2004

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Interactions between Small Heat Shock Protein Subunits and Substrate in Small Heat Shock Protein-Substrate Complexes*

Received for publication, October 8, 2003, and in revised form, October 21, 2003
Published, JBC Papers in Press, October 22, 2003, DOI 10.1074/jbc.M311104200

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Small heat shock proteins (sHSPs) are dynamic oligomeric proteins that bind unfolding proteins and protect them from irreversible aggregation. This binding results in the formation of sHSP-substrate complexes from which substrate can later be refolded. Interactions between sHSP and substrate in sHSP-substrate complexes and the mechanism by which substrate is transferred to ATP-dependent chaperones for refolding are poorly defined. We have established C-terminal affinity-tagged sHSPs from a eukaryote (pea HSP18.1) and a prokaryote (Synechocystis HSP16.6) as tools to investigate these issues. We demonstrate that sHSP subunit exchange for HSP18.1 and HSP16.6 is temperature-dependent and rapid at the optimal growth temperature for the organism of origin. Increasing the ratio of sHSP to substrate during substrate denaturation decreased sHSP-substrate complex size, and accordingly, addition of substrate to pre-formed sHSP-substrate complexes increased complex size. However, the size of pre-formed sHSP-substrate complexes could not be reduced by addition of more sHSP, and substrate could not be observed to transfer to added sHSP, although added sHSP subunits continued to exchange with subunits in sHSP-substrate complexes. Thus, although some number of sHSP subunits within complexes remain dynamic and may be important for complex structure/solubility, association of substrate with the sHSP does not appear to be similarly dynamic. These observations are consistent with a model in which ATP-dependent chaperones associate directly with sHSP-bound substrate to initiate refolding.

Small heat shock proteins (sHSPs) are ubiquitous stress proteins of 12–42 kDa that share a conserved C-terminal domain of ~100 amino acids (the α-crystallin domain) (1). The conserved α-crystallin domain is flanked by a variable length non-conserved N-terminal arm and a short C-terminal extension. In vivo and in vitro sHSP monomers assemble into native oligomers of 9 to >30 subunits, depending on the specific sHSP (2). sHSPs and the related vertebrate α-crystallins are proposed to function as molecular chaperones by preventing irreversible aggregation and insolubilization of denatured proteins (3–11). Many experiments have shown that sHSPs and α-crystallins have a large binding capacity for a variety of heat and chemically denatured model protein substrates (8, 10, 12–15). The models for sHSP function have been extended to suggest that substrates in sHSP-substrate complexes (hereafter referred to as “complexes”) are held in a folding competent state, such that substrates can be reactivated by the ATP-dependent chaperone HSP70 (DnaK) and its respective co-chaperones (6, 9, 13, 14, 16).

The x-ray structures of HSP16.5 from Methanococcus jannaschii, a 24-subunit oligomer (17), and HSP16.9 from Triticum aestivum (wheat), a 12-subunit oligomer (17, 18), reveal common features of sHSP structure. Despite limited sequence identity, these structures have a 1.5-Å root mean square difference between C-α atoms in the conserved α-crystallin domain, which consists of a β-sandwich of two anti-parallel β-sheets (18). In both oligomers the most extensive interface is between the subunits of a dimer, and the oligomers are stabilized through an interaction of hydrophobic residues in the C-terminal extension of each subunit and a hydrophobic groove on the α-crystallin domain of a subunit in another dimer (17, 18). In HSP16.9 the resolution of the N-terminal arms from six subunits shows that the wheat oligomer is also stabilized by extensive N-terminal arm interactions (18).

Despite the extensive subunit contacts in sHSP oligomers and the predominance of the oligomeric state at optimal temperature, sHSP subunit interactions are highly dynamic. Rapid subunit exchange between sHSP oligomers has been observed for a number of sHSPs (11, 19–22). Fluorescence energy transfer experiments conducted with the polydisperse mammalian αA- and αB-crystallin and HSP27 revealed fully exchanged oligomers within a few hours at 37 °C (19, 21). Even the well ordered wheat and Methanococcus sHSP oligomers exhibit rapid subunit dissociation and reassociation (11, 22). The dynamic nature of sHSP subunit interactions is further seen in the reversible dissociation of the wheat HSP16.9, yeast HSP26, Synechocystis HSP16.6, and M. tuberculosis HSP16.3 oligomers into a smaller subassembly upon heating (8, 18, 23, 24). Heating also induces structural changes that result in the exposure of hydrophobic sites, believed to be substrate-binding sites (10, 14, 25, 26). Increased rates of subunit exchange and/or oligomer dissociation upon heating have also been proposed to expose hydrophobic substrate-interacting sites, suggesting that the suboligomeric species, most likely the dimer, binds substrate (8, 18, 23, 24).

Although substrate is thought to bind to a suboligomeric species, when sHSPs are heated with substrate proteins large complexes of sHSPs and substrate form (6, 8–10, 12–16), rang-
of sHSPs were then used to examine the dynamic organization of complexes (6, 29, 30). However, we and our colleagues failed to observe spontaneous release of malate dehydrogenase (MDH) bound to HSP16.6 (from Synechocystis sp. 6803) in large complexes (>3000 kDa), as measured by substrate transfer to a GroEL trap mutant or to added Synechocystis HSP16.6 or pea HSP18.1 (27). A second model is that a direct interaction between complexes and the refolding machinery is necessary. Consistent with this idea, complexes of sHSPs with HSP70 and HSP100 proteins have been observed, although their functional relevance has not been demonstrated (16, 31). Depending on the specific sHSP and substrate, as well as complex organization, both mechanisms of substrate transfer could operate. However, the fact that substrate refolding can be accomplished by HSP70 family members not related to the sHSP used (9) suggests that HSP70 chaperones do not specifically interact with sHSPs in complexes but rather with the bound substrate.

