Cryo Electron Microscopy Analysis of Small Heat Shock Protein 16.5 (Hsp16.5) Complexes with T4 Lysozyme Reveals the Structural Basis of Multimode Binding

Jian Shi1, Hanane A. Koteiche1, Ezelle T. McDonald1, Tara L. Fox2, Phoebe L. Stewart1,2, and Hassane S. Mchaourab1

1Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, TN 37232, USA
2Current address: Department of Pharmacology, Cleveland Center for Membrane and Structural Biology, Case Western Reserve University, Cleveland, OH 44106 USA

Address correspondence to: Hassane S. Mchaourab, email:hassane.mchaourab@vanderbilt.edu, Phone: 615-322-3307, Fax: 615-322-7236 and Phoebe L. Stewart, email:pls47@case.edu, Phone: 216-368-4349, Fax: 216-368-1300

* Running Title: Structure of Hsp16.5/T4L complexes

**Keywords:** small heat shock proteins, cryoEM, electron paramagnetic resonance, chaperone, T4 lysozyme

**Background:** Small heat shock proteins (sHSPs) bind non-native proteins preventing their aggregation.

**Results:** CryoEM image reconstruction reveals that high affinity binding of T4L occurs on the interior of the Hsp16.5 oligomer shell.

**Conclusions:** Substrate binding by Hsp16.5 involves multiple modes and induces oligomer expansion and structural changes in the N-terminal region.

**Significance:** Our findings provide insight into the mechanism of sHSPs.

**SUMMARY**

Small heat shock proteins (sHSPs) are ubiquitous chaperones that bind and sequester non-native proteins preventing their aggregation. Despite extensive studies of sHSPs chaperone activity, the location of the bound substrate within the sHSP oligomer has not been determined. In this paper, we used cryo electron microscopy (cryoEM) to visualize destabilized mutants of T4 Lysozyme (T4L) bound to engineered variants of the small heat shock protein Hsp16.5. In contrast to wild type Hsp16.5, binding of T4L to these variants does not induce oligomer heterogeneity enabling cryoEM analysis of the complexes. CryoEM image reconstruction reveals the sequestration of T4L in the interior of the Hsp16.5 oligomer primarily interacting with the buried N-terminal domain but also tethered by contacts with the α-crystallin domain shell. Analysis of Hsp16.5-WT/T4L complexes uncovers oligomer expansion as a requirement for high affinity binding. In contrast, a low affinity mode of binding is found to involve T4L binding on the outer surface of the oligomer bridging the formation of large complexes of Hsp16.5. These mechanistic principles were validated by cryoEM analysis of an expanded variant of Hsp16.5 in complex with T4L and Hsp16.5-R107G which is equivalent to a mutant of human αB-crystallin linked to cardiomyopathy. In both cases, high affinity binding is found to involve conformational changes in the N-terminal region consistent with a central role of this region in substrate recognition.

Small heat shock proteins (sHSPs)1 are a class of molecular chaperones that play a critical role in protection of cells and organisms from aggregation of misfolded and unfolded proteins under conditions of stress (1,2). Their role in proteostasis is highlighted by the association of point mutations in human sHSPs with a number of diseases including cataracts (3-5), cardiomyopathy (5) and Charcot-Marie-Tooth disease (6). Expressed as subunits of molecular masses between 12 and 40 kDa, sHSPs assemble into
oligomeric structures of diverse symmetry and different degrees of order and polydispersity (7). At one extreme of the evolutionary spectrum, Hsp16.5 from Methanocaldococcus jannaschii forms ordered 24-subunit oligomers (8) while vertebrate lens \( \alpha \)-crystallin assemblies are dynamic and have a variable number of subunits (7,9). The building block of sHSP oligomers is a dimer of a highly conserved C-terminal domain of approximately 100 amino acids referred to as the \( \alpha \)-crystallin domain (8,10-13). Insights into the assembly of sHSPs, primarily from the more ordered and symmetric sHSPs, reveal an outer shell formed by the conserved \( \alpha \)-crystallin domain while the N-terminal domain, which is variable in size and sequence (14), is sequestered in the interior of the oligomer. Packing of \( \alpha \)-crystallin domain dimers is mediated by a short peptide extension C-terminal to the \( \alpha \)-crystallin domain and containing a highly conserved sequence motif (15). The conformational flexibility of this extension is critical for the versatile architectures and diverse symmetries characteristics of sHSP oligomers (13,16).

Numerous studies investigated mechanistic elements of sHSPs chaperone activity *in vitro* (17-20). In common with other chaperones, sHSP binding to client proteins is driven by the latter’s unfolding and functions to prevent the formation of large aggregates and ensuing precipitation. Studies using T4 Lysozyme (T4L) mutants as model substrates demonstrated that under equilibrium conditions favoring T4L native state, sHSPs act as sensors of protein stability with binding affinities that correlate with the level of T4L destabilization (21). Hence, for a series of T4L mutants differing in their free energy of unfolding, sHSPs bind the more destabilized mutants with higher affinity. Mammalian \( \alpha \)-crystallin (22,23) and Hsp27 (19) as well as archael Hsp16.5 (24) bind T4L in two modes characterized by vastly different affinities. A low affinity mode has a stoichiometry of one substrate molecule per sHSP subunit. In contrast, high affinity binding consistently involves the binding of four sHSP subunits to a substrate protein molecule and is the predominant mode of binding to the more stable mutants of T4L. With this high binding capacity, sHSPs provide a buffering mechanism under conditions promoting extensive unfolding of cellular proteins.

