Cryoelectron Microscopy and EPR Analysis of Engineered Symmetric and Polydisperse Hsp16.5 Assemblies Reveals Determinants of Polydispersity and Substrate Binding

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We have identified sequence and structural determinants of oligomer size, symmetry, and polydispersity in the small heat shock protein superfamily. Using an insertion mutagenesis strategy that mimics evolutionary sequence divergence, we induced the ordered oligomer of Methanococcus jannaschii Hsp16.5 to transition to either expanded symmetric or polydisperse assemblies. A hybrid approach combining spin labeling EPR and cryoelectron microscopy imaging at 10 Å resolution reveals that the underlying plasticity is mediated by a packing interface with minimal contacts and a flexible C-terminal tether between dimers. Twenty-four dimeric building blocks related by octahedral symmetry assemble into the expanded symmetric oligomer. In contrast, the polydisperse variant has an ordered dimeric building block that heterogeneously packs to yield oligomers of various sizes. Increased exposure of the N-terminal region in the Hsp16.5 variants correlates with enhanced binding to destabilized mutants of T4 lysozyme, whereas deletion of this region reduces binding. Transition to larger intermediates with enhanced substrate binding capacity has been observed in other small heat shock proteins including lens α-crystallin mutants linked to congenital cataract. Together, these results provide a mechanistic perspective on substrate recognition and binding by the small heat shock protein superfamily.

In the crowded molecular environment of the cell, five classes of heat shock proteins (HSP) act as molecular chaperones by suppressing protein aggregation through the selective binding of proteins in non-native states (1, 2). Subsequent processing of the bound polypeptides involves either release and refolding or delivery to degradation machineries. Small heat shock proteins (sHSP) are members of a superfamily of oligomeric proteins characterized by a conserved module in the C-terminal region of their sequences referred to as the α-crystallin domain (3, 4). In vitro, sHSP are protein stability sensors that can differentially bind mutants with similar structures in the folded state but different free energies of unfolding (5, 6). Substrates are bound with efficiencies that can reach 1 substrate molecule/subunit of sHSP and without direct input of ATP energy (7–9). Bound substrates are protected against heat inactivation and can be released from sHSP complexes by other members of the chaperone network (7, 10). sHSP play critical roles in maintaining vertebrate lens transparency (11) and in a number of physiologic processes (12–14). Their expression in Caenorhabditis elegans promotes longevity and delays the onset of polyglutamine protein aggregation (15).

The structural basis of substrate recognition and binding by sHSP continues to be enigmatic. Most sHSP assemble into polydisperse and dynamic oligomers, and their heterogeneity is enhanced by substrate binding. Available high resolution structures are for three ordered, symmetrical sHSP assemblies, Hsp16.5 from Methanococcus jannaschii (MJ) (16), Hsp16.9 from wheat (17), and the more distantly related Tsp36 from a parasitic flatworm (18), but there is a paucity of structural information for polydisperse sHSP. Many sHSP oligomers exchange subunits on a relatively fast time scale (19, 20). The functional role of equilibrium dissociation has been controversial (21), with one class of models implicating the dissociated form as a major binding-competent species (6, 17).

Taken together, crystallographic (16, 17), cryoEM (22, 23), and spin labeling EPR (24) analyses of selected sHSP reveal a remarkably versatile protein scaffold amenable to a wide range of oligomeric structures and dynamic behaviors. The oligomer of Hsp16.5 is monodisperse and composed of 24 subunits assembled with octahedral symmetry. On the other end of the spectrum, the mammalian α-crystallins and human Hsp27 form heterogeneous oligomers of different sizes and shapes (22). The basic building block of Hsp16.5 is a dimer of the conserved α-crystallin domain. In wheat Hsp16.9, distinct packing by the dimeric building block, facilitated by two conformations of the C-terminal extension, reshapes the quaternary structure, resulting in a double disk assembly (17).

