Subunit Exchange, Conformational Stability, and Chaperone-like Function of the Small Heat Shock Protein 16.5 from *Methanococcus jannaschii*

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Hsp16.5, isolated from the hyperthermophilic Archaea *Methanococcus jannaschii*, is a member of the small heat-shock protein family. Small Hsps have 12- to 42-kDa subunit sizes and have sequences that are conserved among all organisms. The recently determined crystal structure of Hsp16.5 indicates that it consists discretely of 24 identical subunits. Using fluorescence resonance energy transfer, we show that at temperatures above 60 °C, the subunits of MJHsp16.5 freely and reversibly exchange with a rate constant of exchange at 68 °C of 0.067 min\(^{-1}\). The subunit exchange reactions were strongly temperature-dependent, similar to the exchange reactions of the α-crystallins. The exchange reaction was specific to MJHsp16.5 subunits, as other sHsps such as α-crystallin were not structurally compatible and could not integrate into the MJHsp16.5 oligomer. In addition, we demonstrate that at temperatures as high as 70 °C, MJHsp16.5 retains its multimeric structure and subunit organization. Using insulin and α-lactalbumin as model target proteins, we also show that MJHsp16.5 at 37 °C is a markedly inefficient chaperone compared with other sHsps with these substrates. The results of this study support the hypothesis that MJHsp16.5 has a dynamic quaternary structure at temperatures that are physiologically relevant to *M. jannaschii*. 

MJHsp16.5 is a small heat shock protein (sHsp)\(^1\) from the hyperthermophilic Archaea *Methanococcus jannaschii* that lives at temperatures up to 94 °C (1). sHsps are ubiquitous among all organisms, have subunit sizes that range from 12 to 42 kDa, and show homology in amino acid sequence (2). Most of the conservation between sHsps exists in a region known as the α-crystallin domain (2); this is a stretch of 80–100 amino acids generally located in the C-terminal part of the sequence. A number of studies have identified this domain to be important for subunit-subunit interactions that lead to the assembly of building blocks of oligomeric structure (3–5). Many sHsps exist as high molecular weight assemblies that have dynamic and variable quaternary structures with subunits that can freely and rapidly exchange between oligomers (6–8). Current evidence suggests that the quaternary structure of mammalian sHsps has smaller multimers that exist in a dynamic equilibrium with the larger oligomeric assembly (9–11). Although many sHsps have variable quaternary structures, Hsp16.5 is monodisperse consisting of exactly 24 subunits with a molecular mass of 400 kDa (12). However, the subunit exchange properties of MJHsp16.5 and its multimeric structure at higher temperatures have not been investigated.

The crystal structure of Hsp16.5 has been determined and reveals 24 subunits arranged in octahedral symmetry forming a hollow spherical assembly with small openings (13). The monomer folds into a β-sandwich or immunoglobulin fold with one strand, referred to as β-6, contacting a neighboring subunit at the dimer interfaces. A similar but not identical folding pattern has been shown for the α-crystallin domain in α-crystallin (14). Recently, the crystal structure of Hsp16.9 from wheat was obtained at 2.7-Å resolution where its α-crystallin domains and flanking extensions assemble into a dodecameric double disk (15). The dodecamer of Hsp16.9 consists of two disks, each comprising six α-crystallin domains organized into a trimer of dimers (15). Although wheat Hsp16.9 and MJHsp16.5 from the hyperthermophile *M. jannaschii* are distantly related from an evolutionary standpoint, as well as in amino acid sequence (23%), their α-crystallin domains are similar, and both use a dimer as the fundamental unit of oligomeric assembly (15). However, despite the similarities in their α-crystallin domains, the quaternary structures of MJHsp16.5 and Hsp16.9, as well as the mammalian sHsps are different. This difference in oligomeric assembly may be encoded in the N-terminal domains of sHsps (16); alternatively, it has been hypothesized to result from different orientations of their respective C-terminal extensions caused by a hinge between β-strands 9 and 10 in their α-crystallin domains (15).