Non-equilibrium aggregation-based assays that utilize high temperature or denaturants to promote protein aggregation revealed that sHSPs recognize a broad spectrum of unfolded intermediates ranging from compact, molten globular to extensively unfolded (25-28). Interpretation of these experiments is intrinsically difficult and hindered by the heterogeneity of the substrate proteins under the non-equilibrium conditions typical of aggregation-based assays. To overcome these limitations, Claxton et al. used a spectroscopic approach to characterize the structure of T4L bound to \( \alpha \)-crystallin (29). They demonstrated that native tertiary contacts are disrupted and secondary structure elements are unfolded supporting the contention that the stably bound T4L conformation is extensively unfolded.

In addition to confounding interpretation of substrate-bound conformations, the heterogeneity of sHSP/substrate complexes in aggregation-suppression assays hinders direct structural analysis of bound substrate in the confines of a sHSP oligomer. This is due to changes in the size and symmetry of the sHSP assemblies as well as the lack of control over stoichiometry of bound substrates under these conditions. Within these constraints, current models propose substrate binding at subunit interfaces in the \( \alpha \)-crystallin domain or the C-terminal tail (30,31), and at a groove between two \( \beta \)-strands (32) in the \( \alpha \)-crystallin domain while other studies suggested that the major contacts occur in the N-terminal region (33-36).

To directly visualize the location of substrates bound to Hsp16.5 oligomer, we adopted a cryoEM image reconstruction approach taking advantage of Hsp16.5 variants of well-defined functional and structural properties (24). Hsp16.5-P1 has a 14-amino acid peptide insertion at the junction between its N-terminal region and the conserved \( \alpha \)-crystallin domain (between residues 33 and 34). The inserted peptide, consisting of residues 57-70 in the N-terminal domain of human Hsp27, was selected based on patterns of sequence conservation and functional analysis implicating this peptide in the equilibrium dissociation of Hsp27 (19). A recent crystal structure of Hsp16.5-P1 (16) reveals an oligomer of 48 subunits related by octahedral (24-fold) symmetry, in contrast to
the WT oligomer which consists of 24 subunits with octahedral symmetry. The Hsp16.5-P1 oligomer structure is expanded relative to WT and provides increased access to the N-terminal regions in the interior of the assembly through new square windows, in the outer protein shell (16). The mechanistic significance of this expansion was highlighted by the substantial increase in Hsp16.5-P1 affinity to T4L relative to the WT (24). In contrast, truncation of the N-terminal 32 amino acids in another Hsp16.5 variant, Hsp16.5-TR, reduces its affinity to T4L although the oligomeric assembly, mediated by contacts between α-crystallin domain dimers, remains intact (24,37).

In this paper, cryoEM image reconstruction of Hsp16.5-P1 and Hsp16.5-TR complexes with T4L reveal that bound T4L is primarily localized within the interior of the oligomer presumably in contact with the N-terminal region but also tethered to the α-crystallin domain shell. Analysis of the Hsp16.5-WT complexes identifies the structural arrangements underlying two modes of binding by a sHSP. Our conclusions are supported by analysis of a point mutant of Hsp16.5, R107G, which is equivalent to a disease-causing mutation in human αB-crystallin (5). Thus, the significance of this work extends to the mechanism of disease-linked αA- and αB-crystallin mutants where increased affinity to T4L is accompanied by transition of the ensemble of oligomers to a larger size (38,39).

Experimental Procedures

Protein Expression and Purification. The cloning, expression and purification of Hsp16.5 and its variants (24); T4L and its destabilized mutants (18) were described in previous publications. The Hsp16.5 variants used in this study are: Hsp16.5-TR which lacks the N-terminal 32 amino acids (37) and Hsp16.5-P1 which has a 14-amino acid insertion (residues 57-70 of human Hsp27) between residues 33 and 34 (24). Briefly, Hsp16.5 variants were expressed from pET-derived vectors in E. coli BL21(DE3). A two-step purification protocol consisting of sequential anion exchange and size exclusion chromatography yielded homogenous Hsp16.5 samples by SDS-PAGE. T4L was purified as previously described (40). After elution of a cation exchange column, T4L was incubated with a 10-fold excess of monobromobimane (41) for two hours at room temperature and then stored at 4°C overnight. Unreacted labels were removed in a subsequent size exclusion chromatography (SEC) step on a Superdex 75 column. All samples were concentrated in SEC buffer containing 9 mM Mops, 6mM Tris, 50 mM NaCl, 0.1 mM EDTA at pH 7.2.

Size Exclusion Chromatography of Hsp16.5/T4L Complexes. Labeled T4L mutants were incubated with Hsp16.5 at the appropriate ratios for two hours at 37°C. For bimane labeled samples, the mixtures were loaded on a Superose 6 column (25 ml bed volume) and eluted in the SEC buffer while simultaneously monitoring bimane emission at 465 nm and absorbance at 280 nm at a flow rate of 0.5 ml/min.

Binding of T4L to Hsp16.5-R107G. Samples for binding reactions contained 10 µM of bimane-labeled T4L-D70N mutant. Hsp16.5 and Hsp16.5-R107G were added at the appropriate molar ratio and the samples were incubated at 37°C for 2 hours. Fluorescence intensity was measured by a Photon Technology International (PTI) L-format spectrofluorometer equipped with an RTC2000 temperature controller and a sample holder controlled by a circulating water bath. Samples were excited at 380 nm, and the resulting fluorescence emission spectra were recorded over a 420 nm to 500 nm range.

