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A Mechanism of Subunit Recruitment in Human Small Heat Shock Protein Oligomers

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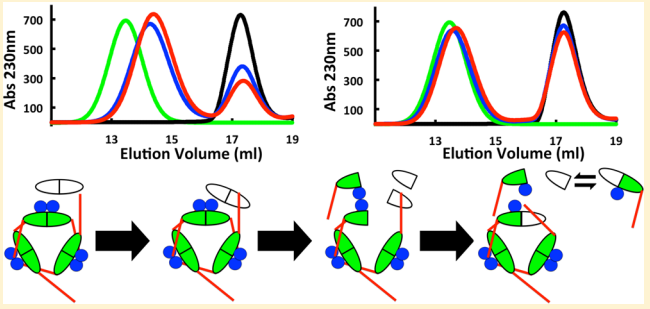
Supporting Information

ABSTRACT: Small heat shock proteins (sHSPs) make up a class of molecular chaperones broadly observed across organisms. Many sHSPs form large oligomers that undergo dynamic subunit exchange that is thought to play a role in chaperone function. Though remarkably heterogeneous, sHSP oligomers share three types of intermolecular interactions that involve all three defined regions of a sHSP: the N-terminal region (NTR), the conserved α-crystallin domain (ACD), and a C-terminal region (CTR). Here we define the structural interactions involved in incorporation of a subunit into a sHSP oligomer. We demonstrate that a minimal ACD dimer of the human sHSP, HSPB5, interacts with an HSPB5 oligomer through two types of interactions: (1) interactions with CTRs in the oligomer and (2) via exchange into and out of the dimer interface composed of two ACDs. Unexpectedly, although dimers are thought to be the fundamental building block for sHSP oligomers, our results clearly indicate that subunit exchange into and out of oligomers occurs via monomers. Using structure-based mutants, we show that incorporation of a subunit into an oligomer is predicated on recruitment of the subunit via its interaction with CTRs on an oligomer. Both the rate and extent of subunit incorporation depend on the accessibility of CTRs within an HSPB5 oligomer. We show that this mechanism also applies to formation of heterooligomeric sHSP species composed of HSPB5 and HSPB6 and is likely general among sHSPs. Finally, our observations highlight the importance of NTRs in the thermodynamic stability of sHSP oligomers.



Small heat shock proteins (sHSPs) are molecular chaperones that maintain protein homeostasis and protect cells from stress.[1−3] Members of this protein family occupy various biological niches and are found in all kingdoms of life.[2] The human sHSP family includes 10 members, each with distinct gene expression patterns and presumably distinct roles.[4,5] Of these, HSPB5 (αB-crystallin) is among the most abundant, with especially high levels of expression in the eye lens, heart, brain, and skeletal muscle.[4] Highlighting its importance in maintaining cellular function, deleterious mutations in HSPB5 cause a number of known congenital disorders, including cataracts, cardiomyopathies, and myofibrillar myopathies.[6−9] Furthermore, overexpression of HSPB5 is linked to the progression of numerous cancers, including prostate and breast carcinomas.[10,11]

Because of its significance, HSPB5 has been the focus of intense study, yet many aspects of its structure and function remain poorly understood. HSPB5 contains structural elements common to all sHSPs, with a highly conserved α-crystallin domain (ACD) that is flanked by variable N- and C-terminal regions (NTR and CTR, respectively). In the absence of the NTR and CTR, ACDs form stably folded dimers.[12−15] The C-terminal region of HSPB5 contains a three-residue isoleucine-proline-isoleucine/valine ("IXI/V") motif that is found in many sHSPs. Though their monomeric mass is relatively small, many metazoan sHSPs form large, polydisperse (oligomers contain variable numbers of subunits), structurally heterogeneous oligomers that undergo dynamic subunit exchange. The distribution of oligomers and exchange dynamics are unique for different sHSPs and can be attributed to the variability observed in N- and C-terminal regions.[16−18]