Many sHsps have been shown to function in a chaperone-like manner (17–20). Chaperones function to bind unfolding proteins, preventing their aggregation and thus promoting their proper folding. In the crowded molecular environment of the cell, aggregation of proteins will lead to impaired function and ultimately cell death. sHsps have been shown to bind unfolding proteins without the hydrolysis of ATP and are remarkably efficient in terms of ratio of chaperone subunit to bound substrate. It has been suggested that sHsps serve as a storage depot for unfolded proteins, which can then be completely re-folded in the presence of other chaperones (21). A number of protein folding diseases such as desmin-related myopathies and cataracts have been shown to result from specific point mutations in sHsps (22–24). MJHsp16.5, as well as sHsps from other hyperthermophiles, confer protection against thermally induced stress when over-expressed in *Escherichia coli* cells

\* This work was supported in part by National Eye Institute Grant R37-EY3897 (to J. H.) and by the Bank of America Giannini Foundation (to M. P. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1724 solely to indicate this fact.

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The abbreviations used are: sHsp, small heat-shock protein; MJHsp16.5, small heat shock protein from *Methanococcus jannaschii*; AIAS, 4-acetamido-4′-(iodoacetyl)aminostilbene2, 2′-disulfonic acid; LYL, lucifer yellow iodoacetamide; DTT, dithiothreitol; wt, wild-type; MOPS, 4-morpholinepropanesulfonic acid.

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Received for publication, June 5, 2002, and in revised form, August 8, 2002

Published, JBC Papers in Press, August 9, 2002, DOI 10.1074/jbc.M205594200
and have been shown to prevent the thermally induced aggre-
gation of certain model target proteins in vitro (12, 25).
In this report, we use fluorescence resonance energy trans-
fer, circular dichroism, and size exclusion chromatography
with online multi-angle light scattering, absorbance, and
refractive index detectors to characterize the subunit exchange
properties and oligomeric structure of MjHsp16.5 at various
temperatures. In addition, we also investigate the chaperone-
like properties of MjHsp16.5 using a variety of substrates over
a wide temperature range. The results in this paper illustrate
the importance of monitoring the function of MjHsp16.5
at temperatures that are physiologically relevant to
M. jannaschii.

MATERIALS AND METHODS

Materials—Bovine insulin and a-lactalbumin were obtained from
Sigma. Lucifer yellow idoacetamide (LYI) and 4-acetamido-4-(ido-
aecetyl) amino) stilbene-2, 2'-disulfonic acid (AIAS) were purchased
from Molecular Probes, Eugene, OR. The wild-type MjHsp16.5 clone in
the pET 20b+ vector was a generous gift of Dr. Hassane Mchaourab,
Vanderbilt University, Nashville, TN. E. coli strain BL21DE3 was
purchased from Novagen. Trep lipocalin was a generous gift of Dr. Ben
Glasgow. Complete protease inhibitor tablets were purchased from
Roche Applied Science.

Expression and Purification of Hsp16.5 from M. jannaschii—Wild-
type MjHsp16.5 in the pET 20b+ vector was transformed into E. coli
strain BL21DE3. Bacterial cells were grown at 37 °C in LB medium and
induced with isopropyl-D-thiogalactoside when the culture reached an
optical density between 0.6 and 1. Bacterial cells were grown for 3 h
and applied to an Amersham Biosciences Phenyl Sepharose HP column
equilibrated in 50 mM sodium phosphate buffer, pH 6.8. These concentrated fractions were
then applied to an Amersham Biosciences Mono-Q filtration column equilibrated in 50 mM sodium phosphate buffer, pH 8.2 containing 100
mM NaCl. The protein was eluted from the column with a linear gra-
dient of 0–1 M NaCl and applied to an Amersham Biosciences Phenyl Sepharose HP column (1 ml bed volume) equilibrated in 50 mM sodium phosphate buffer, pH 7.0 containing 1 M ammonium sulfate. Protein was eluted from the column using a linear gradient of 0–1 M ammonium sulfate in the same buffer. MjHsp16.5 containing fractions were pooled, concentrated, and applied to an Amersham Biosciences Superose 6 HR 10/30 gel filtration column equilibrated in 50 mM sodium phosphate buffer, pH 7.0 containing 100 mM NaCl. These procedures yielded pure MjHsp16.5 as assayed by SDS-PAGE. aB-Crystallin was purified as previously
described (26).