We have produced C-terminally, affinity-tagged sHSPs from both an eukaryote (pea HSP18.1) and a prokaryote (Synechocystis HSP16.6) as tools to address questions regarding sHSP function and the interactions between the sHSP and substrate in complexes. We first show that the C-terminal tag does not interfere with sHSP oligomerization or chaperone activity in vitro or with sHSP function in vivo. Both wild-type and tagged sHSPs were then used to examine the dynamic organization of sHSP oligomers in the presence and absence of substrate, the consequent effects on substrate refolding, and the transfer of sHSP-bound substrate to added sHSP. We show that subunit exchange in solution is rapid and temperature-dependent. Bound substrate had no observed effect on the time course of HSP18.1 subunit exchange nor did subunit exchange reduce sHSP-bound substrate to added sHSP. We show that subunit exchange can be accomplished by HSP70 family members not related to the sHSP used (9). However, the fact that substrate refolding can be accomplished by HSP70 with the appropriate amount of sHSP, in the same buffer, to bring the final concentrations to 12 μM sHSP and 1 μM Luc. Samples were then heated as for initial complex formation. All the above experiments were performed in sili-

**EXPERIMENTAL PROCEDURES**

**Construction of Strep-tagged HSP18.1 and Strep-tagged HSP16.6—**Addition of the 8-amino acid strep-tag II affinity tag (WSHPQFEK) (Clontech, Palo Alto, CA) to the C terminus of *Pisum sativum* (pea) HSP18.1 was achieved by PCR using primers including the codons for these amino acids (5'-GAAAAATATCGGAGTGTTCTCCAAACGAATCTCATTAA-3' and 5'-GAAGTGGGCGGGGCCTCTGGGTGCTGACCAAGACTTAA-3') to amplify the entire pJC20 hsp18.1 plasmid (AZ516) (32) followed by blunt end ligation. Plasmids with the correct sequence were con-

**Protein Purification—**sHSPs were expressed in BL21 Escherichia coli cells and purified to >95% homogeneity by conventional methods as described previously involving ammonium sulfate precipitation, su-

**Luciferase Reactivation Measurements—**sHSP-Luc complexes were created as described above. Luciferase reactivation experiments were performed as in Lee and Vierling (9). After centrifugation samples were diluted to 480 nm sHSP in 10% (v/v) rabbit reticulocyte lysate (Green Biologic, Madison, WI) at 2.5 μM sHSP, 2 mM ATP, pH 7.5, in siliconized tubes. Refolding was initiated by diluting the samples to 11.5 μM, in the same buffer conditions, in a mixture which was 50% (v/v) rabbit reticulocyte lysate and 2 mM ATP (40 μl total volume). Reconstituted luciferase protein was then heated at 31 °C. Luciferase activity was determined by adding 2.5 μl of the refolding reaction to 50 μl of luciferase assay mix (Promega, Madison, WI) and recorded after emission in a TD-20/20 luminometer (Turner, Sunnyvale, CA). Luciferase activities are expressed as percentages relative to that of an equivalent amount of native Luc measured prior to the heating step in the formation of complexes.

**Synechocystis Strains, Growth Conditions, Heat Shock Assays, and Protein Precipitation—**sHSP, hsp18.1, hsp16.6, or ΔHSP16.6 strains were created by transforming a ΔCLpB1/HK-1, ΔHSP16.6 strain with pNaive (23) vectors carrying the appropriate hsp16.6 alleles, as described by Giese and Vierling (23). Cells were grown and stressed as described previously (23). Briefly, cells were grown on Bg-11/glucose (Clontech, Palo Alto, CA) to the C terminus of *Pisum sativum* (pea) HSP18.1 was achieved by PCR using primers including the codons for these amino acids (5'-GAAAAATATCGGAGTGTTCTCCAAACGAATCTCATTAA-3' and 5'-GAAGTGGGCGGGGCCTCTGGGTGCTGACCAAGACTTAA-3') to amplify the entire pJC20 hsp18.1 plasmid (AZ516) (32) followed by blunt end ligation. Plasmids with the correct sequence were confirmed by DNA sequencing. Strep-tagged HSP16.6, from *Synechocystis* sp. FCC 6803, was constructed in a similar manner, using the pJC20 hsp16.6 plasmid (AZ353) and appropriate primers (5'-GAAAAATAGGGCCAATCTGCTAATCA-3' and 5'-GAAGTGGGCGGGGCCTCTGGGTGCTGACCAAGACTTAA-3').

**Protein Purification—**sHSPs were expressed in BL21 Escherichia coli cells and purified to >95% homogeneity by conventional methods as described previously involving ammonium sulfate precipitation, sucrose gradient centrifugation, and DEAF chromatography (15, 23, 32). A NaCl gradient of 0–350 mM was needed to elute the HSP16.6 proteins from the DEAE resin. An additional step was added to purification of the HSP18.1 proteins. The protein-containing fractions from the DEAE column were loaded on a hydroxypatite column, which was developed with a gradient of 10–400 mM NaPO4, pH 7.5. HSP18.1 and strep-tagged HSP18.1 eluted at ~380 mM NaPO4. Proteins were stored in 25 mM NaPO4, pH 7.5. Protein concentrations were determined from absorbance at 280 nm using the calculated molar extinction coefficients according to Pace et al. (33): HSP18.1, 16,500 M⁻¹ cm⁻¹; strep-tagged HSP18.1, 22,000 M⁻¹ cm⁻¹; HSP16.6, 5960 M⁻¹ cm⁻¹; and strep-tagged HSP16.6, 11,460 M⁻¹ cm⁻¹. Concentrations are expressed as moles of monomer.