The mechanism by which a conserved module such as the α-crystallin domain mediates highly specific contacts to form...
symmetrical assemblies in some contexts and yet can participate in polydisperse assemblies in others is one of the mysteries in the sHSP field. Comparative sequence analysis provides hints regarding the determinants of the quaternary structural variation found in the sHSP family (3). sHSP have undergone extensive sequence divergence with remnants of sequence similarity in the α-crystallin domain, whereas the N-terminal region is highly variable and is generally hydrophobic. The short C-terminal extensions following the α-crystallin domain are of variable length with a conserved sequence motif that serves to tether the dimeric building blocks in Hsp16.5 and Hsp16.9.

Guided by analysis of sHSP sequence divergence, we have transformed the ordered and monodisperse Hsp16.5 assembly into both a larger symmetrical assembly as well as polydisperse oligomers consisting of variable numbers of subunits. The structural basis of oligomeric plasticity is gleaned from a hybrid approach combining cryoEM and site-directed spin labeling (SDSL) EPR (25). The functional consequence of polydispersity is explored using a steady state binding assay to non-native states of T4 lysozyme (T4L) (5). Together, these results identify the sequence motifs responsible for the transition between ordered and variable quaternary structures, define the structural basis of oligomeric plasticity, and elucidate the nature of the intermediates involved in substrate binding and recognition.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—Site-directed mutagenesis of T4L and Hsp16.5-TR (amino acids 34–147) was described previously (26, 27). To generate Hsp16.5-P1 and Hsp16.5-P1N, the Hsp27 P1 peptide sequence was inserted in the Hsp16.5 gene between residues 33 and 34 and residues 12 and 13, respectively, using overlap extension PCR. The cysteine mutants of Hsp16.5-P1 were generated using the QuikChange method (Stratagene). All of the plasmids were sequenced to confirm the substitution and the absence of unwanted changes.

**Expression, Purification, and Labeling**—Expression, purification, and labeling of T4L mutants were carried out as described (27). Hsp16.5 and its mutants were purified by sequential, anion exchange, hydrophobic interaction, and size exclusion (SEC) chromatographies (26). For SEC, a Superdex 200 column was used for Hsp16.5 and Hsp16.5-TR, and a Superose 6 column was used for Hsp16.5-P1 and Hsp16.5-P1N. The buffer for SEC contained 9 mM MOPS, 6 mM Tris, 50 mM NaCl, 0.1 mM EDTA, and 0.02% NaN₃ at pH 7.2 and was used for subsequent binding studies.

Labeling of the cysteine mutants with either bimane or MTSSL was carried out as previously described (9, 26). Single-site mutants are named by specifying the original residue and residue number followed by R1 (Scheme 1).

**Binding of T4L Mutants to Hsp16.5 Variants**—Samples containing 5, 7, 10, or 14 μM T4L and varying concentrations of Hsp16.5 were incubated at 37 °C for 2 h. The fluorescence intensity measurements were carried out on a PTI L-format spectrofluorometer equipped with an RTC2000 temperature controller and a sample holder containing a Peltier heater/cooler. The bimane molecule was excited at 380 nm, and the fluorescence emission spectra were then recorded in the 420–500 nm range. The binding isotherms were fit with the appropriate equations (9) using the program Origin (OriginLab Inc.). The Levenberg-Marquart method was used for nonlinear least squares fits.

**Size Exclusion Chromatography**—Analytical SEC was performed on a Superose 6 column. The samples, at a concentration of 1 mg/ml, were prepared in the SEC buffer described above and injected from 100-μl volumes at a flow rate of 0.5 ml/min.

For molar mass determination, a multi-angle laser light-scattering detector (Wyatt Technologies) was connected in line with the absorption detector. The signal at the 90° angle was analyzed to obtain molar mass values.

**EPR**—EPR spectra were collected on a Bruker EMX spectrometer. The microwave power was 5 milliwatt, the modulation amplitude was 1.6 G, and the scan width was 200 G. Distances were determined by nonlinear least squares fitting of the EPR spectra based on the convolution approach (28), assuming one or two Gaussian distance distributions (see supplemental Fig. S2).