Though homomeric sHSP oligomers have been best characterized, many human sHSPs are known to form heterooligomers (containing more than one type of sHSP). HSPB5 is known to form heterooligomeric complexes with HSPB1, HSPB4, and HSPB6, sHSPs that are expressed in tissues where HSPB5 is also present.[19−21] That heterooligomeric sHSPs exist in cells provides an impetus to understand the properties of sHSPs in the context of mixed oligomers. Defining structural details of heterooligomer assembly proves to be complicated because of the inherent heterogeneity observed in sHSP oligomers.[16,22] We postulated that interactions that drive heterooligomer formation are common to those that drive homooligomer formation, which have been more thoroughly characterized. In this way, we can draw on the insights gained from many previous studies of homooligomers.

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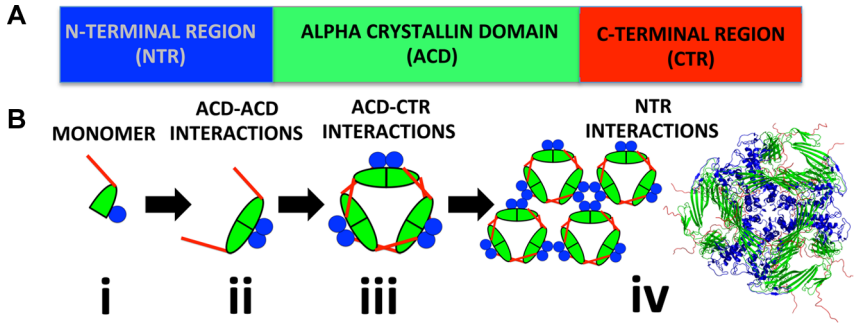

Figure 1. (A) Three regions of sHSPs are the conserved α-crystallin domain (ACD, green), flanked by variable N-terminal regions (NTRs, blue) and C-terminal regions (CTRs, red). (B) Cartoon diagram of interactions observed in pseudoatomic models of HSPB5 (same colors as defined in panel A). sHSP monomers (i) assemble into dimers through ACD−ACD interactions (ii). Higher-order assemblies occur through CTR−ACD interactions (iii, hexamer shown), and poorly defined NTR interactions drive the assembly of the final oligomer (iv). Rather than the formation of an expanded array (iv, left cartoon), sHSPs assemble into oligomers with defined assemblies and/or distributions (iv). A pseudoatomic model of a 24mer HSPB5 oligomer is shown in the colors described above.[12]

Because of their size and polydispersity, HSPB5 oligomeric complexes have proven to be challenging to study using conventional biophysical and structural techniques. Gaining structural information about HSPB5 has thus proceeded through a piecemeal approach, beginning with domain-level structures of the HSPB5-ACD solved by NMR and X-ray crystallography.[12−15] Pseudoatomic models of HSPB5 oligomers have been generated using hybrid approaches that combine information from solid-state NMR, small-angle X-ray scattering, and electron microscopy.[22,23]

Although they differ in certain respects, the two pseudoatomic models of HSPB5 share several important features that highlight putative intermolecular contacts that may occur within oligomers. In both cases, dimers form the basic building block of an HSPB5 oligomer, where the dimer interface is formed by the antiparallel alignment of the β6+7 strands of the ACD. Dimers form hexameric rings through the interaction of a C-terminal IXI motif from one dimer with a hydrophobic groove formed by the β4/β8 strands in an ACD of a neighboring dimer. A model of a 24mer with tetrahedral symmetry can be generated through the assembly of four hexameric rings through extensive contacts between NTRs (Figure 1).[22,23] The resulting models represent a considerable advancement in our understanding of HSPB5 structure. However, each represents only a single state within the ensemble of HSPB5 oligomers that exist in solution and does not explain dynamic subunit exchange properties.