**Formation of sHSP-Substrate Complexes—**sHSP-Luc complexes were formed by mixing the indicated sHSPs with firefly luciferase (Luc) (Fishier) at the concentrations specified in each experiment and heating for 85 min at 42 °C. sHSP-MDH complexes were formed by mixing the indicated sHSPs with mitochondrial MDH (Roche Applied Sci-

**Synechocystis Strains, Growth Conditions, Heat Shock Assays, and Protein Precipitation—**sHSP, HSP18.1, or ΔHSP16.6 strains were created by transforming a ΔCLpB1/HK-1, ΔHSP16.6 strain with pNaive (23) vectors carrying the appropriate hsp16.6 alleles, as described by Giese and Vierling (23). Cells were grown and stressed as described previously (23). Briefly, cells were grown on Bg-11/glucose (Clontech, Palo Alto, CA) to the C terminus of *Pisum sativum* (pea) HSP18.1 was achieved by PCR using primers including the codons for these amino acids (5'-GAAAAATATCGGAGTGTTCTCCAAACGAATCTCATTAA-3' and 5'-GAAGTGGGCGGGGCCTCTGGGTGCTGACCAAGACTTAA-3') to amplify the entire pJC20 hsp18.1 plasmid (AZ516) (32) followed by blunt end ligation. Plasmids with the correct sequence were confirmed by DNA sequencing. Strep-tagged HSP16.6, from *Synechocystis* sp. FCC 6803, was constructed in a similar manner, using the pJC20 hsp16.6 plasmid (AZ353) and appropriate primers (5'-GAAAAATAGGGCCAATCTGCTAATCA-3' and 5'-GAAGTGGGCGGGGCCTCTGGGTGCTGACCAAGACTTAA-3').

**Protein Purification—**sHSPs were expressed in BL21 Escherichia coli cells and purified to >95% homogeneity by conventional methods as described previously involving ammonium sulfate precipitation, sucrose gradient centrifugation, and DEAF chromatography (15, 23, 32). A NaCl gradient of 0–350 mM was needed to elute the HSP16.6 proteins from the DEAE resin. An additional step was added to purification of the HSP18.1 proteins. The protein-containing fractions from the DEAE column were loaded on a hydroxypatite column, which was developed with a gradient of 10–400 mM NaPO4, pH 7.5. HSP18.1 and strep-tagged HSP18.1 eluted at ~380 mM NaPO4. Proteins were stored in 25
Results

Strep Affinity-tagged sHSPs Are Active Chaperones in Vitro—A strep affinity tag (WSHPQFEK) was added to the C termini of the pea HSP18.1 and Synechocystis HSP16.6, in order to establish tools to examine the mechanism of sHSP function. We initially determined whether the strep tag affected monomeric size, oligomeric size and structure, substrate interactions, and chaperone activity of recombinant protein. Characterization of strep-tagged HSP18.1 or HSP16.6 by non-denaturing and SDS-PAGE revealed that the strep tag increases the size of the monomer as expected but does not interfere with formation of the native oligomers (Fig. S1A and not shown). The structure of HSP16.9 from wheat, a homologue of the dodecameric HSP18.1, shows that the C-terminal extensions of all 12 subunits are surface-exposed and point away from the body of the oligomer (18). Thus the added strep-tagged residues most likely extend away from the body of the oligomer, explaining the negligible effects of the strep tag on oligomeric structure.

Strep-tagged HSP18.1 and HSP16.6 were found to be efficiently retained on the streptactin affinity resin, with the majority recovered in the eluted fraction (Fig. S1B and not shown). In contrast, wild-type protein did not interact with the streptactin affinity resin, except for a small amount of nonspecific binding of wild-type HSP18.1 (Fig. S1B). Additional pre-blocking of the column with BSA or 1% dried milk did not eliminate this residual nonspecific binding of wild-type HSP18.1.

The strep-tagged sHSPs were tested for ability to protect model sHSP substrates by forming complexes, as shown previously for wild-type HSP18.1 (14). Wild-type HSP18.1, strep-tagged HSP18.1, or 1:1 and 2:1 (wt/strep) sHSP mixtures were incubated at 12 μM sHSP monomer (1 μM oligomer) with 1 μM Luc. Samples were maintained at room temperature (22 °C) or heated at 42 °C for 8.5 min to form complexes and then analyzed by SEC (Fig. 1A and B). Unheated, strep-tagged HSP18.1 was observed to form oligomers that are slightly larger than wild-type HSP18.1. Mixtures of the two proteins at ratios of 1:1 and 2:1 (wt/strep) eluted as single peaks with elution times inbetween wild-type and strep-tagged proteins.
This suggested that the wild-type and tagged sHSPs formed mixed dodecameric oligomers.