**CryoEM Imaging**—CryoEM grids were prepared by applying 2.5 μl of (0.5–1 mg/ml) protein, to R2/4 Quantifoil grids (Quantifoil Micro Tools GmbH). The grids were frozen in ethane slush using either a homemade vitrification device or a Vitrobot (FEI, Eindhoven, The Netherlands). CryoEM data collection was performed for all of the samples on an FEI Tecnai 12 (120 kV, LaB₆) electron microscope (120 kV; 67,000× nominal magnification; underfocus range, 0.7 to 2.90 μm) equipped with a Gatan cryo-holder and UltraScan 2kx2k CCD camera (Gatan Inc., Pleasanton, CA). The 120-kV data sets include 66 micrographs for Hsp16.5-TR, 377 for Hsp16.5-P1, and 233 for Hsp16.5-P1N. The Angstrom-to-pixel ratio on the molecular scale for the 120-kV micrographs is 1.5 Å and 3.1 Å after binning for image processing.

A high resolution cryoEM data set was collected for Hsp16.5-P1 on an FEI Polara (300 kV, FEG) electron microscope (liquid nitrogen temperature, 300 kV; 310,000× nominal magnification; underfocus range, 0.6–1.9 μm) with an UltraScan 4kx4k CCD camera. The data set contains 809 micrographs. The Angstrom-to-pixel ratio on the molecular scale for the 300-kV micrographs is 0.4 Å and 1.5 Å after binning for image processing. The microscope magnifications were calibrated to within ±0.5% with a negatively stained TMV grid.

**Image Processing**—For the 120-kV micrographs, contrast transfer function (CTF) correction was performed with the CTFIT routine in the EMAN v1.7 package (29). For the 300-kV micrographs, the CTF parameters were determined with CTFFIND3 (30), and CTF correction (phase flipping only) was performed by the EMAN routine APPLYCTF. The BOXER semi-automatic routine in EMAN was used to select particle
Flexible Peptide Insertions in Hsp16.5 Lead to Novel Structural Assemblies—Sequence alignment of members of the sHSP family (17) identifies a unique 14-residue proline- and alanine-rich sequence at the junction between the N-terminal and \( \alpha \)-crystallin domains of Hsp27, labeled P1 in Fig. 1a. We have previously demonstrated that the P1 peptide is involved in equilibrium dissociation of the Hsp27 oligomer (6). The peptide is expected to have a highly flexible backbone; therefore we predicted that its insertion might affect the dynamic properties of the ordered Hsp16.5 oligomer. Indeed, insertion of this sequence at two positions in the Hsp16.5 sequence alters the equilibrium dissociation of the Hsp27 oligomer (6).

Flexible Peptide Insertions in Hsp16.5—Several possible conformational states of the Hsp16.5-P1 oligomer were modeled using the pseudoatomic model derived from the cryoEM map. The backbone torsion angles of the B-chain C-terminal extension were adjusted using a Swiss-PDB viewer (34).

RESULTS

Flexible Peptide Insertions in Hsp16.5 Lead to Novel Structural Assemblies—Sequence alignment of members of the sHSP family (17) identifies a unique 14-residue proline- and alanine-rich sequence at the junction between the N-terminal and \( \alpha \)-crystallin domains of Hsp27, labeled P1 in Fig. 1a. We have previously demonstrated that the P1 peptide is involved in equilibrium dissociation of the Hsp27 oligomer (6). The peptide is expected to have a highly flexible backbone; therefore we predicted that its insertion might affect the dynamic properties of the ordered Hsp16.5 oligomer. Indeed, insertion of this sequence at two positions in the Hsp16.5 sequence alters the size and polydispersity of the oligomer as revealed by molecular mass determination across the SEC peaks (Fig. 1b). Introduction of the P1 peptide after residue Glu12 in a helix in the N-terminal region of Hsp16.5 (25) induces the formation of a highly polydisperse oligomer (Hsp16.5-P1N) with an average molecular mass of \( \sim 650 \) kDa and a mass distribution reminiscent of that of mammalian sHSP. In contrast, insertion of P1 at the junction between the N-terminal and \( \alpha \)-crystallin domains leads to the formation of a monodisperse oligomer (Hsp16.5-P1) with a molecular mass approximately double that of WT (\( \sim 850 \) kDa). Considering the additional mass of the inserted peptide, this indicates that the oligomer consists of 48 subunits, or twice the number of subunits in the WT oligomer. Simply deleting the N-terminal region of Hsp16.5 (variant Hsp16.5-TR

