 μM as monomer) and IPMDH (36.0 μM as monomer) were incubated at 90°C for 20 min, cooled on ice for 10 min, and then centrifuged at 15,600g 4°C for 10 min. The supernatant was applied to grids as described in Materials and Methods. Scale bar, 50 nm.

fractionated by size exclusion chromatography, and then aliquots of the fractions were analyzed by SDS-PAGE with immunoblotting using anti-StHsp19.7 antibody. Recombinant StHsp19.7 was also size fractionated after being incubated at 80°C, and analyzed by SDS-PAGE with Coomassie brilliant blue staining.

The elution profile of recombinant StHsp19.7 shows two broad peaks, which correspond to short filaments and spherical particles as shown in Figure 2C (Fig. 6A). SDS-PAGE analyses of the fractions showed that StHsp19.7 in the cellular lysate eluted in almost the same manner as recombinant StHsp19.7 (Fig. 6B). The results indicate that native StHsp19.7 may exist as filaments and spherical particles in the cells.

Discussion

There are two sHsp genes in the genomic sequence of the thermoacidophilic crenarchaeon *S. tokodaii* strain 7. We have expressed both of them, StHsp19.7 and StHsp14.0, in *E. coli* and performed biochemical characterizations. Electron microscopic images showed that StHsp19.7 exists as a filament of spherical complexes (Fig. 2B). This is the first report on the formation of filaments by sHsps. It was reported that chaperonin from the cognate archaeon *Sulfolobus shibatae* formed filaments in the presence of Mg^{2+} and nucleotides (Trent et al. 1997). It is proposed that chaperonin filaments exist in vivo and play a cytoskeletal role in archaea. We have shown that StHsp19.7 exists as filaments
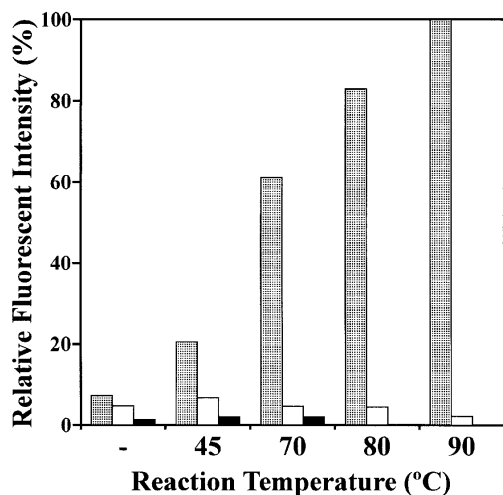
Figure 5. Effect of temperature on subunit exchange of StHsp14.0. The subunit exchange reaction was performed with or without incubation at 45°C, 70°C, 80°C, or 90°C for 20 min. The reaction solution contained fluorescence-labeled StHsp14.0 and biotin-labeled StHsp14.0 (stippled bars), biotin-labeled StHsp14.0 and free fluorescence label (white bars), or fluorescence-labeled StHsp14.0 and free biotin label (black bars). The fluorescence intensity of the magnetic beads fractions was measured as described in Materials and Methods. The strongest intensity equals 100%.

and spherical particles in the cell, by size exclusion chromatography with immunoblotting (Fig. 6A,B). Although more detailed study is necessary, StHsp19.7 might exist and function as filaments in vivo.

StHsp14.0 prevented thermal aggregation of IPMDH from *T. thermophilus* HB8 at 87°C (Fig. 3A). This result shows that StHsp14.0 has molecular chaperone activity similar to other sHsps. However, the chaperone activity was low when the assay was carried out at lower temperature, ~45°C (Fig 3B). This indicates that the chaperone activity of hyperthermophilic sHsp is activated only at high temperature. In contrast, other molecular chaperones from hyperthermophilic archaea, group II chaperonin, and prefoldin could protect CS from porcine heart at 45°C with high efficiency (Iizuka et al. 2001; Okochi et al. 2002). We have shown that an exchange of subunits occurs between StHsp14.0 complexes and the efficiency of the exchange is highly dependent on temperature (Fig. 5). Recently, subunit exchange of a hyperthermophilic archaeal sHsp, MjHsp16.5, was also reported (Bova et al. 2002). The eukaryotic sHsp complex dissociates into small species at the heat shock temperature (Haslbeck et al. 1999; van Montfort et al. 2001). The dissociation of the complex is likely to be required for the molecular chaperone activity of sHsp, and recently it was reported that dynamic change during oligomerization is essential to the function of sHsp in vivo (Giese and Vierling 2002).