At room temperature there is no evidence that native Luc interacts significantly with either wild-type or strep-tagged HSP18.1 oligomers, as the Luc peak is constant and separated from the sHSP peak (Fig. 1A). In the heated samples the expected sHSP-Luc complex peak is observed in all samples, along with a loss of free sHSP and Luc (Fig. 1B). Heating the sHSP alone does not change its elution behavior, and heating of Luc alone results in insolubilization of the denatured Luc such that it does not enter the column (data not shown). HSP18.1-Luc complexes formed with increasing amounts of the strep-tagged protein elute at increasingly earlier times, demonstrating an increase in complex size (Fig. 1B). This size difference may be due in part to the increased size of the strep-tagged sHSP monomers or an alteration in the stoichiometry or structure of the complexes. A similar increase in complex size is observed if the ratio of Luc to sHSP is increased (see Fig. 8), as has also been observed for complexes formed with other substrates (14). Thus, whereas strep-tagged HSP18.1 is effective at binding Luc, the complexes formed are slightly altered in some manner.

HSP16.6, which forms polydisperse oligomers with 12–26 subunits (even integers only with ≤ 50% of oligomers containing 20 or 22 subunits), was compared with strep-tagged HSP16.6 in an identical set of experiments. To fully protect Luc, a ratio of 24 μM HSP16.6 to 1 μM Luc was determined to be optimal (data not shown). Results similar to those for HSP18.1 were observed for HSP16.6 (Fig. 1, C and D). In contrast to the results with HSP18.1, unheated samples of HSP16.6, strep-tagged HSP16.6, and hybrid oligomers eluted at identical times as did complexes formed between Luc and any mixture of these two versions of HSP16.6. The strep tag does not appear to increase the size of strep-tagged HSP16.6 oligomers, but an increase may be masked by the larger size and greater heterogeneity of the oligomers.

To confirm that the sHSP-bound Luc could be refolded, regardless of the subunit composition in an oligomer, complexes identical to those examined above were added to a reticulocyte refolding mixture. Approximately 80% of the starting Luc activity was recovered from all the types of HSP18.1-Luc complexes at the same rate (Fig. S2). Thus the presence of strep-tagged HSP18.1 in the complexes does not affect the amount of Luc refolded in the reaction. 12 μM strep-tagged and wild-type HSP18.1 were able to protect completely up to 3 μM Luc. However, the maximum amount of Luc refolding (80%) was seen only at ratios of 12 to 1 μM (sHSP/Luc) or higher (data not shown). Reactivation of Luc from HSP16.6-Luc complexes, in similar experiments, reaches levels of about 70% after 2 h of refolding (23). However, 24 μM HSP16.6 was not able to fully protect levels greater than 1 μM Luc. Strep-tagged HSP16.6 was found to behave almost identically in similar experiments (data not shown). Taken together these results show that in vitro, strep-tagged HSP18.1 and HSP16.6 are active sHSP chaperones that behave almost identically to their wild-type counterparts.

Strep Affinity-tagged HSP16.6 Is Functional in Vivo—Because HSP16.6 is required for heat tolerance in *Synechocystis*, we were able to compare HSP16.6 and strep-tagged HSP16.6 in *vivo* using a stress-tolerance assay developed by Giese and Vierling (23). Strep-tagged HSP16.6 was expressed in *Synechocystis* cells and shown to accumulate to wild-type levels at 42 °C (Fig. 2A). At 30 °C, cells expressing wild-type HSP16.6 or strep-tagged HSP16.6, as well as ΔHSP16.6 cells, grow identically (Fig. 2B). However, treatment for 8 h at 44 °C resulted in HSP16.6 and strep-tagged HSP16.6 surviving to 32 and 18%, respectively, whereas ΔHSP16.6 survived to less than 0.01% (Fig. 2C). Thus strep-tagged HSP16.6 is able to replace HSP16.6 *in vivo*. The *in vitro* and *in vivo* data together demonstrate that strep-tagged HSP16.6 is essentially functionally equivalent to HSP16.6. Thus, the strep-tagged proteins are a valid model for further studies of sHSP function.

**Time Course of Subunit Exchange**—We first used strep-tagged HSP18.1 to investigate the time course of HSP18.1 subunit exchange in solution at room temperature (22 °C). Wild-type HSP18.1 and strep-tagged HSP18.1 were mixed at either a 1:1 or 2:1 ratio (wt/strep), at a total concentration of 12 μM sHSP, and incubated at room temperature for 0–60 min prior to strep-affinity chromatography. The amount of wild type that was retained on the resin with strep-tagged HSP18.1 was determined. At both ratios, some amount of wild-type protein is retained on the resin and is recovered in the elution fraction of the 0-min sample. Because the addition of resin effectively stops subunit exchange (data not shown), this binding was likely due to a small amount of mixing that occurs before the addition of resin to the mixture, as well as a small amount of nonspecific binding. After 15 min virtually all of the wild-type protein is in the elution of the 1:1 mixture, and the majority is in the elution of the 2:1 mixture (Fig. 3). Failure to retain all of the wild-type subunits on the column in the 2:1 mixture probably reflects a larger number of oligomers with fewer strep-tagged subunits. In total these results show that subunit exchange is rapid and approaches equilibrium by 15 min, again indicating the affinity tag does not compromise sHSP structure and function. Similar experiments with HSP16.6 at 22 °C indicated that subunit exchange was much slower and was not complete after 3 h. However, when incubated at 30 °C (optimal growth temperature for *Synechocystis*) the process was effectively complete at 1 h (data not shown). No subunit exchange for either protein was seen when incubated at 0 °C for 30 min or 5 h for HSP18.1 and HSP16.6, respectively (Fig. 3 and data not shown).

