The N-terminal Arm of Small Heat Shock Proteins Is Important for Both Chaperone Activity and Substrate Specificity

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Small heat shock proteins (sHSPs) are a ubiquitous class of molecular chaperones that interacts with substrates to prevent their irreversible insolubilization during denaturation. How sHSPs interact with substrates remains poorly defined. To investigate the role of the conserved C-terminal α-crystallin domain versus the variable N-terminal arm in substrate interactions, we compared two closely related dodecameric plant sHSPs, Hsp18.1 and Hsp16.9, and four chimeras of these two sHSPs, in which all or part of the N-terminal arm was switched. The efficiency of substrate protection and formation of sHSP-substrate complexes by these sHSPs with three different model substrates, firefly luciferase, citrate synthase, and malate dehydrogenase (MDH) provide new insights into sHSP substrate interactions. Results indicate that different substrates have varying affinities for different domains of the sHSP. For luciferase and citrate synthase, the efficiency of substrate protection was determined by the identity of the N-terminal arm in the chimeric proteins. In contrast, for MDH, efficient protection clearly required interactions with the α-crystallin domain in addition to the N-terminal arm. Furthermore, we show that sHSP-substrate complexes with varying stability and composition can protect substrate equally, and substrate protection is not correlated with sHSP oligomeric stability for all substrates. Protection of MDH by the dimeric chimera composed of the Hsp16.9 N-terminal arm and Hsp18.1 α-crystallin domain supports the model that a dimeric form of the sHSP can bind and protect substrate. In total, results demonstrate that sHSP substrate interactions are complex, likely involve multiple sites on the sHSP, and vary depending on substrate.

The small heat shock proteins (sHSPs), and the related α-crystallins comprise a superfamily of chaperones defined by a conserved C-terminal domain of ~90 amino acids referred to as the α-crystallin domain (1). Flanking this domain is a short C-terminal extension and an N-terminal arm of variable length and highly divergent sequence (1–3). Although the monomeric size of the sHSPs ranges from 15 to 42 kDa, in their native state most sHSPs assemble into large oligomers of 8 to >32 subunits, although there are also dimeric and tetrameric sHSPs (1, 4).

Studies with numerous sHSPs from different organisms have shown that in vitro these proteins act as chaperones by binding to partially unfolded proteins in an ATP-independent manner, preventing their irreversible aggregation (1, 5). Substrates that are denatured in the presence of sHSPs can then be refolded and reactivated by the ATP-dependent chaperone DnaK/Hsp70 with the participation in some cases of ClpB/Hsp100 and GroEL (1, 6, 7). In vivo, increased expression of these ubiquitous stress proteins can enhance cellular tolerance to a variety of stresses, such as heat, salt, drugs, and oxidants (1). sHSPs have also been reported to act as negative regulators of apoptosis, to modulate cellular redox state, and to be linked to increased organism longevity (8–10). Consistent with their proposed chaperone function, sHSPs are associated with protein aggregates in a number of human diseases, including cataract, neurogenerative diseases, and myopathies (11). Defining the molecular mechanism of sHSP action is key to understanding how these chaperones can participate in such diverse cellular processes.

Critical to their proposed mode of action as chaperones is the ability of sHSPs to interact with denaturing substrates. sHSPs have been shown to have a very large capacity for substrate binding, maintaining an equal weight of some denatured substrates in a soluble form (1). Model substrates protected in vitro as well as proteins found associated with sHSPs in vivo include proteins of a wide range of molecular weights, pI values, and structures, with no obvious common characteristics (12, 13). The most well documented aspect of sHSP-substrate interaction comes from multiple experiments showing that sHSP substrate binding capacity is enhanced by structural changes that expose hydrophobic surfaces that are normally occluded in the native sHSP oligomeric structure (1). Putative substrate binding sites may become available through dissociation of sHSP oligomers to dimers as a result of the dynamic equilibrium of sHSP subunits between oligomeric and suboligomeric species or through more subtle environmentally induced (e.g. high temperature) changes in sHSP tertiary structure (1, 5). The potential overlap of oligomeric interfaces with substrate interaction sites has proven problematic for separating these two functions. Determining specific substrate binding sites on sHSPs is further complicated by the large size and heterogeneity of
sHSP-substrate complexes. sHSP-substrate complex architecture, as observed by electron microscopy or size exclusion chromatography, varies with the identity of the sHSP, the ratio of sHSP to substrate, substrate identity, and how substrate is denatured (6, 14 – 16).