Therefore, we speculate that the dissociation of the StHsp14.0 complex into suboligomeric species at elevated temperatures is indispensable to its molecular chaperone activity. Although we have tried to detect dissociated StHsp14.0 suboligomeric complexes by gel filtration chromatography after heat shock treatment, we could not detect any oligomers with slower retention times (data not shown). This result indicates that suboligomeric complexes of StHsp14.0 immediately reform into a complex after heat shock in the absence of unfolded proteins.

StHsp14.0 forms large complexes with denatured proteins at elevated temperatures (Fig 4B,C). Similar to the case for other sHsps (Ehrnsperger et al. 1997; Lee et al. 1997), the association between StHsp14.0 with IPMDH was not specific, and the complex existed as a more irregular shape as observed in the Hsp26–CS complex (Haslbeck et al. 1999). We propose that dissociated StHsp14.0 with exposed hydrophobic regions binds randomly to intermediate-state IPMDH during heat shock.

We showed that the thermoacidophilic archaeal sHsp StHsp14.0 had chaperone properties at temperatures higher than the optimal growth temperature like the sHsps of other organisms. This study supports the prediction that the heat-induced dissociation of the StHsp14.0 oligomer would result in a smaller species that initiates the interaction with substrate, followed by reassembly into a larger StHsp14.0–substrate complex. The molecular chaperone system of thermophilic archaea is very simple compared with that of other organisms. In the *S. tokodaii* strain 7 genome sequence, among major chaperone families, only genes encoding Hsp60s, three group II chaperonin subunits, and two sHsps have been found, but genes for other principal chaperones, Hsp70/DnaK, Hsp104/ClpB, and Hsp90, are absent (Kawarabayasi et al. 2001). Therefore, this report indicated that StHsp14.0 plays the main role in the molecular chaperone system in *S. tokodaii* strain 7 rather than bacteria and eukarya. However, the mechanism of sHsp chaperone activity is still vague. StHsp14.0 may be useful for understanding the relationships between the molecular structure and function of sHsps from not only archaea but also others.

Materials and methods

Strains, enzymes, and reagents

The thermoacidophilic crenarchaeon *Sulfolobus tokodaii* strain 7 was obtained from Tairo Oshima of Tokyo University of Pharmacy and Life Science. *E. coli* BL21(DE3) harboring the plasmids for expression of 3-isopropylmalate dehydrogenase (IPMDH) from the thermophilic bacterium *Thermus thermophilus* HB8 was a gift from Akihiko Yamagishi of Tokyo University of Pharmacy and Life Science. Ex *Taq* DNA polymerase and other reagents for gene manipulation were obtained from TAKARA Bio Inc. Citrate synthase (CS) from porcine heart was purchased from Sigma Chemical Co.

Plasmid constructs for Sulfolobus *sHsps expression*

ORFs ST0555 and ST1653 of the thermoacidophilic crenarchaeon *S. tokodaii* strain 7 were searched for sequence homology with
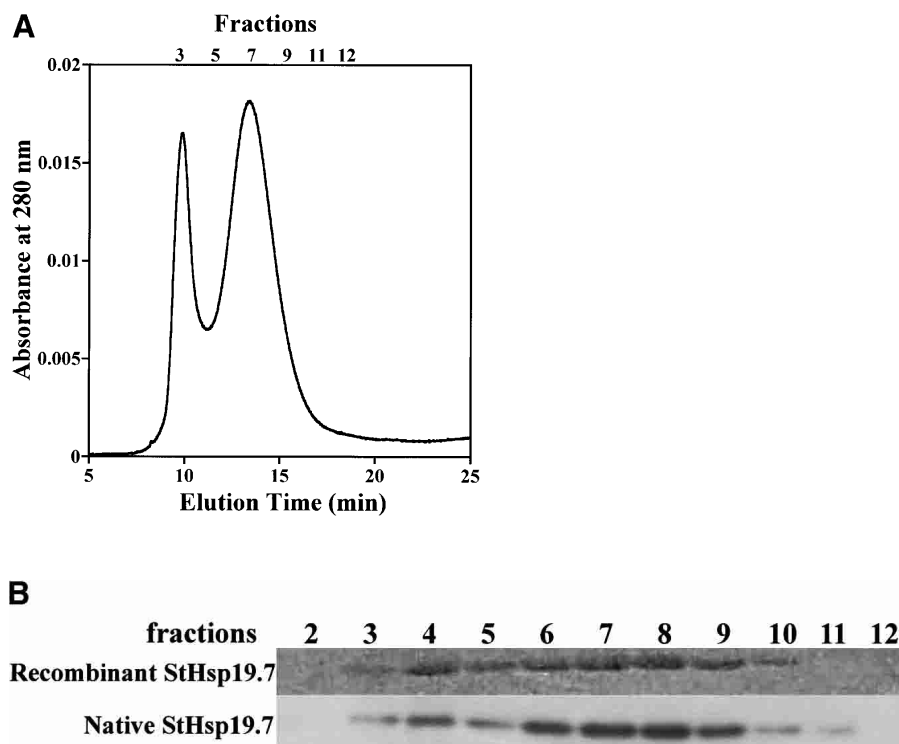
A