images with 100\(^2\) pixels (120-kV data sets) or 160\(^2\) pixels (300-kV data set). This resulted in 1,232 particle images for Hsp16.5-TR; 7,908 for Hsp16.5-P1 at 120 kV; 3,570 for Hsp16.5-P1 at 300 kV, and 4,980 for Hsp16.5-P1N. The particle images were low pass filtered to 19 Å (120 kV) or 6 Å resolution (300 kV). The IMAGIC-5 package (31) was used to calculate initial class sum images and reconstructions.

Octahedral symmetry was imposed on the Hsp16.5-TR data set based on resemblance of the class sum images to wild type (WT) Hsp16.5. For the Hsp16.5-P1 120-kV data set C4, D4, and octahedral symmetries were tested, and the results indicated octahedral symmetry. Reconstructions were calculated for the Hsp16.5-TR and Hsp16.5-P1 120kV data sets with IMAGIC-5 and refined with EMAN. The resolution of the final Hsp16.5-TR reconstruction is 27 Å as assessed by the conservative 0.5 Fourier shell correlation threshold. For Hsp16.5-P1 the best reconstruction from the 120-kV data set was used as the starting model for the 300-kV data set. The resolution of the final Hsp16.5-P1 reconstruction is 10.3 Å as assessed by the 0.5 Fourier shell correlation threshold. Image processing was performed on the Vampire linux cluster at Vanderbilt. Molecular graphics images were produced using the UCSF Chimera package (32).

Docking of the \( \alpha \)-Crystallin Domain Dimer—A dimer of \( \alpha \)-crystallin domains (amino acids 33–135) was extracted from the Hsp16.5 crystal structure (Protein Data Bank code 1SHS) (16) and fit within the Hsp16.5-P1 reconstruction with the CoLoRes routine in the Situs v2.2 package (33). Octahedral symmetry was applied to the coordinates for the best dimer position resulting in a pseudoatomic model for the oligomer (24 dimers or 48 monomers). Three dimers around a 3-fold window in the pseudoatomic model and crystal structure were compared, and a root mean square deviation of 1.9 Å was found with Chimera. The pseudoatomic model was converted to a density map with the PDBe2MRC routine in EMAN and subtracted from the cryoEM density to generate a difference map. The backbone torsion angles of the B-chain C-terminal extension were adjusted using a Swiss-PDB viewer (34).
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CryoEM reconstructions show that Hsp16.5-TR has an outer shell similar to WT but without any internal density, whereas Hsp16.5-P1 has a larger outer shell with weak internal density (Fig. 1c). Comparison of the Hsp16.5-TR reconstruction with that of WT along with previous SDSL-EPR (25) supports the assignment of the internal density to the N-terminal region, which was not visible in the crystal structure. The Hsp16.5-TR reconstruction also confirms previous spin labeling EPR data indicating that the α-crystallin domain and C-terminal extension encode all of the subunit interactions necessary for oligomeric assembly (26).

CryoEM Reconstructions Reveal a Highly Facetted and Symmetrical Structure for Hsp16.5-P1—CryoEM class sum images reveal nearly perfect 2-, 3-, and 4-fold symmetry indicative of an octahedral assembly for Hsp16.5-P1 (Fig. 2a). This is the same symmetry observed for WT, and yet the subunit number is doubled in Hsp16.5-P1. A reconstruction of Hsp16.5-P1 was generated from a data set collected on a Polara (300 kV, FEG) microscope (Fig. 2b). The resolution is 10.3 Å as assessed by the conservative 0.5 Fourier shell correlation threshold. The Hsp16.5-P1 structure is more facetted than WT, and there is a significant expansion with an increase in diameter from 120 Å for the roughly spherical WT, to a maximum of 182 Å for Hsp16.5-P1. Furthermore, Hsp16.5-P1 has relatively large openings in the outer protein shell at both the 3- and 4-fold symmetry axes compared with the WT structure that has large windows only at the 3-fold axes.