CryoEM and Image Processing. Concentrated protein samples (0.5 to 1 mg/ml Hsp16.5) were applied in 2.5 µl drops to either Quantifoil grids (Quantifoil Micro Tools GmbH) or 2/2 C-Flat grids (ProtoChips Inc.). The sample grids were blotted and frozen in ethane slush using either a homemade vitrification device or a Vitrobot (FEI, Eindhoven, The Netherlands). Data acquisition was performed on an FEI Tecnai 12 (120kV, LaB6) electron microscope using a Gatan 626 cryo-holder with a nominal magnification of 67,000X and an underfocus range of 0.7-2.9 µm. Digital micrographs were collected on a Gatan UltraScan 2k x 2k CCD camera (Gatan Inc., Pleasanton, CA). The semi-automatic SAM data acquisition package was used to facilitate rapid data collection (24). The Angstrom-to-pixel ratio on the molecular scale is 1.5 Å in the micrographs, and 3 Å after pixel binning performed prior to image processing. Particle images were digitally selected with the Batchboxer routine in EMAN.
(42) and CTF parameters were determined with CTFIND3 (43). Initial class sum images and 3D reconstruction were calculated with IMAGIC (44). The cryoEM datasets were further refined using Frealign (45) with imposed octahedral symmetry. To confirm the reliability of the internal density observed in the octahedral structures, the central density within the protein shell was masked. The resulting masked density maps were used as input 3D volumes for an additional round of Frealign refinement for each sample. In each case, the internal density returned and was nearly identical to that of the original octahedral structure (data not shown). Each dataset was also reprocessed without imposed symmetry using the centrally masked structures as the starting models. Asymmetric processing in Frealign involved a global orientational search, followed by one round of parameter refinement and generation of asymmetric reconstructions for each sample. The resulting asymmetric reconstructions were also symmetrized for comparison with the octahedral reconstructions. All of the symmetrized structures closely resemble the original octahedral structures. Molecular graphics images were produced using the UCSF Chimera package (Pettersen et al., 2004).

EPR Spectroscopy. Construction, purification and labeling of Hsp16.5-P1 single cysteine mutants were previously described (24). Briefly, the purified mutants were incubated with 10-fold excess of MTSSL ((1-Oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl Methanethiosulfonate) for two hours at room temperature and then overnight at 4°C (40,46). The protein was then purified by SEC to remove unreacted spin labels. For analysis of T4L binding, 100 or 50 μM Hsp16.5-P1 was incubated with 50 or 25 μM T4L (Hsp16.5-P1:T4L ratio of 2:1) at 37°C for 2 h in SEC Buffer. Hsp16.5-P1/T4L complexes were both monitored and purified by SEC. This SEC purification step ensured the removal of uncomplexed Hsp16.5-P1 and T4L. Samples of Hsp16.5 and T4L-L99A at the molar ratio indicated in Figure 1 were incubated at 37°C for two hours. Prior to cryoEM analysis, T4L complexes with Hsp16.5 variants were purified by SEC to remove unbound T4L if any (Data not shown). Similar to observations reported for other sHSPs (47), Hsp16.5/T4L complexes are relatively stable and do not dissociate during or following SEC analysis.

The red trace in Figure 1 is the SEC profile of Hsp16.5 in the absence of T4L monitored by absorbance at 280nm. When Hsp16.5 is incubated with bimane-labeled T4L and analyzed by SEC, a fluorescence peak migrating at or near the retention volume of Hsp16.5 is detected indicating the formation of a stable complex between the Hsp16.5 and T4L. For Hsp16.5-WT, 3 different types of complexes each of different size and hydrodynamic radius (Figure 1A, blue trace) are observed. The relative abundance of these three complexes depends on the molar ratio of T4L to Hsp16.5 and corresponds qualitatively to the binding mode. The first peak runs with the void volume of the column and its population can be increased by reducing the molar ratio of sHSP to T4L or increasing the binding temperature which destabilizes T4L (Data not shown).
shown). Thus in these complexes, T4L is bound in both low and high affinity modes. The second species has a similar retention time to that of the Hsp16.5-P1/T4L complex (see below) and appears to be heterogeneous. The third peak has similar retention time as Hsp16.5 in the absence of the substrate. The second and third peaks are favored by low T4L to Hsp16.5 molar ratio and therefore represent high affinity binding complexes. Cryoelectron micrographs of the three Hsp16.5-WT/T4L peaks confirm that peak-1 contains large aggregates, peak-2 heterogeneous complexes, and peak-3 complexes with diameters similar to that of Hsp16.5-WT without substrate (Figure 2).

In contrast, the retention times of the Hsp16.5-P1/T4L (Figure 1B) and Hsp16.5-TR/T4L complex peaks (Figure 1C), identified from the bimane fluorescence, are almost identical to their peaks in the absence of bound T4L (red traces) indicating little change in the hydrodynamic properties of the oligomers when T4L is bound at this ratio of substrate to Hsp16.5. One model wherein binding of T4L would not significantly change the shape of the particle has it localized in the core of the Hsp16.5 particle in contact with the N-terminal region and the interior surface of the α-crystallin shell. Furthermore, the rather homogeneous SEC profile reflects the lack of extensive polydispersity of the sHSP/substrate complex for the two Hsp16.5 variants.

Hsp16.5-WT oligomer expands to accommodate substrate molecules. Cryoelectron micrographs of the SEC peak-1 (Figure 2A) of the Hsp16.5-WT/T4L sample show that this fraction consists of clustered Hsp16.5 particles. Previous studies suggested that low affinity bound T4L are likely to be on the outer shell of the oligomer exposed to solvent (29). These molecules can then mediate the clustering of Hsp16.5 particles. Interestingly, individual Hsp16.5 assemblies (~120 Å in diameter) can be discerned within these large aggregates.