A variety of experimental approaches with different sHSPs or α-crystallins assayed with different model substrates has been used to identify potential substrate binding sites on these chaperones but has not led to any coherent model. The conserved α-crystallin domain has been implicated in substrate binding in a number of studies. There is good evidence that the α-crystallin domain shares a common structure in all members of this family consisting of a seven-stranded, IgG-like β-sandwich with topology identical to p23 (1, 17, 18). Experiments concluding that this domain interacts with substrates have included cross-linking to substrate (19, 20), identifying peptides that bind the hydrophobic probe (1,1'-bi(4-anilino)naphthalene-5,5'-disulfonic acid) (Bis-ANS) (20 – 22), single amino acid substitutions (23 – 26), substrate binding to α-crystallin peptide arrays (27), and antibody fragments corresponding to a region of the α-crystallin domain to effect substrate protection (20, 27, 28). In addition, high resolution structures of the Methanococcus jannaschii Hsp16.5 (17) and Triticum aestivum (wheat) Hsp16.9 (18) oligomers predict specific regions of the α-crystallin domain would be exposed only by alterations in sHSP oligomeric structure, consistent with the model of sHSP action.

Similarly, a range of studies point to the evolutionarily variable N-terminal arm as essential for substrate interactions. sHSPs with a naturally short N-terminal arm or an N terminus truncated by mutagenesis most often lack chaperone function (29 – 32). However, because the oligomeric structure of most of these variants is also disrupted (or naturally limited to dimer or tetramer), discriminating between differences in structure versus substrate binding as the primary cause of the defect remains a problem. Because current models suggest sHSP subunits must reassemble in some fashion after substrate binding, disruption of oligomeric structure could also impact this step (1, 5).

Site-directed mutagenesis identified specific N-terminal Phe residues (Phe-24, Phe-27) in human αB-crystallin as necessary for chaperone activity and potentially involved in substrate binding (33). Bis-ANS also binds the N-terminal arm, reducing sHSP chaperone activity potentially by blocking substrate interactions (21, 32). The evolutionary variation in the N-terminal arm could also be interpreted as accounting for species or sHSP-specific functions of these proteins, pointing to a role in substrate interactions, and sHSP structural data reveal properties of the N-terminal arm that would be advantageous for this function. Half or all of the N-terminal arms of sHSP subunits in Hsp16.9 (18) and Hsp16.5 (17) are unresolved in the crystal structure, and cryo-EM images of α-crystallins indicate the arms are disordered (34), implying flexibility to accommodate interaction with different substrates. The N-terminal arms would also be free to bind substrates when the sHSP oligomer dissociates (18).

To further address the question of the relative importance of the N-terminal arm and α-crystallin domain in substrate interactions, we created chimeras of two very closely related dodecameric sHSPs, wheat Hsp16.9 and pea (Pisum sativum) HSP18.1. Studies of these proteins individually have suggested that there are significant differences in their relative abilities to protect firefly luciferase (Luc) and malate dehydrogenase (MDH) from insolubilization (15, 21). Availability of the 2.65 Å resolution x-ray structure of the Hsp16.9 dodecamer (18) along with data demonstrating that these two sHSPs are fully compatible to form heterododecamers (35) made this an attractive system for constructing chimeric sHSPs. We created four chimeric proteins, switching either the entire N-terminal arm or the first 10 residues of Hsp18.1 with the corresponding region of Hsp16.9, and characterized the oligomeric structure and chaperone activity of the purified recombinant proteins compared with the parental wild types. We observed decreased oligomeric stability of some of the chimeras, illustrating the importance of contacts within an sHSP monomer for proper assembly of sHSP quaternary structure. However, oligomeric stability did not correlate with chaperone activity as analyzed with three different substrates (Luc, citrate synthase (CS), and MDH). Furthermore, the role of the N-terminal arm in substrate protection was not the same for the different substrates. The identity of the N-terminal arm determined the effectiveness of Luc and CS protection by the sHSP but was not the determining factor in MDH protection. In total the results provide new insight into the complexity of sHSP-substrate interactions.