Preservation of the Dimeric Building Block in Hsp16.5-P1—The reorganization of the quaternary structure in Hsp16.5-P1 does not involve disruption of the dimeric building block. We observed strong spin-spin couplings at residues near the 2-fold symmetry axis of WT. EPR spectra at the same sites in the context of either Hsp16.5-P1 or WT shown in Fig. 3 are almost superimposable, suggesting that the overall architecture of the dimer is preserved. For instance at site 42 located at the monomer-monomer interface, spectral components arising from spin labels separated by less than 8 Å are evident in both WT and Hsp16.5-P1 (indicated by brackets). The change in the sharp spectral components represents ~5% increase in the population of singly labeled dimers for Hsp16.5-P1 compared with the WT. Notable also is the close proximity between residues 46 and 97, which reflects the strand swapping between monomers.

A Pseudoatomic Model of Hsp16.5-P1 Reveals Two Non-equivalent Subunit Positions—On the basis of the EPR data, a dimer of α-crystallin domains (amino acids 33–135) was extracted from the crystal structure of Hsp16.5 and fit within the Hsp16.5-P1 cryoEM density by the quantitative docking tool CoLoRes in the Situs software package (33). Both possible hands of the cryoEM density were tested, and a significantly better correlation (12% higher) was found for the hand shown in Figs. 2 and 4. The best fit dimer position was selected, and octahedral symmetry was imposed, resulting in the generation of a pseudoatomic model for all of the α-crystallin domains in Hsp16.5-P1 (Fig. 4a). The atomic fitting indicates that the outer protein shell of the Hsp16.5-P1 oligomer is formed by 48 α-crystallin domains. The α-crystallin monomers are found in two independent positions, referred to as chains A and B. The pseudoatomic model has three dimers around the opening at the 3-fold symmetry axis (Fig. 4b) and four dimers around the larger opening at the 4-fold axis (Fig. 4c). The high degree of accuracy of the pseudoatomic model is highlighted by the delineation observed between α-crystallin monomers (Fig. 4, b and c) and by the superposition of the three dimers around the 3-fold symmetry axis on three dimers from the WT Hsp16.5 crystal structure with an root mean square deviation of 1.9 Å.

Validation of the Hsp16.5-P1 Pseudoatomic Model by SDSL-EPR—To further validate the pseudoatomic model of Hsp16.5-P1, we determined proximities between spin labels near the symmetry axes. At sites along the 2-fold symmetry axis of Hsp16.5-P1 such as Gly34, the spin labels are predicted to undergo strong dipole-dipole interactions, and the EPR spectra

FIGURE 2. CryoEM structure of Hsp16.5-P1 at 10 Å resolution. a, three selected class sum images based on a 120-kV cryoEM data set showing 2-, 3-, and 4-fold symmetry from left to right. b, cryoEM reconstruction of Hsp16.5-P1 at 10 Å resolution based on a 300-kV FEG cryoEM data set of 3080 particle images. Three surface views are shown aligned along the symmetry axes to resemble the class sum images shown directly above. The radial color coding is the same as in Fig. 1c. The scale bar represents 50 Å.
are similar to those of spin labels introduced at the same sites in WT (Fig. 5a). In contrast, the increase in the spectral intensity observed at site 102 reflects an increase in distance between symmetry related copies as expected based on the location of these residues at the symmetry interfaces of Hsp16.5-P1. One independent \(\alpha\)-crystallin monomer (chain B) in the pseudoatomic model has residue 102 facing the 3-fold opening as in the WT structure (Fig. 5b). The other independent monomer (chain A) has this residue facing the larger 4-fold opening that is only present in the Hsp16.5-P1 structure. Quantitative analysis of the EPR spectrum for residue 102 of Hsp16.5-P1 (28) (supplemental Fig. S2 and supplemental Table S1) reveals two spectral components each representative of a \(\sim 50\%\) spin population. One population represents dipolar-coupled spin labels as in WT, and the other population represents noninteracting spin labels consistent with a larger distance between sites around the 4-fold symmetry axes of Hsp16.5-P1. Similarly, the change in the EPR spectrum of spin label pair 51/80, which reports on the interaction between dimers, is consistent with a change in the relative orientations of residues 51 and 80 for approximately half of the sites in the Hsp16.5-P1 model (Fig. 5b).