Multiple studies have demonstrated that subunit exchange occurs between HSPB5 oligomers with subunit exchange rates on the scale of 10^{-3} s^{-1} at 37 °C.[18,24] Recent studies using native mass spectrometry and solution-state NMR attribute the global exchange rate of HSPB5 subunits to structural changes that occur on a millisecond time scale.[25] On the basis of these kinetic models, the rate of dissociation of the subunit from the oligomer is proposed to be limited by interactions involving CTRs within the oligomers. These observations are a first step in linking structural details to the observed subunit exchange. However, the kinetic data do not speak to oligomer assembly and recruitment of a subunit into an oligomer.

Here we demonstrate that an ACD-only (i.e., the domain without its flanking NTR and CTR) can interact with an HSPB5 oligomer, identifying the minimal and most conserved domain of a sHSP as being sufficient for interactions with a sHSP oligomer. The interaction is detected only when the ACD-only can first bind to a CTR from an HSPB5 oligomer and accessibility of CTRs in the HSPB5 oligomer dictates the extent and rate of exchange. Further, we demonstrate that an ACD-only can exchange at the dimer interface within an oligomer, but only when an ACD-only can first bind the CTR, illustrating interactions that dictate oligomer formation are interdependent. Finally, we extend the observations made using an HSPB5 ACD-only and oligomers to the formation of the HSPB5/HSPB6 heterooligomers to gain further insight into the roles of the ACD, CTR, and NTR in oligomer assembly.

EXPERIMENTAL PROCEDURES

HSPB5 and HPB5-ACD-only constructs and mutants have been previously described.[22,26] ΔN-HSP20 (residues 65−160) was expressed from the pPROEX HT(b) vector. HSPB6 was expressed from the pET23b vector. Under nonreducing conditions, the formation of disulfide bonds is apparent in HSPB6. HSPB6 has one native cysteine (C46), which was mutated to serine (C46S) in all constructs. Mutagenesis was accomplished using the primer 5′-TGGCTGCGCTCAGCCCCACCACG-3′. Additionally, the S134Q mutation was introduced into HSPB6 constructs using the primer 5′-GGCTGCCGTGACGCAGGCGCTGTCCCCCG-3′.

Protein Expression and Purification. All sHSP constructs were expressed in *Escherichia coli* BL21(DE3) cultured in Luria-Bertaini medium containing 100 μg/mL ampicillin. Protein expression was induced with the addition of isopropyl thio-β-D-thiogalactoside to a final concentration of 0.5 mM at 22 °C for 16 h. All sHSP constructs were purified from *E. coli* BL21(DE3) by methods previously described.[12,17,26] Briefly, wild-type (wt) and mutant full-length HSPB5 and HSPB6 were purified by an ammonium sulfate precipitation, followed by anion exchange chromatography. Full-length proteins were further purified by size exclusion chromatography in 25 mM sodium phosphate and 150 mM sodium chloride (pH 7.5) (PBS 7.5). Purification of HSPB5-ACD-only (residues 64−152), HSPB5-ACD-only mutants, and ΔN-HSPB6 (residues 65−160) was accomplished by methods previously described.[12,26] Briefly, TEV cleavable, N-terminally His-tagged HSPB5-ACD-only or ΔN-HSPB6 was initially purified from lysate using a Ni^{2+} affinity column. The His tag was removed by TEV-protease and separated from the ACD using a Ni^{2+} affinity column. Proteins were further purified by anion exchange chromatography and size exclusion chromatography in PBS 7.5.

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Analytical Size Exclusion Chromatography (aSEC). All aSEC experiments were performed on an GE akta Purifier equipped with a Superose 6 10/300 GL column (GE Life Sciences) and a 200 μL sample loop in PBS 7.5. aSEC experiments were performed at room temperature (~25 °C) with samples preincubated at 37 °C. Elution volumes for molecular weight standards and proteins in this study are available in Figure S1 of the Supporting Information.

HSPB5 and ACD-only aSEC. Mixtures of 200 μM HSPB5 oligomers and 200 μM ACD-only in PBS pH 7.5 were incubated at 37 °C for 1 h prior to aSEC (monomer concentrations). Mixtures of mutant HSPB5 oligomers and mutant ACD-only constructs were made in the same manner. Fractionated mixtures from these experiments were visualized by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).