**MATERIALS AND METHODS**

Construction of Chimeric sHSPs—Chimeric sHSPs were created in which the entire N-terminal arms of wheat Hsp16.9 (accession number CAA45902) and pea Hsp18.1 (accession number P19243) were exchanged (see Fig. 1). A site-specific mutation was made in the Hsp16.9 coding sequence in the pJC20 expression plasmid (AZ388) to remove an Aval site in the C-terminal region of the gene (the third base of Glu-143 codon was changed from G to A). The third base of the Gly-63 codon in Hsp16.9 was then changed from C to G to create a new Aval site at this position, and the third base of the Pro-69 codon in Hsp18.1 (also in the pJC20 expression plasmid (AZ316) was changed from T to C) to create a complementary Aval site for the exchange of the N termini. The new plasmids were cut with Aval, which also cuts at a site in the DNA beyond the coding region in both plasmids, and the smaller of the two fragments from each digest was extracted from the gel and ligated to the opposite plasmid. The resulting plasmids encoded chimeric proteins that were designated N18C16 and N16C18. Note that because of amino acid sequence identity between residues 47 and 62 of Hsp16.9 with residues 54 and 69 of Hsp18.1 the effective exchange was of 46 residues of Hsp16.9 for 53 residues of Hsp18.1 (Fig. 1).

Two other chimeric proteins were created to switch only the first four residues of Hsp16.9 with the first 10 residues from Hsp18.1 as shown in Fig. 1. These resulting chimeric proteins were designated “short” N18-C16 (sN18-C16) or short N16-C18 (sN16-C18). The chimeric sN16-C18 was created using the primers 5’-AATCGACATATGGATCATCCTGCTT-3’ and 5’-GTCCGAAGAGGACTAATTTTGC-3’ to amplify the entire Hsp18.1 plasmid followed by ligation to recircularize the plasmid. The N18-C16 chimera was created using AGTTTCT-
state of the sHSPs, sHSPs at the stated concentrations were centrifuged to remove any aggregated protein, and the supernatant was loaded over the SEC column the same way. Standards were thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa) (Bio-Rad).

**Western Analysis of SEC Fractions**—sHSP-Luc complexes were prepared and subjected to SEC as described above. Fractions were collected every 30 s from 5.5 to 11.5 min. Samples were mixed with an equal volume of 2× SDS sample buffer and separated on 15% SDS-PAGE. Regions from the same gel, which corresponded to the molecular weight of either Luc or the sHSPs, were processed for Western analysis using either Luc antibody (the upper part of the gel) or both Hsp18.1 and Hsp16.9 antibodies (the lower part of the gel). Antibody reactions were visualized by chemiluminescence (Amersham Biosciences).

**Luc Refolding Assay**—Luc (1 μM) was incubated with different sHSP concentrations in the previously described buffer at 42 °C for 9 min. Luc reactivation was carried out as described in Giese and Vierling (36).

**RESULTS**

**Creation of Chimeric sHSPs**—Amino acid sequence alignment (Fig. 1A) revealed that from residue 45 or 54 of wheat Hsp16.9 or pea Hsp18.1, respectively, through the C terminus, the proteins are 80% identical and 86% similar. In contrast, the remaining N-terminal region was only 41% identical and 50% similar, and the Hsp18.1 N terminus had a 6-residue insertion (residues 5–10) that is missing in Hsp16.9. The high conservation of the C-terminal α-crystallin domain compared with the variation in the N terminus suggests that differences in chaperone activity of these sHSPs may result from N-terminal differences and implicates the N-terminal arm in substrate interactions. To address this hypothesis we created two sets of chimeric proteins between Hsp16.9 and Hsp18.1. These two sHSPs have been shown previously to co-assemble into fully heterogeneous oligomers, with a random equilibrium distribu-
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The relative importance of the N-terminal arm and α-crystallin domains in sHSP chaperone activity.

In the first set of chimeras the entire non-conserved N-terminal region was switched to create the two chimeras N18-C16 and N16-C18, as shown in Fig. 1B. A second set of chimeras was designed to test the importance of the six-residue insertion in Hsp18.1 by switching the first 10 amino acids of Hsp18.1 with the first 4 amino acids of Hsp16.9, creating sN18-C16 and sN16-C18. All four of these chimeras were soluble when expressed in E. coli and could be purified to >95% homogeneity.