**The C-terminal Extension Is a Flexible Tether That Enables Repacking**—To identify the structural features that mediate oligomer repacking, a difference map was calculated between the Hsp16.5-P1 cryoEM reconstruction and the pseudoatomic model that includes just the \(\alpha\)-crystallin domains. Difference density was observed on the outer surface of both independent \(\alpha\)-crystallin domains (chains A and B) in a groove between the two \(\beta\)-sheets of the \(\alpha\)-crystallin domain where the C-terminal extension docks in the WT structure. Given that the three
dimers around the 3-fold opening can be nearly superimposed on the WT Hsp16.5 crystal structure, it seems likely that the conformation of the C-terminal extensions that stabilize these three dimers is maintained in Hsp16.5-P1 as it is in WT. This accounts for the conformation of half of the 48 C-terminal extensions in Hsp16.5-P1, those of the A-chain monomers.

In view of the change in the interface angle at the corner of the 4-fold symmetry window, the C-terminal extensions of the B-chain monomers must have a modified conformation to stabilize the ring of four α-crystallin dimers. Difference density is observed extending from near residue 135 of the B-chain monomer over the A-chain α-crystallin domain forming an interlocked ring of four dimers (Fig. 6a). Simply attaching amino acids 136–147 in the WT conformation onto the B-chain monomer leads to a clash with the side of the A-chain α-crystallin domain. Therefore, we positioned the C-terminal extension over the A-chain α-crystallin domain so as to preserve the numerous hydrophobic interactions between residues 141–147 and the underlying α-crystallin domain. Small adjustments (12–37°) in the backbone torsion angles, staying within the allowed helical region of the Ramachandran plot, for residues 137 and 138 in the short α-helix enabled the extension to be connected to the rest of the B-chain monomer (Fig. 6b).

Plasticity at the Dimer-Dimer Interface Can Lead to Polydispersity—Structurally, the insertion position of P1 in Hsp16.5-P1 maps to the interface between α-crystallin domain dimers that is held together by only two ionic interactions (Asp51/Arg80 and Glu78/Arg93) (16). In the WT structure neighboring α-crystallin domain dimers meet and form a 60° angle, thus creating the triangular openings at the 3-fold symmetry axes. The same angle is also found in the Hsp16.5-P1 pseudoatomic model around the 3-fold openings (Fig. 7a); however, a larger angle of 90° is also found around the 4-fold opening (Fig. 7b). The combination of these two packing angles between α-crystallin domain dimers leads to the larger diameter octahedral assembly observed for Hsp16.5-P1. The sparseness of contacts between dimers around the 3-fold opening can be nearly superimposed on the WT Hsp16.5 crystal structure, it seems likely that the conformation of the C-terminal extensions that stabilize these three dimers is maintained in Hsp16.5-P1 as it is in WT. This accounts for the conformation of half of the 48 C-terminal extensions in Hsp16.5-P1, those of the A-chain monomers.

In contrast, when the Hsp27 P1 sequence is inserted between residues 12 and 13 to form the Hsp16.5-P1N variant, polydisperse assemblies are created. Cryoelectron micrographs of Hsp16.5-P1N show variably sized and shaped assemblies. In a data set of 5,000 particle images classified into 500 class sum images, less than 5% of the class sum images showed 2-, 3-, or 4-fold symmetry, and they displayed only loose symmetry (Fig.
In comparison, most of the Hsp16.5-P1 class sum images displayed essentially perfect 2-, 3-, and 4-fold symmetry (Fig. 2a). The more irregular class sum images of Hsp16.5-P1N often show apparent gaps in the outer protein shell (Fig. 8b). Given the heterogeneity in the Hsp16.5-P1N data set, it was not possible to calculate a three-dimensional reconstruction.