HSPB5 and HSPB6 aSEC. Mixtures of 50 μM HSPB5 oligomers and 50 μM HSPB6 mutants were incubated at 37 °C for 1 and 2 h prior to aSEC (monomer concentrations). All full-length HSPB6 proteins contained the C46S mutation to eliminate confounding disulfide bond formation. Mixtures of HSPB5 and ΔN-HSPB6 were made in the same manner.

Detection of ACD Interface Exchange. To detect mixing at the ACD−ACD interface, E117C-HSPB5 and E117C-ACD-only were reduced in PBS pH 7.5 containing 15 mM DTT for 1 h at 37 °C. Reduced E117C-HSPB5 and E117C-ACD-only were then mixed for 1 h at 37 °C. The reducing agent was removed by a 16 h dialysis into PBS pH 7.5, at room temperature (~25 °C). Additionally, mixing experiments with E117C-HSPB5 and/or E117C-ACD-only containing the S135Q mutation were conducted using the same methodology. Nonreduced mixtures were then visualized by nonreducing SDS−PAGE. The extent of exchange at the ACD−ACD interface was quantified by the appearance of a band corresponding to a disulfide-locked mixed FL/ACD-only dimer. Mixing experiments were conducted in triplicate, and gels were imaged using a LI-COR Odyssey CLx instrument and band intensities quantified using the imaging software ImageJ.[27] Band intensities were normalized to the mean band intensity observed for the E117C-HSPB5/E117C-ACD-only mixture.

NMR. ^{1}H, ^{15}N TROSY-HSQC spectra of [^{15}N]HSPB5 oligomers were recorded on a Bruker Avance II 600 MHz spectrometer equipped with a triple-resonance, z-gradient cryoprobe. Spectra were recorded on samples containing 300 μM [^{15}N]HSPB5 in the presence and absence of 600 μM HSPB5-ACD-only in PBS pH 7.5 and 10% D_2O at 22 °C. ^{15}N HSQC spectra of 500 μM ΔN-HSPB6 were recorded under identical buffer conditions with a Bruker 500 MHz AVANCE II NMR spectrometer. HSQC spectra of ΔN-HSPB6 were recorded in the presence of a 6-fold excess of a peptide with the sequence PERTIPITREEK that mimics a region HSPB5 CTR, by methods described previously.[26] Spectra were processed using NMR-Pipe/NMRDraw and visualized with NMRView.[28,29]

RESULTS

An ACD-only Can Interact with the HSPB5 Oligomer. On the basis of previous structural and biochemical studies, interactions within HSPB5 oligomers can be classified into three types, shown schematically in Figure 1.[22,23] Two of the interaction classes involve the ACD, namely, (1) interactions across the ACD dimer interface ("ACD−ACD") and (2) interactions between the β4/β8 groove at the edge of an ACD β-sandwich and the IXI motif within the C-terminal region ("ACD−CTR"). The third type of interaction is poorly defined: the N-terminal region interacts with other NTRs and/or with an ACD. Both the "ACD−ACD" and "ACD−CTR" interactions observed in HSPB5 oligomers can be recapitulated with an ACD devoid of NTRs and CTRs (ACD-only).[12−15,26] As at least two classes involve the ACD, we asked whether ACD-only can be incorporated into an oligomer and, if so, which interactions are critical.