Circular Dichroism Measurements of MjHsp16.5—All near and far
UV CD spectra were obtained using a Jasco J-600 spectropolarimeter
interfaced with a Luda water circulator. Far UV CD measurements
were performed using a cell with a 0.2-mm path length, whereas the
near UV CD cuvette had a path length of 1 cm.

Labeling of Hsp16.5 with Fluorescent Probes—MjHsp16.5 was di-
luted to 1 mg/ml in 20 mM MOPS buffer, pH 7.9 containing 100 mM
NaCl. Solid LYI or AIAS was added to the solution to final concen-
trations of 8.5 and 3.2 mM respectively. The reactions were allowed to
proceed at room temperature in the dark for 2 h. Subsequently, AIAS-
and LYI-containing solutions were heated at 37 °C for 3 and 5 h,
respectively. Covalently bound probe was separated from free, unre-
acted probe using gel filtration with a Sephadex G-25 column. Fluores-
cent-labeled Hsp16.5 was electrophoresed on a 12.5% acrylamide gel
and imaged using an AlphaImager 2000 (AlphaInnotech).

Measurements of Subunit Exchange Using Fluorescence Resonance
Energy Transfer—To initiate the subunit exchange reaction, 30 µM
concentrations of AIAS- and LYI-labeled MjHsp16.5 were mixed to-
gether in a reaction tube at 68 °C unless otherwise noted. At the time
points indicated in the figures, a 20-µl aliquot was taken from the
reaction mixture and diluted 1:100 with room-temperature phosphate-
buffered saline to quench the reaction. Because subunit exchange does not take place at temperature below 50 °C, upon dilution of the sample
to room temperature the subunits that were exchanged are now “fro-
en.” The sample was subsequently excited at 335 nm in a 1-cm square
cuvette, and the fluorescence emission spectra were taken from 360–
600 nm at room temperature. It should be emphasized that the subunit exchange reactions occur at 69 °C or higher. However, the fluorescence emission spectra are recorded at room temperature. The emission in-
tensity at 412 nm was recorded and plotted. The normalized donor fluorescence intensity as a function of time, F(t), was fit to the equation
F(t) = C1 + C2e−kt. The constants C1 and C2 were determined by the conditions that C1 + C2 = 1 at F(0) and F(∞) = C1. The rate constant k of the subunit exchange reaction was determined using the Bio- 

dynamical analysis package. All fluorescence spectra were recorded using a Spex Fluorolog Fluorimeter model FL3-11, interfaced with an Iso-
temp 1016S water circulator.

Monitoring the Oligomeric Structure of MjHsp16.5 at Various Tem-
peratures—To find the native molecular weight of MjHsp16.5 at various
temperatures, size-exclusion chromatography with online light scatter-
ning, absorbance, and refractive index detectors were used (27-29).
Brielly, size-exclusion chromatography was carried out with a Pharma-
cis BioFPLC system using Bio-Sil SEC 400–5000 (300 × 7.5 mm) column
with a Bio-Sil 400 guard column (80 × 7.8 mm). The columns were
mounted in an Eppendorf CH500 column heater. The column was
connected in line with 1) uv detector Pharmacia LKB UV-M-11, 2) multi-angle laser light scattering detector Down-EOS (Wyatt-

Technology, Santa Barbara CA), 3) refractive index detector, Optilab DSP (Wyatt Technology, Santa Barbara CA). Both the UV detector and
the refractive-index detector have a Peltier heating/cooling system
for controlling and obtaining the desired temperature.

Aggregation Assays—These were assayed as previously described
(26). Bovine insulin and a-lactalbumin were unfolded with 20 mM DTT
at 37 °C. This resulted in the aggregation of the B-chain of insulin, and
a-lactalbumin that was monitored by measuring the apparent absorp-
tions due to light scattering in a spectrophotometer. MjHsp16.5 or aB-crystallin was added to the various target proteins at concentrations
indicated in the figure legends.