We explored the subunit arrangement in Hsp16.5-P1N by determining the pattern of proximity between spin labels near the axes of symmetries. At site 42 at the monomer-monomer interface, the EPR spectrum is unchanged from that of Hsp16.5-P1 (Fig. 8c), indicating that the dimeric building block is intact. The lack of spectral change at site 102 is consistent with the preservation of 3- and 4-fold windows in Hsp16.5-P1N. In contrast, the EPR spectrum at site 34 reveals increased distance heterogeneity at the dimer-dimer interface. We speculate that the highly variable quaternary structure of Hsp16.5-P1N can be explained by a continuum of angles between \( \alpha \)-crystallin domain dimers. Although the flexible C-terminal extension can adapt to many of these dimer packing angles, there are likely to be regions within the assembly where dimers cannot be tethered. This would lead to gaps in the outer shell as suggested by the cryoEM images and to highly irregular assemblies of varying size and subunit copy number.

**Oligomer Expansion Enhances Substrate Binding**—Transition to either large or polydisperse oligomers is accompanied by a significant increase in the apparent affinity to destabilized mutants of T4L (5). Substrate affinity was determined using an assay where Hsp16.5 is presented with T4L mutants of progressively lower free energies of unfolding (\( \Delta G_{\text{unf}} \)). At 37 °C, \( \Delta G_{\text{unf}} \) of T4L-L99A is 4.7 kcal/mol, whereas that of T4L-D70N is 6.0 kcal/mol (6). Thus, under the assay conditions, T4L is predominantly in the folded state and does not aggregate. Steady state association between Hsp16.5 and dynamically populated non-native T4L is detected directly via changes in the fluorescence property of a bimane probe attached on the surface site, 151, of T4L (9). The binding isotherms of Hsp16.5 and the Hsp16.5-P1 variant to T4L-L99A (Fig. 9, a and b) are biphasic, indicative of two-mode binding as previously reported for \( \alpha \)-crystallin. Binding was confirmed by SEC of Hsp16.5/T4L mixture followed by fluorescence detection (data not shown) (35). The quenching observed at low molar ratio of Hsp16.5 to T4L has been shown to reflect the extensive unfolding of T4L when bound with low affinity (9). Truncation of the N-terminal region leads to a right-shifted monophasic binding isotherm, indicating a reduced level of binding. We used a phenomenological two-mode binding model previously derived from analysis of \( \alpha \)-crystallin and Hsp27 interaction with T4L (9) to rank the affinity of the Hsp16.5 variants for T4L. The binding isotherms of Hsp16.5 and the Hsp16.5-P1 variant to T4L-L99A (Fig. 9) are biphasic, indicative of two-mode binding as previously reported for \( \alpha \)-crystallin. Binding was confirmed by SEC of Hsp16.5/T4L mixture followed by fluorescence detection (data not shown) (35). The quenching observed at low molar ratio of Hsp16.5 to T4L has been shown to reflect the extensive unfolding of T4L when bound with low affinity (9). Truncation of the N-terminal region leads to a right-shifted monophasic binding isotherm, indicating a reduced level of binding. We used a phenomenological two-mode binding model previously derived from analysis of \( \alpha \)-crystallin and Hsp27 interaction with T4L (9) to rank the affinity of the Hsp16.5 variants for T4L. Dissociation constants obtained from nonlinear least squares fits demonstrate that the Hsp16.5-P1 variant has significantly higher affinity than the Hsp16.5 homologue.
higher affinity than the WT, whereas the N-terminal truncation Hsp16.5-TR is best modeled with a single low affinity mode (supplemental Table S2). High affinity bound T4L has a higher quantum yield in the Hsp16.5-P1 variant and a larger blue shift of its maximum intensity wavelength relative to the WT, suggesting a more hydrophobic environment of bound T4L.