To determine whether an ACD-only can interact with an oligomer, mixtures of HSPB5-ACD-only and HSPB5 oligomer were incubated at 37 °C for 1 h, and the distribution of the two species was characterized by analytical size exclusion chromatography (aSEC) (Figure 2A). The mixture reaches equilibrium within the 1 h incubation (data not shown). As shown in Figure 2A, the elution profile obtained for the mixture is different from that of the oligomer and ACD-only in isolation in two ways. When HSPB5 oligomers are mixed with an ACD-only, the earliest-eluting peak that corresponds to HSPB5 oligomers elutes slightly later, indicating a smaller average hydrodynamic radius, and the slow-eluting peak, associated with the ACD-only dimer, is broader and elutes earlier. Although the differences are subtle, they are reproducible and indicate that the two species interact with sufficient affinity to alter the SEC distributions. SDS−PAGE analysis of fractions from the elution confirms that ACD-only is present in earlier-eluting fractions in the mixture than when applied and eluted on its own (Figure 2D). Thus, aSEC provides a simple way to detect an interaction between an ACD-only and an oligomer.

As mentioned above, there are two well-defined ways in which an ACD-only might interact with an oligomer: (1) an ACD-only subunit could exchange with a full-length subunit at the ACD−ACD interface, and/or (2) an ACD-only subunit could bind the CTR of an oligomer through ACD−CTR interactions. To determine whether either of these known interactions mediates the ACD-only−oligomer binding detected in the mixing experiment, we performed additional mixing experiments using mutations in ACD-only that either inhibit CTR binding in the ACD β4/β8 groove or lock the ACD-only into a dimer unable to exchange subunits. It has been previously demonstrated that mutation of Ser135 to Gln in the ACD effectively blocks binding of a CTR IXI motif without distorting the ACD structure.[26] Remarkably, the IXI binding mutation in the ACD-only subunit (S135Q-ACD-only) abrogates observable interactions with the oligomer. The elution profile of the mixture is indistinguishable from that of the individual components, and there is no sign of mixing in the SDS−PAGE analysis of fractions (Figure 2B,D).

To block exchange that might occur though the dimer interface, a Cys residue was introduced at the center of symmetry in the ACD dimer interface (E117C-ACD-only). E117C-ACD-only robustly forms a disulfide-locked dimer under nonreducing conditions.[30] While not as dramatic as the S135Q-ACD-only mixing result, the aSEC profile obtained for thw wt HSPB5 oligomer incubated with locked ACD-only dimers is reproducibly different from that from the mixing experiments shown in Figure 2A. The elution volume of the oligomer species does not shift upon mixing with E117C-ACD-only, but there is a slight broadening and shift of E117C-ACD-only dimers toward earlier elution (Figure 2C,D). This suggests that a locked ACD-only dimer retains some ability to interact with large oligomers, but not in a way that detectably changes the hydrodynamic properties of the oligomer (as seen in Figure

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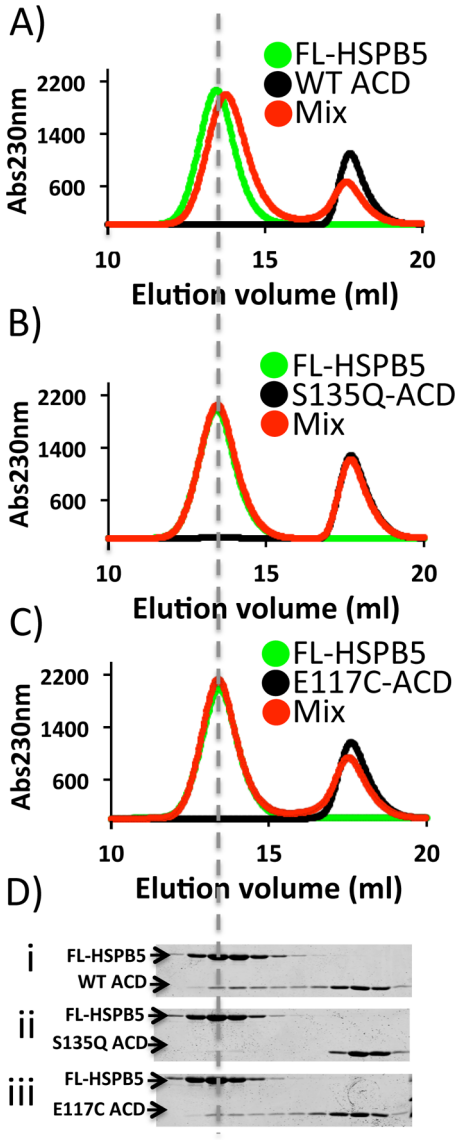