In contrast, the particles in peak-2 appear to be heterogeneous complexes with a wide range of sizes (Figure 2B) reminiscent of Hsp16.5-P1N, an Hsp16.5 variant wherein P1 was inserted in the middle of the N-terminal helix following residue 13 (24). The observation of heterogeneous particles is consistent with previous results of Hsp16.5-WT activated by elevated temperature (48,49). Particle images of peak-3 (Figure 2C) are similar in size to that of Hsp16.5-WT without T4L. The bimane fluorescence signal from peak-3 confirms that T4L substrate is bound to these complexes despite their similar outer diameter to Hsp16.5-WT without bound T4L (Figure 1).

The cryoEM images of all three SEC peaks of the Hsp16.5-WT/T4L sample indicate that Hsp16.5-WT is capable of binding T4L either with or without expansion of the α-crystallin domain shell. We suggest that the expanded and non-expanded form differ by the number of T4L molecules bound. In contrast, SEC analysis of Hsp16.5-TR demonstrates that truncating Hsp16.5 N-terminal region abolishes its ability to expand as a consequence of T4L binding. Insertion of the human Hsp27-P1 peptide, as in Hsp16.5-P1, leads to an expanded sHSP oligomer that can accommodate T4L without further changes in the oligomeric structure.

Hsp16.5-WT can bind T4L without changing the outer α-crystallin shell. CryoEM reconstructions were conducted for samples corresponding to the homogenous peak-3 of Hsp16.5-WT/T4L (1:4 T4L:sHSP subunit molar ratio), as well as for Hsp16.5-WT as a control, using the previous 13Å Hsp16.5-WT density map as an initial model (7). Briefly, datasets for Hsp16.5-WT and Hsp16.5-WT/T4L were collected on a Tecnai 12 (120kV, LaB6 filament) cryoelectron microscope. They each included about 12,000 particle images. Refinement with imposed octahedral symmetry produced reconstructions with nominal resolutions of 9 Å (FSC 0.5). Separate refinement of these datasets without imposed symmetry resulted in structures at about 13Å resolution. However, visual comparison of the cryoEM structures with filtered representations of the Hsp16.5-WT crystal structure (PDB-ID 1SHS) and the Hsp16.5-P1 crystal structure (PDB-ID 4ELD) indicates that 15 Å is more reasonable approximation for the resolution. The data collection and refinement statistics are shown in Table 1.

The Hsp16.5-WT and Hsp16.5-WT/T4L cryoEM reconstructions are compared in Figure 3 and show comparable features for the outer α-crystallin protein shell. When processed with symmetry the structures of both complexes appear similar in the interior as well (Figure 3A,B). However, when processed without symmetry the two structures show subtle differences in their internal density (Figure 3C,D). Imposing
octahedral symmetry on structures after processing without symmetry produces weak density in the interior of each cavity (Figure 3 E,F). Quantitation of the internal density regions indicates that Hsp16.5-WT/T4L has 47% more mass within the cavity. The density within Hsp16.5-WT is 83% of that expected for 24 copies of the N-termini with the flexibility of the N-terminal regions likely accounting for the lower than expected volume. The density within Hsp16.5-WT/T4L is 95% of that expected for 24 copies of the N-termini plus one molecule of T4L. This is consistent with volume calculations indicating that the Hsp16.5-WT cavity is only large enough to accommodate one molecule of T4L in addition to 24 N-terminal regions.

**Bound T4L is sequestered in the interior cavity of Hsp16.5-TR.** Based on the observation of homogenous SEC peaks, we predicted that substrate complexes of Hsp16.5-TR and Hsp16.5-P1 would also be amenable to cryoEM three-dimensional image reconstruction. Samples of the two Hsp16.5-variant/T4L complexes were collected following SEC and used for analysis. For consistency, we re-analyzed Hsp16.5-TR and Hsp16.5-P1 in the absence of substrate in a side by side fashion on the same microscope, collected similar number of particle images with and without T4L substrate and carried out the same analysis protocol. The cryoEM data collection and image processing protocol was similar to that used for Hsp16.5-WT and Hsp16.5-WT/T4L.

The Hsp16.5-TR and Hsp16.5-TR/T4L reconstructions have a similar external surface (Figure 4). The diameter of the Hsp16.5-TR oligomer with and without substrate is ~120 Å, which is the same as Hsp16.5 WT indicating a lack of expansion of the α-crystallin domain shell upon substrate binding. When the reconstructions processed with imposed symmetry are cropped in half to reveal the interior cavity, it is clear that Hsp16.5-TR/T4L has weak internal density that is not present in Hsp16.5-TR alone. We attribute the weak internal density to bound, unfolded or partially unfolded T4L substrate.

The localization of unfolded T4L in the inner cavity of Hsp16.5-TR suggests that T4L must be tethered through contacts with the α-crystallin domain shell. Indeed, processing of the Hsp16.5-TR/T4L dataset without imposed symmetry reveals that the internal density is in close proximity to the inner surface of the protein shell (Figure 4D). The moderate resolution, as well as presumed structural heterogeneity between Hsp16.5-TR/T4L complexes, hinders the identification of the contact residues.

Symmetrizing the asymmetric structures produces appreciable density only in the cavity of Hsp16.5-TR/T4L (Figure 4E,F). Volume calculations indicate that the Hsp16.5-TR cavity could accommodate up to six molecules of T4L, however the observed internal density is more consistent with just one or two bound T4L molecules.