Addition of Hsp16.5-P1N to 7 or 10 μM T4L-L99A leads to visible aggregation and light scattering. The aggregates consist of both Hsp16.5 and T4L, which is indicative of saturation binding where the sHSP oligomer becomes insoluble. Therefore, the level of binding was reduced by obtaining the binding isotherm at 5 μM T4L-L99A (Fig. 9b) and by using T4L-D70N, which is more stable than T4L-L99A by ~2 kcal/mol (Fig. 9c). Hsp16.5-P1N shows a significantly higher level of binding than the WT. In the presence of T4L-D70N, little or no binding is observed for the WT, whereas both insertion mutants display biphasic binding at substrate concentrations as low as 7 μM. These qualitative results indicate that both Hsp16.5-P1 and Hsp16.5-P1N represent activated forms of Hsp16.5 displaying significant enhancement of binding to non-native states of T4L. As discussed below, the mechanism of activation may involve the increased exposure of the N-terminal region.

DISCUSSION

By combining rational insertion mutagenesis with a hybrid cryoEM and EPR structural approach and a unique binding assay, we derive a model for the interplay between the three sequence modules (N-terminal domain, α-crystallin domain, and C-terminal extension) that underlies the transition to polydispersity and defines the role of polydispersity in substrate binding. The finding that disruption of the Hsp16.5 N-terminal region profoundly affects the packing interactions of the α-crystallin domain establishes the paradigm that sequence divergence in the N-terminal domain of sHSP is a primary mechanism for tuning the oligomer symmetry and degree of order. An extreme manifestation of this mechanism is the evolved role of the N-terminal domain of αA-crystallin and Hsp27 in controlling the global assembly of the native oligomer and its dynamic properties (20, 36, 37). Multiple assemblies were also recently reported from cryoEM analysis of Hsp26

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FIGURE 9. Hsp16.5 and its variants display differences in binding to destabilized mutants of T4L. a, truncation of the N-terminal region leads to a loss of two-mode binding, whereas Hsp16.5-P1 shows an increased level of binding relative to WT. The Hsp16.5 and Hsp16.5-P1 curves were generated with 10 μM T4L-L99A and the Hsp16.5-TR curve with 7 μM T4L-L99A. The binding-induced changes in the maximum emission wavelengths are as follows: Δλmax,WT = 10 nm, Δλmax,Hsp16.5-P1 = 14 nm, Δλmax,Hsp16.5-TR = 10 nm. b and c, enhancement of Hsp16.5-P1 and Hsp16.5-P1N binding to two T4L destabilized mutants with two different free energy of unfolding. The curves were generated with 5 μM T4L-L99A in a and 14 μM T4L-D70N in c. All of the curves were generated at pH 7.2 and 37 °C.
the consequence of the dynamic properties of this peptide, its detailed sequence character, or the structural disruption of the N-terminal region cannot be conclusively established.

This mechanistic model for chaperone activity is consistent with negative stain EM studies reporting an expansion of the Hsp16.5 oligomer following binding to single chain monellin (40). Furthermore, exposure of the oligomer to the binding temperature of 65 °C in the absence of the substrate results in a similar increase in diameter without change in the secondary structure (41). Transition to either large symmetrical or polydisperse assemblies is accompanied by increased accessibility of the N-terminal region as demonstrated by the large 4-fold windows observed in the Hsp16.5-P1 structure. The incomplete and irregular shells of Hsp16.5-P1N may provide even greater access to the interior of the assembly. We propose that WT binding-competent intermediates may resemble the expanded Hsp16.5-P1 and Hsp16.5-P1N variants and thus provide substrate increased access to the internal N-terminal regions.

From a more global perspective, our results resolve the conundrum between the α-crystallin domain encoding specific subunit contacts and the overall polydispersity of the oligomers. The polydisperse quaternary structures observed for mammalian shSP such as α-crystallin can be explained in the same manner as Hsp16.5-P1N. Hsp16.5-P1 may also provide a structural and mechanistic model for the expansion of oligomers observed in mutants of α-crystallin associated with congenital cataract. It is noted that these mutants also display higher affinity to their substrates (42).

It is likely that the N-terminal sequences of αA- and αB-crystallin induce polydisperse oligomers and simultaneously confer and modulate the ability to undergo dynamic subunit exchange. Subunit dissociation provides transient exposure of the N terminus beyond the steady state level of accessibility associated with polydispersity. The steady state fraction of dissociated multimers may be an added mechanism by which mammalian shSP control their activation state.

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