Figure 2. Interactions of ACD-only with HSPB5 oligomers detected by aSEC. (A) aSEC traces of HSPB5 oligomers (green) and ACD-only alone (black), as well as an equimolar mixture of HSPB5 oligomer and ACD-only incubated at 37 °C for 1 h prior to aSEC (red). (B) aSEC traces of HSPB5 oligomers, S135Q-ACD-only, and their equimolar mixture (same colors as in panel A). (C) aSEC traces of HSPB5 oligomers, E117C-ACD-only, and their equimolar mixture (same colors as in panel A). (D) SDS–PAGE gels of aSEC fractions from HSPB5 oligomer/ACD mixtures: (i) HSPB5 oligomers with wt ACD-only, (ii) HSPB5 oligomers with S135Q-ACD-only, and (iii) HSPB5 oligomers with E117C-ACD-only.

2A). An explanation for the combined observations is that a CTR–ACD interaction is required for the recruitment of subunits to an oligomer (i.e., ACD-only associates with the oligomer, without altering the distribution of the oligomer) but is not sufficient for incorporation of a subunit into an oligomer, which requires exchange at the ACD–ACD interface and results in an altered distribution of the oligomer.

To further define and confirm the CTR–ACD interaction is responsible for subunit recruitment, NMR spectra of ^{15}N-labeled HSPB5 oligomers were collected in the presence and absence of ACD-only. In solution, the large oligomers tumble slowly, and therefore, most of the resonances are too broad to observe. There are, however, approximately 20 peaks visible in a TROSY-HSQC spectrum that are derived from the highly mobile and dynamic CTR.[31] Consistent with this, the spectrum contains a single peak in the region that uniquely contains glycine resonances. The final glycine in the HSPB5 sequence, Gly154, is 21 residues from the C-terminus. This indicates that residues as far back as Gly154 are flexible and dynamic and can be observed in the TROSY-HSQC spectrum. As illustrated in Figure 3A, addition of HSPB5-ACD-only to a [^{15}N]HSPB5 oligomer sample results in broadening of ∼14 resonances, confirming that the interaction between ACD-only and the oligomer involves the flexible C-terminal region. Among the observable peaks are 10 resonances that have been previously assigned to residue 164 to the C-terminus, residue 175.[31] Ile159 and Ile161 of the CTR IXI motif precede the assigned region of the spectrum. In the presence of ACD, assigned resonances proximal to the IXI motif (residues 164–170) are broadened significantly, while the last five C-terminal resonances (residues 171–175) are relatively unaffected (Figure 3B). Additionally, the Gly154 peak is among those affected by ACD-only, providing additional support that the region most proximal to the structured portion of the oligomer including the IXI motif (Ile159-Pro-Ile161) is involved in the binding of ACD to oligomers. Additionally, eight of the nine unassigned peaks are broadened significantly with the addition of ACD-only (Figure 3B, red bars). These peaks are likely resonances from residues falling between Gly154 and the first assigned residue, Glu164, but these resonances have not been unambiguously assigned. This region is where we would predict ACD-only to bind on the basis of previous studies.[14,26,32]

To directly survey the extent of exchange at the dimer interface between an ACD-only and an oligomer, the "locked" dimer mutation E117C was introduced into both full-length (oligomeric) HSPB5 (E117C-FL) and the ACD-only construct (E117C-ACD-only). The two species were mixed under reducing conditions for 1 h to allow exchange and then dialyzed into nonreducing conditions at room temperature, where the disulfide bond can form. Exchange at the dimer interface was detected by the presence of a disulfide-linked FL/ACD-only heterodimer and visualized by nonreducing SDS–PAGE. By this method, it is apparent that exchange at the dimer interface occurs between an ACD-only subunit and a full-length subunit (Figure S2 of the Supporting Information). Because we observed that the initial recruitment of ACD-only into oligomers required the ACD–CTR interaction, we introduced the ACD–CTR-blocking mutation into E117C-ACD-only to determine if recruitment was required for exchange at the ACD–ACD interface observed by this method. When E117C-FL is equilibrated with the E117C/S135Q-ACD-only double mutant, very little heterodimer is formed, indicating that exchange at the dimer interface does not occur without the binding of a C-terminus from the oligomer (Figure 4A and Figure S2 of the Supporting Information).