It is possible that equilibrium has not yet been reached in these experiments and that every wild-type oligomer contains only one strep-tagged subunit, which allows for its retention on
the resin. However, similar experiments performed at higher ratios of wild-type to strep-tagged sHSP, such as 11:1 and 10:2, showed very little retention of the wild-type protein on the resin (data not shown). This suggests that to be retained on the resin, oligomers must contain multiple strep-tagged subunits. Therefore, one may deduce that in the lower ratio experiments (wt/strep) a high degree of mixing is occurring over the course of 15 min, in order to retain most of the wild-type sHSP on the resin.

Rapid Subunit Exchange Continues in the Presence of Bound Luc and Does Not Affect the Folding Competence of Luc—To determine whether sHSP subunit exchange could occur when Luc was associated with the sHSP in a folding-competent state, sHSP-Luc complexes were formed with either wild-type HSP18.1 or strep-tagged HSP18.1 (12 μM sHSP, 1 μM Luc). These experiments were performed with HSP18.1 because it has a higher capacity for Luc and forms more distinct complexes than HSP16.6 (compare Fig. 1, B and D). The sHSP-Luc complexes were incubated with an equal amount of free wild-type or strep-tagged HSP18.1 for 1 h, to ensure adequate time for exchange, and then subjected to strep-affinity chromatography. When strep-tagged sHSP was present in mixtures with wild-type sHSP, the wild-type sHSP and Luc were recovered in the bound fraction regardless of whether Luc was initially bound to wild-type or strep-tagged sHSP (Fig. 4A, lanes 7–12). The control mixtures (Fig. 4A, lanes 1–6) behaved as expected. The Luc observed in the flow-through lanes, when strep-tagged HSP18.1 is present, is presumably Luc that did not stably bind to the sHSP initially (see Fig. 1B). The presence of Luc in lane 12 shows that strep-tagged subunits are exchanging with wild-type subunits from the wild-type HSP18.1-Luc complexes and not just with wild-type subunits from the free sHSP pool. Using MDH as the substrate instead of Luc or increasing the amount of substrate in complexes, up to 12:3 (sHSP/Luc), yielded the same results and did not visibly affect the time course of subunit exchange (data not shown). Thus, sHSP subunits in complexes continue to exchange, and substrate has no observable effect on the time course of exchange compared with free sHSP.

To confirm that wild-type subunits were exchanging with strep-tagged subunits from the strep-tagged HSP18.1-Luc complexes, and not just with strep-tagged subunits from the free sHSP pool, identical samples were separated by size exclusion chromatography following the exchange reaction. After exchange, complexes eluted at a similar position to complexes before exchange. Electrophoresis and Western blotting revealed that both types of sHSP subunits were now present in every fraction from the complex peak as well as the free sHSP peak (Fig. 4B). Similar results were observed when wild-type HSP18.1-Luc complexes were incubated with free strep-tagged HSP18.1 (data not shown).

The folding competence of substrate from complexes that had been incubated with free sHSP to allow subunit exchange was tested by adding complexes to the reticulocyte refolding system. As observed in Fig. 5, ~80–85% of the Luc activity was recovered, and refolding rates were similar no matter which complexes were added to the refolding reaction. Thus, substrate was still folding competent after subunit exchange, indicating that subunit exchange does not significantly alter the interactions between sHSP and substrate or the interactions with the refolding machinery.

Pre-formed sHSP-Substrate Complexes Can Bind Additional Substrate—The dynamic nature of the sHSP-substrate complexes with regard to sHSP subunit exchange challenges the model that complexes are very stable. To investigate further the nature of complexes between sHSP and substrate, we first tested if additional substrate could be incorporated into pre-formed complexes using wild-type HSP18.1 and Luc. In testing the capacity of HSP18.1 to bind and protect Luc, we noted that the smaller the ratio of sHSP to substrate, the larger the complexes. Fig. 6 shows the ~1-min difference in SEC elution times between complexes formed at a ratio of 12 μM HSP18.1 to 1 μM Luc and 12 μM HSP18.1 to 3 μM Luc. Thus, it was possible to test for incorporation of more Luc into pre-existing complexes by starting with smaller complexes and assaying for a shift to larger size complexes with the addition of more Luc.
do this, HSP18.1-Luc complexes were formed at a ratio of 12 μM HSP18.1 to 1 μM Luc. Luc was added to these complexes in order to bring the final Luc concentration to 3 μM, and the sample was heated as before and subjected to SEC. The chromatograms revealed that the complexes had rearranged to form larger complexes, which looked very similar to complexes originally formed at a ratio of 12 μM HSP18.1 to 3 μM Luc (Fig. 6, compare open squares and circles). If the mixtures were not subject to the heating step, an increase in the free Luc peak was the only change observed (data not shown). The additional Luc is not just binding to, and being protected by, the free sHSP that was not incorporated into the original complexes. If that were the case the right-hand side of the complex peak should match the 12 μM HSP18.1 to 1 μM Luc complex peak, because the original complexes would be unaffected. However, because there is less of the smallest complexes, we conclude that Luc is being incorporated into pre-existing complexes causing them to become larger.