*The flexible N-terminal region of Hsp16.5-P1 is involved in substrate binding.* CryoEM reconstructions of Hsp16.5-P1 both with and without T4L have similar external surfaces indicating the absence of major structural rearrangements in the outer α-crystallin domain shell upon substrate binding (Figure 5). Hsp16.5-P1 is composed of 48 subunits and has a larger outer diameter (~182 Å) than Hsp16.5-TR and WT (~120 Å). The volume of the internal cavity of Hsp16.5-P1 is large enough to enclose up to ~10 unfolded T4L molecules, in addition to 48 copies of the N-terminal region. When the cryoEM reconstructions of Hsp16.5-P1 and Hsp16.5-P1/T4L that were processed with imposed symmetry are cropped in half, both reveal internal density. In the absence of T4L, the internal density is attributable to 48 copies of the N-terminal region (amino acids 1-33) and the inserted 14-amino acid P1 peptide. The P1 peptide sequence is rich in Ala and Pro residues and is presumably flexible (19,24). The N-terminal region may have defined interactions between subunits, as is indicated by EPR analysis (24), but if the P1 linker peptide is flexible then the cryoEM reconstruction will not reveal defined density for the N-terminal regions. Indeed this is the case, as the internal density within Hsp16.5-P1 is not as well resolved as that of the α-crystallin domains.

Comparison of the symmetric cryoEM reconstructions of Hsp16.5-P1 with and without bound T4L indicates a considerable rearrangement of the N-terminal regions upon T4L binding (Figure 5 A,B). The internal cryoEM density within Hsp16.5-P1/T4L is weaker than in Hsp16.5-P1 and shifted more towards the outer α-crystallin domain protein shell. This suggests that the bound T4L molecules may be interacting with
the N-terminal regions of Hsp16.5-P1 as well as the inner surface of the α-crystallin domains. Processing of the Hsp16.5-P1 and Hsp16.5-P1/T4L datasets without imposed symmetry leads to similar results (Figure 5C,D), further supporting the conclusion of conformational changes in the N-terminal region. When the asymmetric structures were symmetrized they closely resembled the octahedral structures (Figure 5E,F).

Previously, we used Site Directed Spin Labeling EPR (50) combined with cryoEM density to build an atomic model for the N-terminal region of Hsp16.5-WT (51). In this model each N-terminal region has an α-helix (amino acids 6-19) and 4 α-helices interact at the 4-fold symmetry axes of the oligomeric assembly. EPR measurements indicate that the N-terminal regions of Hsp16.5-WT and Hsp16.5-P1 have similar conformations (24). If the EPR/cryoEM N-terminal model originally built for Hsp16.5 WT is expanded to include 48 copies of the N-terminal region, we obtain a model for Hsp16.5-P1 internal segments that agrees reasonably well with the cryoEM density (Figure 6). Twelve clusters of 4 α-helices form a sphere ~70Å in diameter, matching the internal cryoEM density observed in Hsp16.5-P1. This leaves a gap of ~15Å between the cluster of α-helices and the external α-crystallin domain protein shell. This gap could easily be spanned by the P1 peptide insert.

To explore structural rearrangements in the N-terminal domain induced by T4L binding, we collected EPR spectra of spin labels introduced at selected residues in Hsp16.5-P1 (50). These residues have been shown previously to be at or near interfaces of the N-terminal region (24). Spin label proximity is manifested by broadening of the EPR lineshapes of residue 18. Figure 7 shows that upon T4L binding, there is a detectable reduction in spectral broadening at residue 18 consistent with changes in subunit packing. At residues 10 and 14, the spin label motion becomes more restricted indicating either direct contact with T4L or local structural changes. The effect of substrate binding is somewhat attenuated by the use of molar excess of Hsp16.5 necessary to keep the sample from aggregating at the concentrations required for EPR. By comparison, there is no change in the EPR spectra of G34 in the α-crystallin domain which is located at a 2-fold interface or at residue 102 which is located at the 3- and 4-fold windows (24). This result reinforces the conclusions that the N-terminal domain is directly involved in T4L binding and that the outer shell remains intact in the Hsp16.5-P1/T4L complex.

Substrate binding requires conformational changes in the N-terminal region: evidence from analysis of Hsp16.5-R107G mutant. The critical role of the N-terminal region in substrate binding, deduced from image reconstruction of Hsp16.5-P1/T4L complexes, was further reinforced by cryoEM analysis of Hsp16.5 R107G mutant. R107G is the equivalent mutant to human αB-crystallin R120G, a mutation associated with desmin-related cardiomyopathy (5). The R120G substitution changes the overall size and polydispersity of the αB-crystallin oligomer. In aggregation assays, the protein co-precipitates with its substrates suggesting increased affinity and/or loss of solubility (39). More recently, a crystal structure of truncated αB-crystallin bearing the R120G mutation suggested changes at the dimer interface of its α-crystallin domain (32).

We find that Hsp16.5-R107G has higher affinity to destabilized T4L mutants than Hsp16.5-WT. We illustrate this property in Figure 8 for T4L mutant D70N which marginally bind Hsp16.5-WT (24). The replacement of aspartate 70 which is involved in a partially buried salt bridge leads to a moderate change in the free energy of unfolding of T4L (18). A unique cysteine was inserted at position 151 enabling the selective attachment of a bimane group to monitor binding. Incubation with Hsp16.5-WT does not change the fluorescence intensity (Figure 8) or the anisotropy (data not shown) of bimane consistent with lack of extensive binding to this particular mutant of T4L. In contrast, the corresponding isotherm for Hsp16.5-R107G indicates robust two-mode binding emphasizing a large change in Hsp16.5 affinity to T4L induced by the R107G substitution. This finding parallels results from analysis of cataract-linked αA-crystallin mutants which have an order of magnitude higher apparent affinity than their WT counterpart (22). For α-crystallin and Hsp27, the proposed mechanism for activation is increased equilibrium dissociation to smaller oligomers which exposes the presumably buried N-terminal region (22). In the absence of evidence that Hsp16.5 undergoes subunit
exchange at the temperature of the binding assay, we sought to define the structural basis of the activation of the R107G mutant.