Analytical Methods—Protein concentrations of MjHsp16.5 and aB-

crystallin were determined from their absorption spectra using their published extinction coefficients at 280 nm, which are ε280nm = 0.565 for
MjHsp16.5 (30), and 19,000 M−1 cm−1 for aB-crystallin (26). A specific
refractive index increment value of 0.186 ml/g was used (28). The
concentrations of fluorophore were determined from their absorption
spectrum using extinction coefficients of 11,000 m−1 cm−1 at 425 nm for
LYI and 39,000 m−1 cm−1 at 335 nm for AIAS. In addition, to calculate the stoichiometry of fluorescent probe bound to MjHsp16.5, we have
used extinction coefficients at 280 nm for AIAS of 11,000 M−1 cm−1
and 26,000 m−1 cm−1 for LYI.

RESULTS

Effect of Temperature on the Secondary and Tertiary Struc-
ture of MjHsp16.5—There was little difference in the far UV
spectra of MjHsp16.5 taken at 25 °C, 37 °C, or 70 °C (Fig. 1A).
The far UV spectra of MjHsp16.5 had a pronounced minimum at
217 nm, characteristic of β-sheet-containing protein and consistent
with what is known from the crystal structure of MjHsp16.5. The near UV CD spectra of MjHsp16.5 is shown in
Fig. 1B. The vibronic transition at 295 nm arises from the
single tryptophan residue, whereas the bands at 285 and 275
nm reflect contributions from both the tryptophan residue and
the two tyrosine-residue side chains. The fact that the near UV
spectra at 25 °C and 70 °C are very similar indicates that the
environment of aromatic amino acids did not change. Taken
together, the CD data imply that both the secondary and tery-
ary structure did not change in this temperature range.

Effect of Temperature on the Oligomeric Structure of MjHsp16.5—It has recently been shown that increase in tem-
perature can lead to changes in the oligomeric state of several
sHsps (15, 31). Because conventional methods for estimating
molecular weight such as standard gel filtration, and ultrasen-
trifugation cannot be easily performed at temperatures above
50 °C, we used size-exclusion chromatography with online multi-
angle laser light scattering, absorbance, and refractive
index detectors to characterize the oligomeric structure of
MjHsp16.5 at various temperatures. The advantage of this
spectra of MjHsp16.5 at 25°C and 70°C. Each spectrum represents the average of 16 scans. Protein concentration was 0.72 mg/ml. The path length was 0.2 mm.

At 25°C, the average molecular mass was found to be 397 kDa. The average molecular mass at the peak at 25°C was 3.9 mg/ml. Each spectrum represents the average of 32 scans.

**Fig. 1.** Far and near UV CD spectra of MjHsp16.5 taken at 25°C and 70°C. A, far UV CD spectra of MjHsp16.5 at 25°C and 70°C. The protein concentration was 0.72 mg/ml. The path length was 0.2 mm. Each spectrum represents the average of 16 scans. B, near UV CD spectra of MjHsp16.5 at 25°C and 70°C. The protein concentration was 3.0 mg/ml. Each spectrum represents the average of 32 scans.

The system is that the molecular mass determination is independent of the elution volume, the shape of the macromolecule, or the possible interaction between the column matrix and the protein (27–29). Fig. 2 shows the molar mass distribution of MjHsp16.5, which is being eluted from the column at 25°C and 70°C. The average molecular mass at the peak at 25°C is 403 kDa, whereas at 70°C the average molecular mass was found to be 397 kDa. The average molecular mass of three separate experiments yielded a value of 400 kDa. At 70°C, the average molecular mass of three separate experiments was 397 kDa. The predicted molecular weight from the protein sequence is 394,848. It should be noted that with this system, the molecular mass is continuously being determined independent of the column properties.

**Fig. 2.** Molar mass distribution plot and light scattering from the 90° detector for MjHsp16.5 eluted from the gel-filtration column at 25°C and 70°C. Eighty micrograms of the protein were injected into the Bio-Sil SEC 400-5 column, and size-exclusion chromatography was carried out as described under "Materials and Methods." The solid line (—) and the dashed line (—) indicate the trace obtained from the light-scattering detector situated at 90° to the sample. The open circles and triangles are the $M_r$ for each slice calculated directly from the absolute light scattering using the Debye fitting method and ASTRA software (Wyatt Technology, Santa Barbara, CA) For details see Ref. 29. The elution buffer was 50 mM sodium phosphate, pH 7.0 with 100 mM NaCl. Elution was performed at 0.5 ml/min.