It should be noted that HSP18.1 has a limited capacity to bind and protect substrate and becomes saturated at ratios between 3:1 and 4:1 (sHsp:substrate). This saturation has two consequences; first, the complexes start to become insoluble, and second, the amount of substrate that can be refolded in the reticulocyte lysate system is severely decreased (data not shown). These results show that complexes are competent to bind additional substrate but have a limited capacity to bind substrate in a soluble form suitable for refolding by HSP70.

sHSP-Substrate Complexes Retain Their Size Distribution after Fractionation or Addition of Free sHSP—Considering that free sHSP subunits and those incorporated in complexes are capable of exchange, we speculated that complexes could change size through the addition or loss of sHSP subunits or substrate, and that the size distribution of complexes represented a system at equilibrium. We performed three sets of experiments to test this possibility. First, HSP18.1-Luc complexes were separated into three fractions, and these fractions were examined to determine whether complexes redistributed with respect to size. Complexes made at a ratio of 12:3, HSP18.1:Luc, were used instead of complexes made at 12:1, HSP18.1:Luc, in order to increase the difference in the elution times between the far left- and right-hand sides of the complex peak (see Fig. 7A). Three fractions from the elution of complexes made at a ratio of 12:3 (HSP18.1:Luc) were collected, concentrated, and then reinjected onto the SEC column (see Fig. 7A). The original complex had a peak time of 7.3 min, whereas fractions 1–3 had peak times of 6.9, 7.3, and 7.9 min, respectively. No change in these peak times was seen even after >24 h at room temperature. Some broadening of the complex peaks was expected and seen when rerunning the three fractions. This may indicate a small amount of redistribution of complex sizes, but due to the lack of change in the peak elution times seen for the three fractions after long periods of incubation, we believe the most likely explanation for peak broadening is interaction of the complexes, which expose a significant amount of hydrophobicity (10, 14, 25, 26, 34, 35), with the hydrophobic column resin.

The second test of the size distribution of complexes being a system at equilibrium involved combining the first and third fractions and determining if complexes redistributed with respect to size. Fig. 7B shows the chromatograms of the fractions 40 h post-mixing, as well as the individual unmixed fractions. The expected chromatogram, if no rearrangement in the size distribution of the complexes were to occur after mixing, is also included. The actual and expected traces are almost identical, indicating that the size distribution of the complexes in a fraction did not redistribute following separation from the rest of the complex peak or recombination with a different fraction of the peak. From these results we concluded that complexes do not change size, and the size distribution of complexes is not a system in equilibrium.

We had observed that the ratio of sHSP to substrate determined the size distribution of the complexes formed. Considering this, we speculated that if the size distribution of complexes represents an equilibrium, adding free sHSP may perturb the equilibrium such that the sizes of complexes would change. Therefore, as a third test, HSP18.1-Luc complexes were initially made at a ratio of 12 μM to 3 μM (sHSP/Luc), and free HSP18.1 was added to bring the final concentration of sHSP to 36 μM. Then the entire mixture was diluted to 12 μM HSP18.1 to 1 μM Luc and subjected to SEC. The chromatograms revealed that the complexes had not rearranged to form complexes equivalent to complexes originally formed at a ratio of 12 μM HSP18.1 to 1 μM Luc (Fig. 7C). Only an increase in the free sHSP peak was seen. No change in the size of the complexes was observed even if the mixtures were incubated at 42 °C for 8.5 min or if mixtures were allowed to sit for >48 h at room temperature, after the addition of free sHSP. However, Western analysis of fractions collected from SEC runs of these mixtures revealed that if strep-tagged HSP18.1 were added instead of wild-type HSP18.1, every fraction contained both types of sHSP (data not shown), confirming that sHSP subunit exchange continues in the presence of bound...
substrate (as seen in Fig. 4B) and providing evidence that complex size does not appear to affect the exchange of sHSP subunits. Thus, the size distribution of complexes is unaffected by the addition of free sHSP, even though subunit exchange is occurring. These results confirmed our conclusions from the other two tests, that the complexes do not appear to change size, and the size distribution of complexes does not represent an equilibrium system.

Substrate Is Not Observed to Transfer from the sHSP-Substrate Complex—The lack of complex rearrangement with respect to size, unless more substrate is added, and ability of sHSP subunits to freely dissociate from and associate with complexes led us to examine if the denatured substrate could also freely dissociate from complexes. By utilizing the strep-tagged proteins, a substrate transfer assay was developed to test for the ability of substrate to dissociate from complexes. At present we do not have an assay to directly observe substrate dissociation, but considering that the strep-tagged sHSPs can bind and protect substrate, transfer of substrate from one sHSP to another was a means to indirectly assay for substrate dissociation.

To test for the ability of substrate to transfer between complexes, we first made complexes of HSP18.1 or HSP16.6 with Luc using conditions under which ~95% of the substrate was incorporated into the complex peak (Fig. 8). Thus, no free substrate is available to complicate the assay for substrate transfer. Each sHSP and substrate reproducibly form substrate complexes with distinct elution profiles.