For this purpose, a cryoEM reconstruction was calculated for Hsp16.5-R107G and compared with that of Hsp16.5-WT (Figure 9). Both assemblies have similar outer protein shells with openings at the 3-fold axes. When octahedral symmetry is imposed on the Hsp16.5-R107G dataset, the interior density of the reconstruction is displaced from the center of the assembly toward the outer protein shell. This indicates a different average conformation for the N-terminal region in Hsp16.5-R107G compared to Hsp16.5-WT. The results of processing the Hsp16.5-R107G dataset without imposed symmetry (Figure 9C), as well as imposing octahedral symmetry on the asymmetric structure (Figure 9D), are both consistent with an alteration in the structure of the N-terminal region.

In the crystal structure of Hsp16.5, R107G makes an intra-subunit bifurcated hydrogen bond with Gly 41(8) which is 7 residues away from the N-terminal region and provides a plausible mechanism for transmission of the mutation to the N-terminal region. In conjunction with the binding isotherms, this result indicates that by adopting a binding-competent conformation in the N-terminal region, the R107G mutation increases the apparent affinity to T4L.

**Discussion**

The mechanism by which sHSPs bind their substrates and the location of the binding elements within the oligomeric structure are central questions in the sHSP field. By taking advantage of Hsp16.5 variants where substrate binding does not lead to heterogeneous assemblies, we were able to directly visualize the location of bound T4L. Both SEC and cryoEM analyses indicate that Hsp16.5-WT can undergo expansion of the α-crystallin domain shell by incorporation of additional subunits, which leads to an increase in the volume of the interior cavity and presumably enables binding of more substrate molecules per oligomer. We propose that high affinity binding involves sequestration of the substrate to the inside of the oligomer where it interacts with the N-terminal region as well as the inner surface of the α-crystallin domain shell which is 50% non-polar (16). The cryoEM structures of Hsp16.5-WT/T4L, Hsp16.5-TR/T4L and Hsp16.5-P1/T4L show differences within the internal cavity compared to the assemblies without T4L although the differences are most apparent for Hsp16.5-TR and Hsp16.5-P1. The internal density within Hsp16.5-TR/T4L demonstrates that low affinity binding can occur in the absence of the N-terminal region suggesting involvement of the α-crystallin domain. The surface of this domain accessible to the inside of Hsp16.5 particle is largely non-polar as noted in the crystal structures of the WT (8) and P1 (16). This may provide a complementary surface for substrate interactions in addition to the N-terminal region.

The cryoEM structures of the engineered expanded Hsp16.5 variant, Hsp16.5-P1, with and without substrate reveal a conformational change of the N-terminal region, with the internal density shifting closer to the α-crystallin protein shell (Figures 5). Our cryoEM/EPR based model of 48 copies of the N-terminal region is in agreement with the observed internal density in the Hsp16.5-P1 structure without substrate (Figure 6). In this model hydrophobic sidechains within the N-terminal region are buried at the interface with other N-termini. We propose that upon substrate binding the N-termini splay towards the α-crystallin protein shell and expose these hydrophobic sidechains (Figure 10). Our working hypothesis based on the results of this study is that sHSP activation includes, in part, conformational change of the N-terminal regions exposing potential hydrophobic substrate binding sites.

We tested this model by determining the cryoEM structure of Hsp16.5-R107G. Hsp16.5-R107G has higher affinity to destabilized T4L mutants than Hsp16.5-WT. Comparison of the symmetric Hsp16.5-WT and Hsp16.5-R107G cryoEM structures (Figure 9A and B) reveals a shift for the density corresponding to the N-terminal region from a more central position in the WT, to a position closer to the α-crystallin domain protein shell in the mutant form. Our interpretation of this result is that Hsp16.5-R107G is in a partially activated state even in the absence of substrate. We propose that the mutation induces a change in the packing of the N-terminal regions that leads to exposure of hydrophobic regions.

A previous cryoEM study comparing wild-type and the R120G mutant form of human αB-crystallin found that the mutant assemblies are more irregular in shape and without a well-defined central cavity than the wild-type (Bova et al,
The structural characteristics of the mutant are similar to those observed by cryoEM for wild-type αB-crystallin after addition of a representative unfolded target protein, α-lactalbumin (Haley et al., 2000). These earlier results suggest that the structure of the R120G mutant form of human αB-crystallin more closely resembles a substrate-bound form than a substrate-free form of αB-crystallin. The same observation can be made for the cryoEM data presented here on Hsp16.5-R107G. The structure of Hsp16.5-R107G more closely resembles a substrate-bound form than a substrate-free form of Hsp16.5. A recent crystal structure of the αB-crystallin dimer bearing the R120G mutation reveals that the loss of the charged side chain results in rearrangement of an extensive array of charged interactions at the dimer interface (32). Because α-crystallin and Hsp16.5 have distinct dimer interfaces, it is possible that the mutations have additional structural and functional consequences in the human protein.

Two models have been advanced to describe structural changes in sHSP oligomers upon substrate recognition (1,2,52). The first, referred to as the “dynamic” model of activation (2,38,53), builds on evidence that equilibrium dissociation of subunits, a hallmark of eukaryotic sHSP oligomers, mediates recognition and binding of substrates. In this model, dissociation serves to expose otherwise buried contact sites required for high affinity binding of substrates. Studies of Hsp27 demonstrated that dissociation of the oligomer exposes the N-terminal domain in a flexible and dynamic conformation (53). The dynamic model does not exclude the possibility of low affinity binding by the larger oligomers through structural changes distinct from those associated with dissociation.