**Chimeras with the Hsp16.9 N Terminus Show Concentration-dependent Oligomerization**—The chaperone activity of sHSPs has been linked to the ability of the proteins to oligomerize and to the dynamic equilibrium of sHSP native oligomers with sub-oligomeric forms (1, 5, 35, 36). Therefore, we first examined the oligomeric state of the parent wild-type and chimeric sHSPs by SEC at concentrations of 6, 12, and 36 μM (Fig. 2). As expected, all of Hsp18.1 and most of Hsp16.9 eluted as dodecamers at all concentrations tested, although Hsp16.9 had a small shoulder. The chimeras with the Hsp18.1 N terminus, N18-C16 and sN18-C16, exhibited behavior most similar to Hsp18.1, remaining essentially oligomeric at all concentrations, although they had detectable, more slowly eluting shoulders. In contrast, oligomeric structure of the chimeras with the Hsp16.9 N terminus (N16-C18 and sN16-C18) showed a significant dependence on concentration. N16-C18 is the most impaired protein with respect to oligomerization; at 6 μM all of the protein eluted at a position consistent with a dimeric structure, and even at 36 μM a significant fraction eluted at the position of a dimer. Although sN16-C18 behaved primarily as an oligomer at 36 μM, the oligomer was less stable than either parent sHSP, showing little intact oligomer at 6 μM. Because intact Hsp18.1 and Hsp16.9 readily hetero-oligomerize to form dodecamers, the instability of chimeras with all or part of the N-terminal arm of Hsp16.9 attached to Hsp18.1 suggested that intramolecular contacts are specifically disrupted in these chimeras. This result emphasizes the dependence of sHSP quaternary structure on multiple lower order contacts.

**Proteins with the Hsp18.1 N Terminus Protect Luc More Efficiently than Those with the Hsp16.9 N Terminus**—As mentioned previously, we observed that Hsp18.1 is more effective than Hsp16.9 in protecting firefly Luc against heat insolubilization (14, 15, 21). To determine whether this difference could be due to differences in the N terminus, the wild-type and chimeric proteins were compared for their ability to protect Luc from insolubilization during heating. Luc (1 μM) was incubated at 42 °C for 9 min with 3, 6, 12, 24, or 36 μM sHSP monomer and then separated into soluble and insoluble (pellet) fractions by centrifugation and analyzed by SDS-PAGE (Fig. 3). Under these conditions, Hsp18.1 was found to be more effective than Hsp16.9, protecting Luc against heat insolubilization at significantly lower concentrations. Therefore, the N terminus of Hsp18.1 plays a crucial role in the chaperone activity of sHSPs.
conditions, in the presence or absence of Luc all of the sHSP remained soluble; no significant amount was recovered in the pellet fraction (Fig. 3, left, Pellet). In contrast, when heated in the absence of sHSP all of the Luc was recovered in the pellet fraction (Fig. 3, right, Pellet). The addition of Hsp18.1 essentially fully prevented insolubilization of Luc. Hsp16.9 is significantly less effective; visual estimates show only 20% Luc protection at 24 μM and only 50% protection at 36 μM sHSP. Note that IgG at an equal weight to 36 μM sHSP afforded no protection of Luc from insolubilization (not shown). Therefore, on a molar basis under these buffer conditions Hsp18.1 is at least 6-fold more effective at interacting with Luc to prevent heat-induced insolubilization.

Consistent with an involvement of the Hsp18.1 N terminus in Luc interaction, the N18-C16 chimera showed virtually identical protection of Luc as Hsp18.1 (Fig. 3). Enhanced protection by the Hsp18.1 N-terminal sequence, however, was dependent on presence of the entire N terminus; the sN18-C16 and sN16-C18 chimeras showed protection only intermediate between Hsp16.9 and Hsp18.1, both protecting Luc to -50% at 12 μM sHSP and not completely until 24 μM. This result suggests that both the proximal and distal segments of the N terminus interact with Luc, and/or secondary or tertiary structure formed only by an intact Hsp18.1 N terminus are required for full activity. The importance of some component of higher order structure is supported by the surprising result that the N16-C18 protein protected Luc at least 2-fold better than wild-type Hsp16.9 despite the absence of Hsp18.1 N-terminal sequence. Whatever structure is required does not correlate with the stability of the oligomer, as the N16-C18 chimera is a more effective chaperone than Hsp16.9 despite its destabilized quaternary structure (Fig. 2).