To test for substrate transfer, an equal amount of the same sHSP or strep-tagged version of the other sHSP was added to the complexes shown in Fig. 8. For example, either HSP18.1 or strep-tagged HSP16.6 was added to HSP18.1-Luc complexes. These mixtures were incubated for up to 120 min at 31 °C (the temperature used in refolding reactions) and then analyzed by streptactin chromatography and SDS-PAGE. Fig. 9 clearly shows that when strep-tagged HSP18.1 is added, a small amount of Luc, reaching a maximum at 60 min, is recovered in the elution of every time point in excess of 0 min (lanes 3–7), consistent with transfer to strep-tagged HSP18.1. This does not occur when HSP16.6 was added instead of strep-tagged HSP18.1 (no protein was found in the elution, data not shown). However, when strep-tagged HSP18.1 was added, some of the HSP16.6 is found in the elution lane, which differs from the no Luc controls (lane 1 versus lanes 3–7). This observation sug-
gests that the substrate may not be transferring from the original complexes but is binding the strep-tagged sHSP in addition to maintaining its complex interactions with the wild-type sHSP. When the experiment was performed in the reciprocal direction, very little if any Luc was seen in the elution when strep-tagged HSP16.6 had been added to pre-formed HSP18.1-Luc complexes (Fig. S3), showing that there is no detectable transfer to HSP16.6. Similar results were obtained using MDH as a substrate. Markedly increasing the amount of strep-tagged sHSP added (up to 120 μM) or diluting the original complexes to very low concentrations, as were used in the refolding assay (11.5 μM), also did not change the results (data not shown). In total these data suggest that substrate does not transfer between complexes, which implies that substrate does not dissociate from complexes.

**DISCUSSION**

It is clear that sHSPs are highly dynamic proteins that are able to bind unfolding substrates, forming complexes from which substrate can later be refolded (3–11, 13, 14, 16). However, very little is known about the interactions of sHSPs and substrates within these complexes or how substrate is transferred to the refolding machinery. We have established affinity-tagged sHSPs from both a eukaryote (pea HSP18.1) and a prokaryote (*Synechocystis* HSP16.6) as novel tools to investigate these and other questions. The presence of the affinity tag allowed us to examine the time course of subunit exchange between oligomers, as well as continued exchange of sHSP subunits into pre-existing sHSP-substrate complexes. Surprisingly, despite this rapid exchange of sHSP subunits with free oligomers and complexes, dynamic rearrangement of complexes does not occur, and transfer of bound substrate to added sHSP was not detectable. In contrast, additional substrate was readily incorporated into pre-formed complexes. When considered together, these data indicate that once complexes are formed the amount of sHSP and substrate in a complex is fixed, as well as the arrangement of these components.

It is of significant interest that addition of a C-terminal affinity tag to HSP18.1 or HSP16.6 did not obviously perturb their oligomeric structure or ability to bind substrate and facilitate substrate refolding. Function of the affinity-tagged HSP16.6 was also confirmed in *vivo*. We are not aware of other affinity-tagged sHSPs shown to be functional by all these criteria, although His-tagged sHSPs from *Bradyrhizobium japonicum* could still limit substrate aggregation similarly to wild-type protein (36). The only noticeable difference between wild-type and strep-tagged sHSPs was a small increase in the apparent size of HSP18.1-Luc complexes. From the crystal structure and other evidence, the C-terminal extension has been shown to be important for oligomerization, but there is no evidence that it interacts with substrate (5, 7, 21, 37, 38). The size differences between complexes made with wild type alone and those made with some amount of strep-tagged HSP18.1 indicate that the C-terminal extension may play a role in structuring complexes. Unlike HSP18.1, HSP16.6, and many other sHSPs, mammalian sHSPs have a highly flexible group of residues (10–21 amino acids) beyond the C-terminal extension, referred to as the C-terminal tail, which has been suggested to confer additional solubility (39, 40). The addition of the strep-tag amino acids resembles this C-terminal tail seen in other sHSPs. How the strep tag is specifically affecting or has changed sHSP-substrate interactions remains to be determined, but our data indicate interactions cannot depend on a free, native C terminus. In total, the lack of significant functional differences between wild-type and strep-tagged sHSPs in both *in vitro* and *in vivo* assays establish these sHSPs as excellent tools to dissect further sHSP function. We recently used strep-tagged HSP16.6 to identify specific sHSP-associated proteins in *Synechocystis* cells.

Investigation of the time course of subunit exchange of HSP18.1 and HSP16.6 revealed that this process is temperature-dependent and is rapid at the optimal growth temperature for the organism from which the sHSP originated. At 0 °C no subunit exchange was observed for HSP18.1 or HSP16.6, similar to results with vertebrate sHSPs at 3 °C (19, 21). At 22 °C, a normal growth temperature for pea, subunit exchange of HSP18.1 was very rapid, going to completion between 15 and 30 min. This result is in excellent agreement with the time course of subunit exchange between wild-type HSP18.1 and the related wheat HSP16.9, which was observed by real time mass spectrometry at 24 °C (22). Subunit exchange for *Synechocystis* HSP16.6 was complete within ~1 h at 30 °C, the optimal growth temperature for the cyanobacterium, but was significantly reduced at 22 °C, taking over 3 h to reach completion. Subunit exchange for both proteins is much faster than what has been observed for mammalian αA-crystallin and αB-crystallin, which required ~4 h to reach equilibrium at 37 °C and was even slower at lower temperatures (19, 21). Previous experiments also showed that HSP16.5 from *M. jannaschii* does not exhibit significant subunit exchange until over 50 °C, consistent with the hyperthermophilic lifestyle of this organism. Considering subunit dynamics are probably important for sHSP function, it is logical that sHSPs would be dynamic at temperatures corresponding to the optimum for growth.

sHSP subunit exchange continued in the presence of bound substrate and had no observable effects on the refolding of substrate or on the time course of subunit exchange. Bova et al. (19) found that substrate reduced the rate of subunit exchange by 35% at a 2:1 molar ratio of sHSP to overtransferrin. A similar decrease in the time course of subunit exchange when substrate is bound to HSP18.1 may not have been observed because our assay is less sensitive and the exchange of subunits is much faster than the vertebrate sHSPs at their respective optimal growth temperatures. These results demonstrate that

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The observation that sHSP subunits in complexes exchange with the free oligomeric pool of sHSP subunits suggested that complexes could potentially rearrange or change the amount of sHSP or substrate in each complex. If correct, we would expect that the size of complexes could be changed with the addition of sHSP. No change in the size of complexes was observed in the presence of added sHSP, even after the complexes were re-heated (Fig. 7C). This implied that once formed, complexes have a defined stoichiometry of sHSP subunits and molecules of substrate, which does not change, even at high temperature. This argument is supported by the failure to detect substrate dissociation from complexes. However, a certain portion of the sHSP subunits are able to dissociate from the complexes, and free sHSP subunits can associate with the complexes (Fig. 4A).