The second model, referred to as the “static” model, postulates that substrate binding does not require subunit exchange. The most developed evidence in support of the static model are studies of yeast Hsp26 interactions with substrates (52). Hsp26 mutants defective in subunit exchange can nevertheless suppress aggregation of unfolded proteins. For ordered sHSPs, a conformational change in the intact oligomer occurs to facilitate access to the substrate binding sites in the N-terminal region. Because Hsp16.5 does not undergo subunit exchange at the temperature of T4L binding (54), we propose that oligomer expansion facilitates binding.

Visualizing the substrate bound to Hsp16.5 provides direct insight into the structural mechanism mediating static, dissociation-independent activation. We have previously shown that expansion of Hsp16.5-P1 by the P1 insertion leads to 4-fold windows in the outer shell that have a 50% larger radius than the 3-fold windows (24). Hsp16.5-P1 has an outer diameter of 163Å compared to 115Å for the WT (16). Expansion of Hsp16.5-P1 provides increased access to the N-terminal domain, the putative substrate binding region and thus leads to a higher apparent affinity. Modeling indicates that the larger windows of Hsp16.5-P1 (~45 Å) are suitable for allowing partially denatured T4L to pass through to reach the internal cavity. The smaller holes of Hsp16.5-WT and Hsp16.5-TR (~30 Å) would necessitate more unfolding of T4L. In the WT, expansion of the protein shell can occur as revealed by a separate population in the cryoEM analysis (see Figure 2B), but it requires energetically unfavorable distortion of the oligomer. Thus conformational changes through expansion replace oligomer dissociation in the dynamic model of activation. Furthermore, the results of this paper suggest that in addition to access to the interior of the oligomer, high affinity binding requires conformational changes in the N-terminal region.

By providing evidence of the role of sHSP N-terminal domains in substrate binding, our findings fit into a global model of domain specialization in sHSP structure and mechanism. While the α-crystallin domain mediates the assembly of the dimeric building block across the superfamily, the N-terminal domain appears to be the hub for tuning the oligomeric structure and the site for high affinity binding. The likely hydrophobic nature of the interaction with substrate puts few restrictions on the sequence space available. The divergence in size and sequence reflect its acquired role in the assembly of sHSP oligomers and the fine tuning of their dynamic properties.

Acknowledgment: This work was supported by the National Institutes of Health, National Eye Institute grant EY12018 to Hassane S. Mchaourab. The authors thank Guangyong Yang for help in protein expression and purification.
References


1 **Abbreviations:** sHSP: small heat-shock protein; Hsp16.5: heat-shock protein 16.5; T4L: T4 Lysozyme; WT: wild type; cryoEM: cryo electron microscopy; EPR: electron paramagnetic resonance; SEC: size exclusion chromatography; MTSSL: (1-Oxyl-2,2,5,5-tetramethyl-∆3-pyrroline-3-methyl Methanethiosulfonate).
Figure Legends

Figure 1. SEC analysis of T4L-L99A binding to Hsp16.5: A) WT, B) P1 and C) TR. Samples of Hsp16.5 without T4L (red traces) and with T4L (blue traces) at the indicated molar ratios were incubated at 37°C and then chromatographed as described in the methods section. The red traces monitor the absorbance of unbound Hsp16.5. The blue traces monitor the fluorescence of a bimane probe attached to T4L. Upon complex formation, the bimane fluorescence identifies the peak corresponding to the Hsp16.5/T4L complex. The coincident retention volumes of the absorbance and fluorescence traces demonstrate that T4L binding does not significantly change the hydrodynamic volume of Hsp16.5-P1 and Hsp16.5-TR.

Figure 2. Cryoelectron micrographs of Hsp16.5-WT/T4L complexes. (A) Cryoelectron micrograph of a peak-1 (Figure 1A) sample showing large protein aggregates over 500 Å in diameter. (B) Cryoelectron micrograph of a peak-2 sample showing oligomers ranging from 120 Å to 182 Å in diameter. White circles of these two diameters are shown next to individual oligomers. (C) Cryoelectron micrograph of a peak-3 sample. Most oligomers in peak 3 have a diameter of 120 Å similar to Hsp16.5-WT without substrate. The scale bar represents 200 Å.

Figure 3. CryoEM structures of Hsp16.5-WT and the Hsp16.5-WT/T4L complex. (A) Surface and cropped representations of the Hsp16.5-WT structure with imposed octahedral symmetry viewed along a four-fold symmetry axis. The isosurface level is set to enclose 100% of the expected volume. (B) Similar representations of the Hsp16.5-WT/T4L complex with imposed octahedral symmetry. (C,D) Cropped representations of Hsp16.5-WT and Hsp16.5-WT/T4L processed without imposed symmetry. (E,F) Same structures as in panels C and D but with octahedral symmetry imposed after processing. The crop planes are colored by density value with the strongest density in red and the weakest density in blue. The scale bar represents 50 Å.

Figure 4. CryoEM structures of Hsp16.5-TR and the Hsp16.5-TR/T4L complex. (A) Surface and cropped representations of the Hsp16.5-TR structure with imposed octahedral symmetry viewed along a four-fold symmetry axis. The isosurface level is set to enclose 100% of the expected volume. (B) Similar representations of the Hsp16.5-TR/T4L complex with imposed octahedral symmetry. (C,D) Cropped representations of Hsp16.5-TR and Hsp16.5-TR/T4L processed without imposed symmetry. (E,F) Same structures as in panels C and D but with octahedral symmetry imposed after processing. The crop planes are colored as in Figure 3. The scale bar represents 50 Å.