Characteristics of sHSP-Substrate Complexes Show That Proteins with the Hsp16.9 N Terminus Interact Differently with Luc—Protection of substrates from aggregation has previously been correlated with formation of stable sHSP-substrate complexes (1, 5, 14–16, 21). To test the relationship between substrate protection and sHSP-substrate complex formation, the interaction of wild-type and chimeric proteins with Luc was examined by SEC. sHSPs at 6, 12, and 36 μM were incubated with 1 μM Luc as for experiments in Fig. 3, and the soluble fraction was applied to the column (Fig. 4). Hsp18.1-Luc complexes were observed at all three sHSP concentrations, with apparent size of the complexes decreasing and the uniformity of the complexes increasing with increasing sHSP-substrate ratio. N18-C16 formed complexes with Luc that showed basically the same elution behavior as Hsp18.1-Luc complexes, directly parallel to the ability of N18-C16 to protect Luc from heat-induced aggregation essentially as well as Hsp18.1. The reduced protective ability of the sN18-C16 chimera was dramatically reflected in the SEC elution profile of the heated sHSP-Luc mixtures. Complexes of sN18-C16 with Luc could not be detected at 6 μM HSP, a concentration that provided no Luc protection, but could be detected at 12 and 36 μM, concentrations that support 50 and 100% protection, respectively. The size and heterogeneity of the complexes formed with 36 μM sN18-C16 were most similar to complexes formed with 6 μM Hsp18.1 or N18-C16 (Fig. 4A).

Interaction of Hsp16.9, N16-C18, and sN16-C18 with Luc was distinct from Hsp18.1 and chimeras with the Hsp18.1 N terminus even at 36 μM where Hsp16.9 protected 50% of the Luc, and both N16-C18 and sN16-C18 could protect Luc completely. The only evidence for sHSP-Luc interaction was some material eluting in the void volume in the 12 μM sN16-C18

FIGURE 4. Characteristics of sHSP-Luc complexes. sHSPs at 6, 12, or 36 μM were incubated in the presence of 1 μM Luc at either 4 °C (dotted line) or 42 °C (solid line) for 9 min. Insoluble material was removed by centrifugation, and the supernatant was analyzed by SEC at room temperature. A, Hsp18.1 and its N-terminal chimeras. B, Hsp16.9 and its N-terminal chimeras. Note the difference in absorbance scale for each concentration of sHSP.
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FIGURE 5. Distribution of sHSPs and Luc in fractions from the SEC analysis of sHSP Luc complexes. Luc (1 μM) was incubated with 36 μM sHSP at 42 °C for 9 min as performed in Fig. 4. Soluble fractions were injected onto the SEC column. Fractions from 5.5 to 11.5 min were collected every 30 s and analyzed by 15% SDS-PAGE and Western blotting. Parts of the same gels were probed for either the sHSPs (mixture of both α Hsp18.1 and α sHsp16.9 antibodies) or α Luc. The arrows show where the sHSP oligomer elutes, whereas the arrowheads show where an sHSP dimer elutes.

sample and a tail of increased absorbance starting between 6 and 7 min of elution time in the 36 μM sHSP samples (Fig. 4B).

To examine further how sHSPs interact with Luc, we analyzed SEC fractions (30 s each from 5.5 to 11.5 min) from the 36 μM sHSP, 1 μM Luc-heated samples for the presence of Luc and sHSP by Western blotting (Fig. 5). The Hsp18.1 and N18-C16 data confirm a strong interaction of the sHSP with Luc. In both, maximum Luc was found in the 8.5- and 9-min fractions, whereas the sHSPs peaked in the two fractions that eluted at 9.0 and 9.5 min. The difference in elution time of the sHSP peak relative to Luc is attributed to the presence of free sHSP dodecamer at ~9.5 min (see Fig. 4). For the sN18-C16 chimera, which formed relatively higher molecular weight complexes than the previous two proteins, both Luc and sN18-C16 were found in all the fractions, and the free sHSP peak was also evident. In contrast, the three sHSPs with the Hsp16.9 N terminus did not show a strong interaction with Luc. Luc was present in high molecular weight fractions in proportion to the relative amount protected by 36 μM concentrations of each sHSP, but sHSPs were hardly detectable in these fractions, consistent with the low absorbance in the SEC chromatograms (Fig. 4B).