Given the results presented above, conditions or mutations that enhance ACD–CTR interactions may facilitate subunit exchange. Introduction of the S135Q mutation into full-length E117C-HSPB5 inhibits binding of IXI motifs between full-length subunits within an oligomer and should increase the accessibility of CTRs in the oligomer. We predicted this would enhance formation of mixed dimers, and indeed, we observe an increase in the extent of exchange observed at the dimer interface in the dimer trapping experiments with the S135Q/E117C oligomer (Figure 4A and Figure S2 of the Supporting

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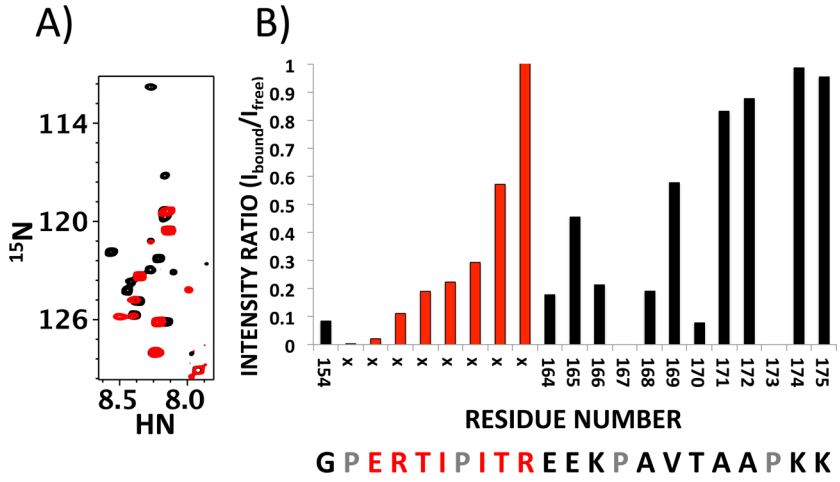

Figure 3. Binding of ACD-only to CTRs of [^{15}N]HSPB5 oligomers. (A) ^{1}H, ^{15}N HSQC spectra of HSPB5 oligomers in the absence (black) and presence (red) of 2 molar equiv of HSPB5-ACD-only. (B) Histogram of intensity ratios (I_{bound}/I_{free}) for HSPB5 oligomer CTR residues, with and without HSPB5-ACD-only bound. Intensity ratios for assigned residues are colored black. Intensity ratios for unassigned residues are colored red in ranked order as the peaks are not attributed to specific residues. Nevertheless, it is likely that unassigned resonances arise from residues 155−163. The CTR sequence is shown below. Prolines (colored gray) are not observed by this method and are denoted with intensity ratios of zero.