Figure 5. CryoEM structures of Hsp16.5-P1 and the Hsp16.5-P1/T4L complex. (A) Surface and cropped representations of the Hsp16.5-P1 structure with imposed octahedral symmetry viewed along a four-fold symmetry axis. The isosurface level is set to enclose 100% of the expected volume. (B) Similar representations of the Hsp16.5-P1/T4L complex with imposed octahedral symmetry. (C,D) Cropped representations of Hsp16.5-P1 and Hsp16.5-P1/T4L processed without imposed symmetry. (E,F) Same structures as in panels C and D but with octahedral symmetry imposed after processing. The crop planes are colored as in Figure 3. The scale bar represents 50 Å.

Figure 6. CryoEM structure of Hsp16.5-P1 and model of 48 N-terminal regions. (A) CryoEM structure viewed along a 2-fold symmetry axis and radially color coded (radius of 35 Å in blue, radius of 75 Å in red). (B) CryoEM structure viewed along a 4-fold symmetry axis. (C) Model of 48 copies of the N-terminal region (aa 6-25) arranged with a 4-helix bundle at every 2-fold axis of the octahedral structure. (D) A 40 Å thick slab of density from the cryoEM structure (mesh) with one 4-helix bundle (purple).

Figure 7. EPR spectra of spin labeled Hsp16.5-P1 at selected residues in the N-terminal domain (10, 14 and 18) and the α-crystallin domain (34, 102). The changes in the EPR lineshape at residues 10 and 14 suggest that the spin labels become more sterically restricted upon addition of T4L. At residue 18, the lineshape changes report a reduction in spin-spin coupling and/or spin label mobility indicative of a
structural change at this interface. In contrast, there is little if any changes in the EPR spectrum at sites 34 and 102 indicating little changes in the α-crystallin domain shell.

**Figure 8.** Binding isotherms of T4L-D70N to Hsp16.5-WT and Hsp16.5-R107G at 37°C. For this T4L mutant, little or no binding is detected for the WT as demonstrated by the lack of change in bimane fluorescence. In contrast, the shape of R107G isotherm is indicative of two-mode binding. The data points are the average of three independent measurements and the error bars represent standard deviations.

**Figure 9.** Comparison of cryoEM structures of Hsp16.5-WT and Hsp16.5-R107G in cropped representations. (A) HSP16.5-WT with imposed octahedral symmetry has weak density throughout the interior cavity. (B) HSP16.5-R107G with imposed octahedral symmetry has density within the interior cavity shifted toward the outer protein shell formed by α-crystallin domains. (C) Hsp16.5-R107G processed without imposed symmetry has similar off-center density. (D) Same structure as in panel C but with octahedral symmetry imposed after processing. The crop planes are colored as in Figure 3. The scale bar represents 50 Å.

**Figure 10.** Proposed models for the N-terminal region of Hsp16.5-P1 with and without substrate. Side view with four N-terminal helices modeled for Hsp16.5-P1 (purple) and the Hsp16.5-P1/T4L complex (red) with sidechains for hydrophobic side chains (F11, F15, F19, M23) displayed.
### Table 1. CryoEM Data Collection and Refinement Statistics

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Molar Ratio (substrate: sHSP subunit)</th>
<th>Particle numbers</th>
<th>Octahedral Final Resolution (FSC 0.5 threshold)*</th>
<th>Asymmetric Final Resolution (FSC 0.5 threshold)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp16.5-WT</td>
<td></td>
<td>12,909</td>
<td>9 Å</td>
<td>13 Å</td>
</tr>
<tr>
<td>Hsp16.5-WT+T4L Peak3</td>
<td>1:4</td>
<td>11,451</td>
<td>9 Å</td>
<td>12 Å</td>
</tr>
<tr>
<td>Hsp16.5-TR</td>
<td></td>
<td>18,799</td>
<td>9 Å</td>
<td>13 Å</td>
</tr>
<tr>
<td>Hsp16.5-TR+T4L</td>
<td>1:4</td>
<td>20,499</td>
<td>12 Å</td>
<td>14 Å</td>
</tr>
<tr>
<td>Hsp16.5-P1</td>
<td></td>
<td>13,798</td>
<td>11 Å</td>
<td>16 Å</td>
</tr>
<tr>
<td>Hsp16.5-P1+T4L</td>
<td>1:2</td>
<td>19,804</td>
<td>12 Å</td>
<td>18 Å</td>
</tr>
<tr>
<td>Hsp16.5-R107G</td>
<td></td>
<td>13,390</td>
<td>9 Å</td>
<td>15 Å</td>
</tr>
</tbody>
</table>

*Visual comparison with filtered representations of the Hsp16.5-WT crystal structure (PDB-ID 1SHS) and the Hsp16.5-P1 crystal structure (PDB-ID 4ELD) indicates that 15 Å is a reasonable approximation for the resolution of all of the cryoEM structures.
Figure 1
Figure 4

A  

Hsp16.5-TR with symmetry

B  

Hsp16.5-TR/T4L with symmetry

C  

Hsp16.5-TR without symmetry

D  

Hsp16.5-TR/T4L without symmetry

E  

Hsp16.5-TR without symmetry then symmetrized

F  

Hsp16.5-TR/T4L without symmetry then symmetrized

50 Å
Figure 5
Figure 7
Figure 8
Figure 9

A  Hsp16.5-WT with symmetry
B  Hsp16.5-R107G with symmetry
C  Hsp16.5-R107G without symmetry
D  Hsp16.5-R107G without symmetry then symmetrized

50 Å
Cryo Electron Microscopy Analysis of Small Heat Shock Protein 16.5 (Hsp16.5) Complexes with T4 Lysozyme Reveals the Structural Basis of Multimode Binding
Jian Shi, Hanane A. Koteiche, Ezelle T. McDonald, Tara L. Fox, Phoebe L. Stewart and Hassane S. Mchaourab

J. Biol. Chem. published online December 30, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M112.388132

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2012/12/30/jbc.M112.388132.full.html#ref-list-1