In this system, the molecular mass determinations are independent of the column properties. The homology between MjHsp16.5 and the mammalian sHsps exists only in the $\alpha$-crystallin domain, and in that region the homology is only 20%. We wanted to determine whether the subunits of MjHsp16.5 also exchange between oligomers. Therefore, we have labeled one population of MjHsp16.5 with AIAS that will serve as the donor, and another population of MjHsp16.5 with LYI the acceptor. These two probes have been previously characterized and used to monitor exchange of $\alpha$-crystallin subunits. The stoichiometry of labeling was found to be 1 mole of LYI and 0.9 mole of AIAS labeled per subunit of MjHsp16.5. It should be emphasized that the stoichiometry of labeling of the fluorophore to MjHsp16.5 most likely represents a statistical distribution of fluorophore per subunit, because wild-type MjHsp16.5 does not contain a cysteine. It is well documented that iodoacetamides at higher pH will label amines and methionine residues as well as tyrosine (32). To ensure that the fluorescent probe associated with MjHsp16.5 was covalently bound, we denatured fluorescein-labeled MjHsp16.5 in 5% SDS with heating at 45°C for 20 min and ran the sample on a gel filtration column equilibrated in 8 M urea. We observed that 98% of the fluorescence co-eluted with protein-containing fractions (data not shown). Because the subunit exchange takes place at temperatures above 50°C but the actual fluorescence measurements are taken at room temperature where the subunit exchange is arrested, it is important to determine that at the higher temperatures the fluorescent probes are not chemically modified.

Fig. 3, A and B show fluorescence emission spectra of AIAS- and LYI-labeled MjHsp16.5 that has been pre-heated at temperatures and times indicated in the figure legend. AIAS-labeled MjHsp16.5 excited at 335 nm had a fluorescence emission maximum at 412 nm whereas LYI-labeled MjHsp16.5 excited at 425 nm had an emission maximum at 515 nm. Pre-heating the AIAS-labeled Hsp16.5 at 72°C for up to 60 min did not change the fluorescence spectra (Fig. 3A). Pre-heating LYI-labeled MjHsp16.5 at higher temperatures led to a small but reproducible increase in fluorescence emission (Fig. 3B). These
experiments indicate that heat alone will not result in marked changes in the fluorescence signal of either fluorophore when attached to MjHsp16.5. In addition, after heating LY-labeled MjHsp16.5 at 68 °C for 90 min, the sample was electrophoresed on a 12.5% acrylamide denaturing gel. This experiment showed that the label migrated with the protein. Thus, heating did not break the covalent linkage of fluorophore to MjHsp16.5 to any major extent. Similar results were observed with heat-treated AIAS-labeled MjHsp16.5 (data not shown.)

**Subunit Exchange of MjHsp16.5**—The subunit exchange reaction was initiated by mixing an equimolar concentration of the donor, AIAS-labeled Hsp16.5, with the acceptor, LYI-labeled Hsp16.5. If MjHsp16.5 has a static structure at 68 °C, then we will not observe a change in the fluorescence emission spectrum over time. At 10 min into the reaction, we observed a significant amount of quenching of donor fluorescence with a concomitant increase in acceptor fluorescence, consistent with an exchange of subunits between oligomers (Fig. 4A). The quenching of donor fluorescence continued until the 90 min time point, after which we did not observe a change in fluorescence emission spectra. The donor fluorescence intensity at 412 nm is plotted versus time in Fig. 4A. Using the BMDP statistical program, the quenching of donor fluorescence was fit to the single exponential function $F(t) = C1 + C2e^{-kt}$, where the rate constant $k$ for subunit exchange was determined to be 0.067 min$^{-1}$ (Fig. 4B).