Proteins That Have the Hsp18.1 N Terminus Promote Luc Refolding Better than Those with the Hsp16.9 N Terminus—sHSPs maintain heat labile substrate proteins in a folding-competent state until conditions are suitable for substrate refolding (1, 5). We, therefore, sought to determine how the differences in Luc interactions observed for Hsp18.1, Hsp16.9, and the chimeras affected the refolding of Luc by ATP-dependent chaperones. As for samples in Figs. 3 and 4, 1 μM Luc was incubated with 12, 36, or 72 μM sHSP at 42 °C for 9 min, which inactivates Luc to less than 3% of the initial activity. Mixtures were then added to reticulocyte lysate plus ATP and incubated at 30 °C for 90 min (Fig. 6). Although the amount of Luc refolding generally paralleled the relative Luc protection efficiency observed in the aggregation assay (Fig. 3), there were some notable deviations from this relationship. First, 12 μM N18-C16 was not as effective in supporting Luc refolding as was 12 μM Hsp18.1 despite virtually identical protection from aggregation. This difference was no longer apparent at 36 μM, and at 72 μM, N18-C16 had even somewhat better refolding yields than Hsp18.1. Also, although N16-C18 was less effective in Luc protection at 36 μM than sN16-C18, the N16-C18 chimera supported a higher level of refolding at this concentration. In addition, refolding from N16-C18 at 72 μM was similar to N18-C16 and higher than Hsp18.1. Therefore, aggregation protection and competence for refolding reflect two different aspects of sHSP function. It is likely that refolding reflects the state of the denatured Luc as well as the affinity of the sHSP for Luc.

Protection of CS Depends on the Presence of Hsp18.1 N-terminal Sequences—To further test the importance of the sHSP N terminus in substrate protection, we examined protection of another substrate, CS, which has a smaller monomeric molecular weight than Luc and slower aggregation kinetics. Even more obvious than with Luc, all three sHSPs with the N-terminal 10 residues of Hsp18.1 protected CS better than proteins with the Hsp16.9 N terminus. At a molar ratio of 6:1 (sHSP:CS), Hsp18.1, N18-C16, and sN18-C16 fully protected CS (Fig. 7). Note that under the conditions used for aggregation, some CS remains soluble in the absence of sHSP, and as seen with Luc, the majority of the sHSPs remain soluble even when the CS maximally aggregates. In contrast to sHSPs with the Hsp18.1 N...
terminus, Hsp16.9 and N16-C18 failed to protect CS even at the molar ratio of 15:1. The sN16-C18 chimera, containing the distal part of the Hsp18.1 N-terminal arm, achieved partial protection at a 9:1 molar ratio and complete protection at a 12:1 molar ratio. As with Luc, IgG at a weight equal to 36 μM sHSP showed no protection of CS (not shown). These data clearly demonstrate the importance of the N-terminal arm in sHSP-substrate interactions, indicating that the 10 N-terminal residues of Hsp18.1 are required to confer full protection of CS. The activity of the sN16-C18 chimera suggests that residues 11–46 of Hsp18.1 interact with CS or that they alter the structure of the four N-terminal residues of Hsp16.9 facilitating interaction with CS.

sHSP Ability to Protect MDH Is Not Determined Solely by the sHSP N Terminus—To extend our analysis of the role of the sHSP N terminus in substrate protection, we investigated a third substrate, MDH, which has been used extensively in analysis of sHSP chaperone activity (6, 14, 16, 21). We showed previously that both Hsp18.1 and Hsp16.9 act as chaperones in protecting MDH (14, 15, 21) but had not directly compared these proteins in the same experiment. In our earlier work we had also obtained evidence from Bis-ANS binding that MDH interacted with two regions of Hsp18.1, an N-terminal peptide (amino acids 1–7) and a peptide that spanned β-strands 3 and 4 (amino acids 66–78) of the α-crystallin domain (21) (see Fig. 1A).

Because Hsp18.1 was found previously to be very effective in MDH protection, we used relatively low molar ratios of sHSP: MDH, from 1:1 to 6:1. Hsp18.1 protected MDH at a 1:1 ratio, whereas Hsp16.9 achieved the same level of protection only at a 4:1 ratio (Fig. 9A). Surprisingly, all of the chimeras protected MDH better than Hsp16.9, and there was no relationship between protection and the presence or absence of N-terminal sequence from Hsp18.1. Thus, essential interactions with MDH cannot be localized solely to the N or C terminus of the sHSP, consistent with our results with Bis-ANS binding (21). It should also be noted that the ability to protect MDH is not at all cor-
related with stability of the oligomer, as the N16-C18 chimera is significantly less stable than Hsp16.9 (Fig. 2), but shows a high level of protection. This observation is consistent with results of Giese and Vierling (36), which suggested that some destabilization of sHSP oligomeric structure could enhance chaperone activity. At a molar ratio of 6:1 (sHSP:MDH), where all of the proteins provided full protection, SEC analysis showed that Hsp18.1 formed moderately sized molecular weight complexes with MDH, whereas Hsp16.9 and all four of the chimeras formed complexes that eluted in the void volume (Fig. 9B).