We suggest that the size of the complex is maintained during this process, because free sHSP subunits only associate with complexes after subunits dissociate, thereby replacing the lost subunits. It has been suggested that dissociation of sHSP from complexes may be necessary for substrate interaction with refolding chaperones (23). We propose that the continuation of substrate exchange in complexes is an important process that potentially allows for interactions of HSP70 and other ATP-dependent chaperones with the denatured substrate while it is still bound to the sHSP, although it may be unavailable in the sHSP-saturated complex.

In contrast to the inability to incorporate more sHSP into pre-formed complexes, addition of more denatured substrate was readily achieved, resulting in an increase in the size of complexes. This suggests that some of the subunits in the complex remain competent to bind more substrate. Binding substrate might initiate on the complex. Alternatively, it might begin with free sHSP subunits, as suggested previously (8, 18, 24), before it is incorporated into the complex. The addition of this substrate changes the content and size of the complexes and might also involve addition of more free sHSP. Once the larger complexes have been formed, the addition of yet more free sHSP had no effect on the size of complexes (data not shown), again suggesting that these complexes have a defined content of sHSP subunits and molecules of substrate.

The inability of complexes to change size or change the amount of sHSP or substrate present in a complex meshes well with what is potentially happening inside living cells. The addition of sHSP to complexes would not be expected to reduce complex size, as that would be equivalent to sHSPs having a type of energy-independent, resolubilizing activity. The ability of complexes to bind additional unfolding substrate would most likely be beneficial to cells. In the absence of adequate amounts of refolding chaperones, the release of denatured substrate could lead to the aggregation of not only these proteins but other proteins in the cell, potentially creating a situation that is detrimental to cells. A stable association of denatured substrate with sHSP in complexes would minimize the chances of such interactions.

Acknowledgments—We thank Dr. K. Nyberg for construction of the strep-tagged HSP18.1 and Dr. A. Hauэрath for helpful discussion and critical reading of the manuscript.

REFERENCES

K. Friedrich and E. Vierling, unpublished data.
sHSP-Substrate Complexes

Supplemental Figure legends

**Fig. S1. The C-terminal strep tag does not interfere with sHSP oligomer formation.** A, purified wild-type and strep-tagged HSP18.1 analyzed by SDS (2 µg/lane) or non-denaturing (3 µg/lane) PAGE. B, SDS gel of wild-type or strep-tagged HSP18.1 subjected to streptactin affinity chromatography. L: protein loaded on the resin; FT: unbound, flow-through fraction; E: bound, eluted fraction. Equal fractions of sample were loaded in each lane. Molecular weight of protein standards (in kDa) are indicated for size comparison. Gels were stained with Coomassie Blue. (Same results obtained with *Synechocystis* HSP16.6 not shown).

**Fig. S2. Wild-type HSP18.1, strep-tagged HSP18.1, and mixtures of wild-type and strep-tagged HSP18.1 bind substrate in a folding-competent state.** Refolding time course of (diamonds and triangles) mixtures of wild-type and strep-tagged HSP18.1 (12 µM total for each sample) with 1 µM Luc. Samples of each mixture were added to the reticulocyte lysate refolding system and put at 31°C. At selected times Luc activity was assayed and compared to activity before heating. BSA control (xs) contained an amount of protein (0.22 mg/ml) equivalent in weight to the sHSP. Each data point is the average of three samples, with standard deviation shown by error bars. (Similar results obtained with *Synechocystis* HSP16.6 not shown)

**Fig. S3. Substrate does not transfer from sHSP/substrate complexes to added sHSP.** SDS-PAGE analysis of HSP18.1/Luc complexes (24 µM sHSP:2 µM Luc) that were incubated at 31°C for the times indicated after the addition of an equal amount of the HSP18.1 (not shown) or strep-tagged HSP16.6 before being subjected to streptactin affinity chromatography. L: protein loaded on the resin; FT: unbound, flow-through fraction; E: bound, eluted fraction. Equal fractions of L, FT, and E samples were loaded in each lane. The No Luc control was treated identically to samples that had strep-tagged HSP18.1 added and was incubated for 120 min, but no Luc was present during the original complex formation step (lanes 1-2, only E and FT shown). The L for samples to which strep-tagged sHSP was added is only shown for the 120 min time point. Gels were stained with Coomassie Blue.
Figure S2

![Graph showing Luc Activity (%) over time (min).]
Figure S3

HSP18.1/Luc complexes

<table>
<thead>
<tr>
<th>No Luc E FT</th>
<th>120 120</th>
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<tbody>
<tr>
<td>16.6 Str Added</td>
<td>0 10 30 60 120 120</td>
</tr>
</tbody>
</table>

Time at 30°C

Luc

18.1
16.6 str

1 2 3 4 5 6 7 8 9