B

Figure 6. Filament formation of StHsp19.7 in *S. tokodaii* strain 7. (*A*) Size exclusion chromatography was performed using a Bio-Prep SE-1000/17 (Bio-Rad) as described in Materials and Methods. Recombinant StHsp19.7 (45.7 μM as monomer) was incubated at 80°C for 20 min and cooled on ice for 10 min. After centrifugation at 15,600*g* at 4°C for 10 min, the supernatant was applied to the column. (*B*) Fractions of size exclusion chromatography of recombinant StHsp19.7 and *S. tokodaii* strain 7 lysate were separated by SDS-PAGE, and analyzed by Coomassie blue staining (*upper*) and immunoblotting with anti-StHsp19.7 antibody (*lower*), respectively.

other sHsps. The proteins encoded by theses genes are named StHsp19.7 and StHsp14.0, respectively. Full-length StHsp19.7 and StHsp14.0 genes were amplified from the total DNA of *S. tokodaii* strain 7 by PCR using primers, 5′-CCA-TAT-GTC-AAG-GAA-ATA-TAG-A-3′, 5′-GGG-ATC-CCT-ACT-CAA-CCT-TAA-TTT-C-3′; and 5′-CCA-TAT-GTA-TTA-TTT-AGG-AAA-AG-3′, 5′-GGG-ATC-CTC-ATT-CTA-TCT-TTA-TAA-CA-3′; and then subcloned into the pT7Blue T vectors, respectively. After sequence confirmation, the genes were excised with *Nde*I and *Bam*HI, and then introduced into the *Nde*I/*Bam*HI site of pET23a to construct pSTSH1E and pSTSH2E, respectively.

Purification of StHsp19.7 and StHsp14.0

E. coli BL21(DE3) transformed with pSTSH1E was grown at 37°C in Luria-Bertani medium containing 100 μg/mL ampicillin. The cells were harvested by centrifugation at 4600*g* at 4°C for 10 min. The harvested cells were resuspended in buffer A (50 mM Tris-HCl at pH 8.0, 0.1 mM EDTA, and 1 mM dithiothreitol) and lysed by sonication. The suspension of disrupted cells was centrifuged at 25,000*g* at 4°C for 30 min, and the supernatant was heated at 75°C for 30 min. The denatured protein was removed by centrifugation at 25,000*g* at 4°C for 30 min. The supernatant was applied to a DEAE-Toyopearl anion exchange column (Tosoh) equilibrated with buffer A. Proteins were eluted with a linear gradient of 0–500 mM NaCl in buffer A. Fractions containing StHsp19.7 were pooled, concentrated by ultrafiltration (Centriprep 10, Millipore), and then applied to a HiLoad 26/60 Superdex

200pg size exclusion column (Amersham Biosciences) equilibrated with buffer B (50 mM Tris-HCl at pH 8.0, 1 mM dithiothreitol, and 150 mM NaCl). Fractions containing StHsp19.7 were collected and then concentrated by ultrafiltration (Centriprep 10, Millipore).

E. coli BL21(DE3), transformed with pSTSH2E, was grown, and StHsp14.0 was purified as described above.

Purification of IPMDH

E. coli BL21(DE3) cells harboring the plasmids for expression of IPMDH from the thermophilic bacterium *T. thermophilus* HB8 were grown at 37°C in Luria-Bertani medium containing 100 μg/mL ampicillin. The cells were harvested by centrifugation at 4600*g* at 4°C for 10 min. The harvested cells were resuspended in buffer A and lysed by sonication. The suspension of disrupted cells was centrifuged at 25,000*g* at 4°C for 30 min, and the supernatant was heated at 65°C for 30 min. The denatured protein was removed by centrifugation at 25,000*g* at 4°C for 30 min. The supernatant was precipitated by the addition of ammonium sulfate to a final concentration of 20%. After centrifugation at 14,000*g* at 4°C for 30 min, the supernatant was applied to a Butyl-Toyopearl hydrophobic interaction column (Tosoh) equilibrated with buffer A containing 20% ammonium sulfate. Proteins were eluted with a linear gradient of 20%–0% ammonium sulfate in buffer A. Fractions containing IPMDH were concentrated and desalted by ultrafiltration (Centriprep 10, Millipore), and then applied to a UNO Q6 anion exchange column (Bio-Rad) equilibrated with buffer A. Pro-