To unequivocally determine that the quenching of donor fluorescence and concomitant rise in acceptor fluorescence is caused only by an exchange of subunits between oligomers, which brings the donor the acceptor fluorophores close to each other, we performed an additional control. We mixed unreacted free AIAS with LYI-labeled MjHsp16.5 at equimolar concentrations at 68 °C. At various time points, an aliquot was taken from the reaction tube, and the fluorescence emission spectrum was recorded. We did not observe changes in the spectra over a 60-min time period (data not shown). This result indicates that the energy transfer that we are observing in the subunit exchange reaction is not caused by minute amounts of free donor or acceptor fluorophores that may be moving between oligomers.

**Reversibility of Subunit Exchange**—To determine whether the subunit exchange reaction is reversible, we mixed 30 μM concentrations of AIAS-labeled MjHsp16.5 and LYI-labeled MjHsp16.5 at 68 °C for 90 min. At this point, the subunits are completely mixed and scrambled. Subsequently, a 10-fold molar excess of unlabeled MjHsp16.5 was added to fluorescently labeled MjHsp16.5 at 68 °C. At various time points, an aliquot was taken from the reaction tube, and the fluorescence emission spectrum was recorded. As expected, at time 0 we observe the maximal amount of quenching of donor fluorescence and concomitant maximal amount of acceptor fluorescence. With time, as the unlabeled MjHsp16.5 subunits are integrating into the fluorescently-labeled MjHsp16.5 oligomers, the distance between the donor and acceptor labeled subunits increase. This leads to an increase in donor fluorescence with a loss of acceptor fluo-
rescence. The data obtained is on concordance with Fig. 4A (data not shown). This result proves that the subunit exchange reaction of MJHsp16.5 is reversible.

In order to determine whether the reversibility of subunit exchange is specific to MJHsp16.5 subunits, we have added tear lipocalin at a 5-fold molar excess to fluorescent-mixed MJHsp16.5 subunits at 68 °C. Tear lipocalin is structurally unrelated to MJHsp16.5, and is thermally stable at 68 °C, and should not integrate into the fluorescent-mixed MJHsp16.5 oligomer. We found that the addition of tear lipocalin did not markedly change the emission spectrum of the fluorescent-mixed MJHsp16.5 oligomer. We determined that the tear lipocalin did not markedly change the emission spectrum of the fluorescent-mixed MJHsp16.5 over a 90 min time frame (data not shown) indicating that the reversibility of the reaction is specific to MJHsp16.5.

Effect of Temperature on Subunit Exchange of MJHsp16.5—To determine the effect of temperature on the subunit-exchange reaction of MJHsp16.5, we performed the exchange reaction over a wide range of temperatures (Fig. 5). At 50 °C, there was no change in donor fluorescence over a 4-h time period, suggesting that Hsp16.5 has a static structure at this temperature with no exchange of subunits. At 65 °C, the reaction was complete in 5 h, with \( k = 0.025 \text{ min}^{-1} \) whereas at 68 °C, the reaction was complete in 90 min with a half-life of 10.9 min. Increasing the temperature to 72 °C dramatically increased the rate of the reaction. At 72 °C, the rate constant of subunit exchange was 0.36 \text{ min}^{-1} with the reaction having a half-life of 1.9 min. We plotted \( \ln(k) \) versus the reciprocal of temperature in the form of an Arrhenius activation energy plot. From the slope of the line, we determined the activation energy for the subunit exchange reaction of MJHsp16.5 subunits to be 84 kcal/mole (data not shown).

Effect of MJHsp16.5 Concentration on Subunit Exchange—In a separate set of experiments, we examined the effects of concentration on the rate of subunit exchange by measuring the decrease in AIAE emission intensity at 412 nm 6 min after mixing at 68 °C different concentrations of AIAE-labeled and LYL-labeled MJHsp16.5 subunits. The concentrations used were 5, 30, and 300 \( \mu \text{M} \) MJHsp16.5. The rate of subunit exchange at 30 \( \mu \text{M} \) and 300 \( \mu \text{M} \) were similar. At the lowest concentrations measured (5 \( \mu \text{M} \)), the rate of subunit exchange was about 20% lower than that at 30 \( \mu \text{M} \) (data not shown).