FIGURE 9. sHSP protection of MDH. A, aggregation protection. MDH (3 μM) was incubated with sHSPs at the indicated molar ratios at 45 °C for 2h. Equal volumes of the soluble and pellet fractions were analyzed using SDS-PAGE. B, characteristics of sHSP-MDH complexes. MDH (1 μM) was incubated with 6 μM sHSP either at 4 °C (dotted line) or at 45 °C (solid line) for 2 h. Insoluble material was removed by centrifugation, and the supernatant was analyzed by SEC at room temperature.

Again, complex structure does not correlate with the sHSP N terminus. The residual peak between the 10.5- and 11-min elution time corresponds to dissociated sHSP, as determined by analyzing each sHSP heated alone at the same concentration before SEC (supplemental Fig. 1). Thus, unlike results with Luc and CS, some form of stable sHSP/MDH complex is formed by each of these sHSPs under conditions that afford full substrate protection.

DISCUSSION

The mechanism by which sHSPs associate with substrates to prevent their irreversible insolubilization during heat denaturation is poorly defined. We analyzed the interactions of three different model substrates, Luc, CS, and MDH, with two closely related plant sHSPs, pea Hsp18.1 and wheat Hsp16.9, and with four chimeras of these two sHSPs, in which all or part of the non-conserved N-terminal arm of the proteins had been switched. Remarkably, switching all or part of the N-terminal arm effectively switched the relative chaperone activity of these sHSPs with the substrates Luc and CS. The fact that chaperone activity was defined by the identity of the N-terminal arm strongly argues that this domain plays a major role in interaction with these substrates. This result suggests that the specificity of sHSPs for different substrates could be in part defined by the properties of the N-terminal arm. The N-terminal arm alone, however, does not determine interaction with every substrate, as the ability of the wild-type and chimeric sHSPs to protect MDH also depended on the identity of the α-crystallin domain. We further observed that variations in oligomeric stability of the wild-type and chimeric sHSPs did not correlate with chaperone efficiency, in contrast to what has been reported in previous studies (36, 37). Even an essentially dimeric chimera (N16-C18) was capable of protecting MDH. In addition, substrate protection by the sHSPs did not result in formation of stable, readily observed sHSP-substrate complexes in every case. In total our results demonstrate that sHSP interactions with substrate are complex, most likely involve multiple sites on the sHSP, and vary significantly depending on the individual substrate as well as the sHSP.

It has been proposed that sHSPs must be able to assemble into oligomers to protect substrates (1, 36–38). However, our data show that whether or not assembly is required for substrate protection is likely dependent on the features of sHSP-substrate interaction. First, we found that the relative stability of Hsp18.1, Hsp16.9, and the chimeric oligomers as estimated from SEC does not correlate with their relative effectiveness in substrate protection. Data analyzing missense mutants of Hsp16.6 from the cyanobacterium Synechocystis sp. PCC6803 also indicate oligomeric stability does not directly correlate with ability to protect Luc (37, 38). In addition, N16-C18, which is primarily dimeric under conditions used for substrate protection, is as effective as Hsp18.1 in protecting MDH. Thus, sHSP oligomer assembly is not required for protection of this particular substrate. We suggest that protection of MDH by a dimeric sHSP is possible because this substrate binds multiple sites on the sHSP, satisfying all of the aggregation prone surfaces of MDH as well as any sites on the sHSP that would other-
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erwise contribute to interaction between sHSP dimers. In contrast, this dimeric sHSP does not effectively protect Luc or CS. The hydrophobic surface presented by these substrates may be too large or inappropriately structured to be accommodated by a single sHSP subunit, as evidenced by the stoichiometry of sHSP to substrate required for protection. In this case, the ability of sHSP subunits to interact to reform an oligomeric structure may be important.