teins were eluted with a linear gradient of 0–500 mM NaCl in buffer A. Fractions containing IPMDH were collected and then concentrated by ultrafiltration (Centriprep 10).

N-terminal amino acid sequencing

The purified StHsp19.7 or StHsp14.0 was subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidine difluoride membranes. The membranes were stained with Coomassie brilliant blue R-250. The bands corresponding to StHsp19.7 and StHsp14.0 were cut out, and analyzed with a 492 Procise Protein sequencing System (Applied Biosystems).

Size exclusion chromatography of sHsps

Size exclusion chromatography was performed using a TSKgel G4000SW$_{XL}$ (Tosoh) with buffer (50 mM Tris-HCl at pH 7.3, 150 mM NaCl, and 1 mM dithiothreitol) at a flow rate of 0.8 mL/min with absorbance monitored at 280 nm. Gel filtration standard protein (Bio-Rad) was used to calibrate the column. To examine the formation of complex, a mixture of StHsp14.0 (216.0 μM as monomer) and IPMDH (36.0 μM as monomer) was incubated at 90°C for 20 min, cooled on ice for 10 min, and centrifuged at 15,600g at 4°C for 10 min, and then the supernatant was applied to TSKgel G4000SW$_{XL}$ (Tosoh).

Electron microscopy

The protein solutions were applied individually onto specimen grids covered with thin carbon support films, which had been made hydrophilic by an ion spatter device (JEOL HDT-400), then negatively stained with 1% uranyl acetate for 30 sec. The images were taken on Kodak electron image films (SO-163) under low electron dose conditions at a magnification of 50,000× in an electron microscope (Philips Tecnai F20) operated at 120 kV.

Thermal aggregation measurements

The effect of StHsp19.7 or StHsp14.0 on the thermally induced aggregation of IPMDH (0.6 μM) from *T. thermophilus* HB8 and citrate synthase (0.15 μM) from porcine heart in 50 mM Tris-HCl (pH 8.0) buffer was monitored by light scattering at 360 nm using a UV-1600PC spectrophotometer (Shimadzu Biotech) equipped with a thermostatted cell compartment preheated to 87°C or 45°C, respectively.

Culture conditions for S. tokodaii *strain 7*

S. tokodaii strain 7 was grown in the medium at 80°C aerobically. This medium contains 1.3 g of $(NH_4)_2SO_4$; 0.28 g of KH_2PO_4; 0.25 g of $MgSO_4 \cdot 7 H_2O$; 0.07 g of $CaCl_2 \cdot 2 H_2O$; 0.02 g of $FeCl_3 \cdot 6 H_2O$; 1.8 mg of $MnCl_2 \cdot 4 H_2O$; 4.5 mg of $Na_2B_4O_7 \cdot 10 H_2O$; 0.22 mg of $ZnSO_4 \cdot 7 H_2O$; 0.05 mg of $CuCl_2 \cdot 2 H_2O$; 0.03 mg of $Na_2MoO_4 \cdot 2 H_2O$; 0.03 mg of $VOSO_4 \cdot 2 H_2O$; 0.01 mg of $CoSO_4$; 1.0 g of yeast extract; 1.0 g of casamino acid; and 1 L of deionized water (Brock et al. 1972). The pH was adjusted to 3.0 with H_2SO_4. For Western blot analysis, *S. tokodaii* strain 7 cells were introduced into 500 mL of the above medium and incubated at 80°C for 3 d.

Antibody preparation, immunoprecipitation, Western blot analysis

Rabbit polyclonal antibodies against the recombinant StHsp19.7 or StHsp14.0 were raised. The IgG fractions were purified using an affinity column (Affi-Gel Protein A, Bio-Rad).