Determination of the Structural Compatibility of MJHsp16.5

duce its disulfide bonds and will lead to its subsequent aggregation at 37 °C (Fig. 8, curve 1). At a 1:1 (wt/wt) ratio of αB-crystallin/α-lactalbumin, αB-crystallin completely prevented the DTT-induced aggregation of α-lactalbumin (Fig. 8, curve 2). In contrast, a 1:1 (wt/wt) ratio of Hsp16.5/α-lactalbumin did not prevent the DTT-induced aggregation of α-lactalbumin (Fig. 8, curve 3). Increasing the concentrations of MjHsp16.5 to 10:1 and 20:1 (wt/wt) ratios of MjHsp16.5/α-lactalbumin partially prevented the DTT-induced aggregation of α-lactalbumin (Fig. 8, curves 4 and 5). We also investigated the chaperone function of MjHsp16.5 using other substrates such as alcohol dehydrogenase at 42 °C and 48 °C (data not shown). With all substrates tested the results were the same: MjHsp16.5 was markedly less effective as a chaperone compared with αB-crystallin.

**DISCUSSION**

A common feature of many sHsps is their dissociation into smaller oligomers due to increased temperatures, phosphorylation, or decreases in concentration (15, 31, 35–40). Present evidence suggests that different oligomerization states of sHsps have different functions. For example, it has been shown that phosphorylation-induced changes in the oligomeric structure of Hsp27 enhance its actin-polymerizing activity, which has a stabilizing effect on microfilaments during stress (40). Furthermore, at physiological temperatures, Hsp26 from *Saccharomyces cerevisiae* exists as a high molecular weight oligomeric protein consisting of 24 subunits (31). However, under heat-shock conditions, the high molecular weight complex dissociates into smaller oligomers, which are the active units in binding unfolding proteins (31). We found that at temperatures up to 70 °C, MjHsp16.5 still retains its native oligomeric structure, as well as its secondary and tertiary structure, and does not dissociate into smaller oligomers. This is also the case with the α-crystallins, which do not dissociate into smaller multimers at higher temperatures (7). However, it is possible that under post-translational modifications such as phosphorylation, or at temperatures higher than 70 °C, MjHsp16.5 may dissociate into smaller oligomers.

MjHsp16.5 has been shown to exist as discrete 24-subunit complex (12). Recently, however, it was suggested that the solution structure of MjHsp16.5 might have more variability than the crystal structure would indicate (6). We have shown that MjHsp16.5 has a dynamic quaternary structure as its subunits freely and reversibly exchange with each other at temperatures relevant for *M. jannaschii*. There are at least two possible mechanisms that account for subunit exchange of MjHsp16.5 subunits. First, subunit exchange could be a collision-dependent process, under which the rate of subunit exchange would increase with concentration. Alternatively, the rate-determining step of subunit exchange could involve the dissociation of smaller oligomers from the larger oligomeric complex. The fact that the rate of subunit exchange did not highly depend on the concentration suggests that subunit exchange of MjHsp16.5 involves a rate-determining dissociation step. Current evidence supports the hypothesis that a dissociation mechanism may be involved for the subunit exchange of many members of the sHsp family. For example, it has previously been shown that the subunits of αA-crystallin exchange through a dissociation mechanism (9) and recently it has been shown that the subunits of Hsp16.9 dissociate into smaller oligomeric forms in equilibrium with the larger oligomeric assembly at higher temperatures (15).

We have previously shown that the structures of human Hsp27 and rat αA-crystallin are compatible, as their subunits can freely and reversibly exchange with each other (9). The results of the present work indicate that the subunits of MjHsp16.5 do not contain the necessary structural determinants to interact with α-crystallin. Although the core structure of the α-crystallin domain in αA and MjHsp16.5 are conserved, structural differences within this core do occur. For example, strand β6 and possibly β1 in MjHsp16.5 do not exist in αA-crystallin (14). These structural differences are thought to have important consequences in terms of oligomeric assembly of these sHsp (14), and may play a role in their subunit-exchange properties. Alternatively, the α-crystallin domain may not be important for subunit exchange of sHsp at all. In support of this hypothesis, it has been reported that the critical structural determinants for exchange of Hsp27 and αA-crystallin subunits, which confer upon these two sHsps their dynamic properties, exist in their N-terminal regions (9, 16).