It is also evident from our experiments that sHSPs can provide equal protection of substrates from insolubilization through the formation of different types of sHSP-substrate complexes as visualized by SEC. Thus, measures of aggregation protection alone do not capture major differences in sHSP-substrate interactions. For example, at concentrations sufficient for N16-C18 or sN16-C18 to protect Luc, most of the sHSP elutes as a free oligomer, and very little sHSP co-elutes with Luc, although the presence of sHSP during heating is necessary for Luc solubility. The interaction between N16-C18 or sN16-C18 and Luc might not be strong enough to persist during SEC. In contrast, at the same concentrations, the major portion of Hsp18.1, N18-C16, and sN18-C16 co-elutes with soluble Luc. Protection of CS or MDH can also occur with apparently little incorporation of sHSP into a stable complex, as evidenced by the presence of significant amounts of free sHSP. The fact that the same substrate at the same sHSP-substrate ratio reproducibly yielded different protective complexes supports the conclusion that the architecture of sHSP-substrate complexes cannot be determined only by the unfolding pathway of the substrate or on the kinetics of substrate aggregation. Rather, affinity and mode of substrate binding to each sHSP must contribute to final complex composition. We cannot rule out the possibility that some sHSP-substrate complexes are either dissociated on the sizing column or that sHSP subunits in rapid equilibrium with the complex are separated from the complex during chromatography. But either case supports the conclusion that sHSPs can protect substrate in complexes assembled in different ways. The differences in sHSP-substrate complex structure are also reflected in the Luc refolding data. Even when Luc is equally protected from aggregation by the different sHSPs, different percentages of Luc are efficiently refolded.

It is not possible from these non-equilibrium experiments to determine free energies or binding constants for sHSP-substrate interaction. Mchaourab and co-workers (39) have measured binding constants for the reversible binding of destabilized T4 lysozyme with sHSPs. They propose that binding for this substrate occurs in two modes, a lower capacity, high affinity binding, and a higher capacity, lower affinity binding. Our data are consistent with multiple modes of sHSP-substrate binding. Results with Luc and CS suggest that these substrates bind to the N-terminal arm of Hsp18.1 with a higher affinity than to the N-terminal arm of Hsp16.9 or to the α-crystallin domain of either sHSP. In the absence of all or part of the higher affinity N-terminal structure, substrate apparently binds to lower affinity sites on other parts of the protein (e.g. N16-C18 with Luc at 72 μM). The clear importance of the N-terminal arm of Hsp18.1 in efficient Luc and CS protection as seen here, however, do not allow the conclusion that these substrates interact with all sHSPs most strongly through the N-terminal arm. Both CS and Luc have been used as model substrates with numerous sHSPs and found to be protected with varying efficiency. The divergent amino acid sequences of sHSP N-terminal arms indicate no common sequence element is responsible for substrate binding.

We can now describe a model for sHSP-substrate interactions that is compatible with our current data as well as previously published work. As proposed before, at normal temperatures hydrophobic residues/surfaces of sHSPs, which could act as substrate binding sites, are buried in the oligomeric structure. These sites become exposed and available for substrate binding at high temperature either upon dissociation of the oligomer or due to a dramatic conformational change in the oligomer (1, 18, 30). We suggest that substrate binding sites comprise both newly exposed hydrophobic surfaces on the α-crystallin domain of the sHSP along with hydrophobic residues of the N-terminal arm. The flexibility of the N-terminal arm in combination with hydrophobic surfaces on the α-crystallin domain would accommodate binding to a variety of substrates with different hydrophobic surface architecture in the denatured state bound by the sHSP. The affinity and compatibility of the denatured substrate with sHSP binding surfaces will determine, in part, the efficiency of substrate protection by the sHSP and whether the N-terminal or α-crystallin domain contributes the major binding interface. With substrates such as MDH, binding to the α-crystallin domain contributes the major stabilizing interaction, whereas for CS and Luc, interaction with the N-terminal arm forms the major stabilizing interaction. The greater ability of Hsp18.1 compared with Hsp16.9 to protect these two substrates can be hypothesized to arise from the increased hydrophobicity of the Hsp18.1 N-terminal arm contributed by Phe residues 7 and 8, which are in the unique insertion in Hsp18.1, and Phe residues 32 and 41, which are unique to the Hsp18.1 the N-terminal arm. These residues may contribute to forming a hydrophobic surface when the N-terminal arm is freed when the Hsp18.1 oligomer dissociates. It is important to include, however, that sHSP-substrate interactions and the efficiency of substrate protection must further depend on the relative kinetics of the heat-induced sHSP conformational change and substrate denaturation as well as the competing kinetics of substrate aggregation versus binding to the sHSP.

In summary, our results demonstrate that there are multiple substrate binding sites on sHSPs and that attempts to pinpoint one critical substrate binding site fail to recognize this complexity. The remarkable ability of sHSPs to protect diverse substrates likely arises from the exposure of multiple hydrophobic surfaces on the sHSP, which present an array of potential substrate interaction sites compatible with different features of partially unfolded substrates.

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