Immunoprecipitation experiments were performed using IMMUNOcatcher (CytoSignal) according to the supplier's instructions. StHsp19.7 or StHsp14.0 and IPMDH were treated at 65°C or 90°C in buffer B for 20 min, cooled on ice for 10 min, and centrifuged at 15,600g at 4°C for 10 min. Then, 10 μL of protein A purified anti-StHsp19.7 or StHsp14.0 antibody was added to 200 μL of the supernatant sample and incubated at room temperature for 1 h. Protein A/G resin (10 μL) was added to the mixture and incubated for 30 min. Proteins bound to the resin were collected by centrifugation, washed, and resuspended in SDS-PAGE sample solution. After incubation for 15 min, bound proteins were collected by centrifugation and then analyzed by SDS-PAGE.

The *S. tokodaii* strain 7 cells grown at 80°C were harvested by centrifugation at 4600g at 4°C for 10 min. The harvested cells were resuspended in buffer A and lysed by sonication. The suspension of disrupted cells was centrifuged at 25,000g at 4°C for 30 min. The supernatant was applied to a Bio-Prep SE-1000/17 size exclusion column (Bio-Rad) equilibrated with buffer B, and eluted at a flow rate of 0.5 mL/min. Fractions were separated by SDS-PAGE using 15% gels and transferred to a polyvinylidine difluoride membrane with a semidry blotter (Nippon Eido). The membrane was used for immunoblotting with anti-StHsp19.7 antibody. Detection was performed using a horseradish peroxidase-linked protein A, and reactive bands were visualized with Enhanced Chemiluminescence (ECL) Detection Reagents (Amersham Biosciences).

Labeling of StHsp14.0 with fluorescent dye or biotin

The labeling of StHsp14.0 with fluorescent dye was performed using an Alexa Fluor 488 Protein Labeling Kit (Molecular Probes), according to the manufacture's instructions. StHsp14.0 was diluted to 2 mg/mL in buffer C (50 mM sodium phosphate at pH 7.5, and 150 mM NaCl), and then sodium bicarbonate was added to the solution (final concentration 100 mM). The solution was transferred to a vial of Alexa Fluor 488 reactive dye. The reaction was performed at room temperature for 1 h in the dark with continuous stirring. Covalently bound probes were separated from free, unreacted probes using the column supplied with the kit. Labeling efficiency was determined by measuring the absorbance of the solution at 280 nm and 494 nm.

The labeling of StHsp14.0 with biotin probe was performed using an EZ-Link Sulfo-NHS-Biotinylation Kit (PIERCE Biotechnology) according to the instructions supplied. StHsp14.0 was diluted to 2 mg/mL in buffer C. Then 1.3 mg of Sulfo-NHS-Biotin was added to the solution. The reaction was run at room temperature for 30 min. Covalently bound probe was separated from free, unreacted probes using the D-Salt Dextran Desalting Column MWCO 5000 supplied with the kit. Biotin incorporation, moles biotin per mole protein, was determined by HABA assay as recommended in the instructions (Green 1965, 1975).

Measurements of subunits exchange by magnetic separation and fluorescence

To initiate the subunit exchange reaction, 3.6 μM each of fluorescence-labeled and biotin-labeled StHsp14.0 monomer in buffer C

was mixed together in a reaction tube at various temperatures for 20 min, and cooled on ice. As a control experiment, biotin-labeled StHsp14.0 (3.6 µM) and free fluorescence label (4.6 times the fluorescence intensity of fluorescence-StHsp14.0; 3.6 µM) or fluorescence-labeled StHsp14.0 (3.6 µM) and free biotin label (2.6 times the absorbance at 500 nm in the HABA assay [Green 1965, 1975] of biotin-labeled StHsp14.0; 3.6 µM) in buffer C were mixed together in a reaction tube. Magnetic beads covalently coupled with streptavidin (Dynabeads M-280 Streptavidin, DYNAL) in an equivalent volume were added to the reaction solution. The suspension was kept on ice for 5 min with occasional pipetting. The magnetic beads fraction was separated from the solution fraction using a magnet, buffer C was added, and the mixture was transferred to a microtiter plate. The fraction was subsequently excited at 532 nm, and the fluorescence emission cut off below 526 nm by a filter was measured using a Typhoon 8600 (Amersham Bioscience).

Other methods

Proteins were analyzed by SDS-PAGE (Laemmli 1970). The separated proteins were visualized by staining with Coomassie brilliant blue R-250. The protein concentration was measured by the Bradford method with a protein assay kit (Bio-Rad) using bovine serum albumin as the standard (Bradford 1976).

Acknowledgments

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