In contrast to the well-characterized large heat shock protein like GroEL whose subunits do not exchange (11), we have shown that subunit exchange is a unifying feature of the sHsp family. There is evidence to suggest that subunit exchange may be important for the ability of sHsps to suppress the nonspecific aggregation of proteins. For example, it has been shown that...
the chaperone-like function of α-crystallin markedly increases with temperature (11, 41, 42). This correlates well with the increase in subunit exchange rates of α-crystallin that also occur with increasing temperatures (7). Interestingly, residues 75–80 and 135–139 or α-crystallin are buried due to subunit-subunit interactions yet have been identified as important for interacting with model substrates (19, 43). The homologous regions in MjHsp16.5 are also buried from subunit-subunit contacts (13). In order to resolve these findings, it has been suggested that the high molecular weight oligomeric form of sHsp serves as a reservoir for the smaller, functional binding units that can be activated by disassembly during times of stress (4, 13, 21, 31). The transient exposure of buried sites that occur in subunit exchange of sHsps is consistent with this mechanism (9). The high activation energy of the subunit exchange reaction could operate as a temperature-controlled switch for chaperone-like function (7, 9). In support of this hypothesis, Hsp26 reversibly dissociates into dimers at increased temperatures that can then bind substrates (31).

The recent elucidation of the crystal structure of wheat Hsp16.9 supports the idea that dimers are the exchangeable units in this dodecameric assembly. Moreover, it was shown that the Hsp16.9 dodecamer dissociates at elevated temperatures, and that the smaller species can efficiently bind model substrates (15). Previous studies by Arrigo et al. (44) have shown that the mammalian Hsp27 undergoes dynamic changes in its structure and intracellular localization after subjecting the cell to a heat shock. It was shown that this protein is accumulated in the nucleus (44). The native molecular weight of Hsp27 is 200,000 to 800,000; the simplest explanation for finding it in the nucleus is the ability of Hsp27 to undergo subunit exchange. Thus, monomers, dimers, or tetramers can then enter the nucleus. Similarly, α-β-crystallin is known to migrate to the nucleus under both stress and non-stress conditions (45). Again, the simplest explanation for the nuclear localization is subunit exchange. In addition to their chaperone-like properties, recent work implicates small Hsps as regulators of cell division and growth as well as regulators of intracellular redox state and programmed cell death (33, 45, 46). More studies are needed to ascertain the role of subunit exchange for the regulatory functions.

Is subunit exchange important for the chaperone-like function of MjHsp16.5? We found that a 20:1 (w/w) ratio of Hsp16.5 to insulin was necessary to completely suppress the aggregation of single-chain monellin at stoichiometric ratios (12). In addition, at temperatures of 70 °C and greater, MjHsp16.5 has been shown to effectively prevent the aggregation of E. coli cell lysate (12). These results suggest that MjHsp16.5 can function as an effective chaperone with selected substrates and that a dynamic structure characterized by an exchange of subunits, may be important for its chaperone-like function. Although the role that subunit exchange plays in chaperone-like function of MjHsp16.5 is not clear, the data in this paper illustrate the importance of assaying for the function of MjHsp16.5 at temperatures that are physiologically relevant to M. jannaschii.

The data presented in this paper support the hypothesis that MjHsp16.5 has a dynamic quaternary structure at higher temperatures. Although there are more differences than similarities between MjHsp16.5 and the mammalian subfamily of sHsps, one common theme that unites these distantly related sHsps is that their subunits exchange at temperatures that are physiologically relevant to the organisms from which they arise.

Acknowledgments—We would like to thank Dr. Octai Gassymov and Adil Abduragimov for their assistance and helpful discussions. We thank Ann Chang for her assistance with the BMDP program.

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Subunit Exchange, Conformational Stability, and Chaperone-like Function of the Small Heat Shock Protein 16.5 from *Methanococcus jannaschii*

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doi: 10.1074/jbc.M205594200 originally published online August 9, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M205594200

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