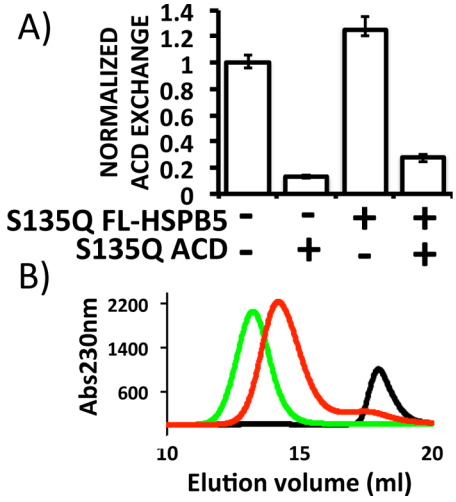

Figure 4. (A) Normalized SDS−PAGE band intensities for the disulfide-locked FL/ACD-only mixed dimer from E117C-FL-HSPB5 and E117C-ACD-only mixtures, incubated under reducing conditions and subsequently dialyzed into nonreducing conditions. Mixtures were visualized by nonreducing SDS−PAGE. Intensities for disulfide-locked FL/ACD-only mixed dimer were determined with mixtures in which either E117C-FL-HSPB5, E117C-ACD-only, or both had the additional mutation, S135Q. Mixing experiments were conducted in triplicate, and intensities were normalized to the mean intensity for the E117C-FL-HSPB5 and E117C-ACD-only mixture (left bar). Mean normalized intensities are plotted for each mixture, as are the minimum and maximum observed normalized intensities (error bars). (B) aSEC traces of S135Q-HSPB5 oligomers (green) and HSPB5-ACD-only (black) alone, as well as an equimolar mixture of the S135Q-HSPB5 oligomer and HSPB5-ACD-only incubated at 37 °C for 1 h prior to aSEC (red).

Information). As shown in Figure 4B, the effect is particularly apparent in aSEC mixing experiments in which the majority of the peak that corresponds to free ACD-only is shifted and broadened, as more ACD-only subunits associate with the S135Q-HSPB5 oligomer than we observe associating with the wt HSPB5 oligomer (Figure 2A). Furthermore, when both the oligomer and ACD-only carry the S135Q mutation, little mixing at the dimer interface is observed, demonstrating that if ACD-only cannot bind the oligomer CTR, subunit exchange does not readily occur (Figure 4A and Figure S2 of the Supporting Information).

Assembly of HSPB5/HSPB6 Heterooligomers. Mammalian HSPB6 is a small heat shock protein originally copurified with HSPB5 from skeletal muscle extract.[19] HSPB5 and HSPB6 are expressed in many other tissues, such as the heart, lungs, kidney, and brain.[4] HSPB6 has been shown to form heterooligomeric complexes with HSPB5, *in vitro*.[21] HSPB6 possesses both an NTR and CTR, although its CTR does not contain an IXI motif. Consistent with the lack of an IXI motif, a peptide with the HSPB6-CTR sequence does not interact with the ACD of HSPB5 in contrast to peptides mimicking CTRs from other sHSPs that contain an IXI motif.[26] Despite lacking its own IXI motif, the sequences that compose the $\beta4/\beta8$ groove are conserved between HSPB6 and other IXI motif-containing sHSPs. This raised the question of whether HSPB6 retains the ability to bind IXI motifs found in heterooligomeric partners. Analogous to those of ACD-only from HSPB5, ^{15}N HSQC NMR spectra can be collected on HSPB6 lacking its NTR (ΔN-HSPB6). To determine whether HSPB6 can bind CTRs containing IXI motifs, ^{15}N HSQC spectra of ΔN-HSPB6 were collected in the absence and presence of a peptide mimicking the HSPB5-CTR. Large perturbations in the HSQC spectrum of ΔN-HSPB6 are observed upon addition of the CTR peptide, demonstrating that though HSPB6 lacks a CTR IXI motif, it is able to bind to peptides mimicking IXI motif-containing CTRs (Figure S3 of the Supporting Information). On the basis of the results in the previous section, we hypothesized that binding the CTR of HSPB5 may be critical in the formation of HSPB5/HSPB6 heterooligomers.

To study incorporation of HSPB6 into HSPB5 oligomers, we performed aSEC mixing experiments similar to those previously described.[21] The aSEC profile of full-length HSPB6 alone shows it forms homooligomers (dimer to tetramer) that are much smaller than those of HSPB5 (Figure 5A). As shown in Figure 5A, aSEC profiles of an equimolar mixture of HSPB5 and HSPB6 reveal the formation of HSPB5/HSPB6 hetero-oligomers. Assembly of the heterooligomer was observed over a

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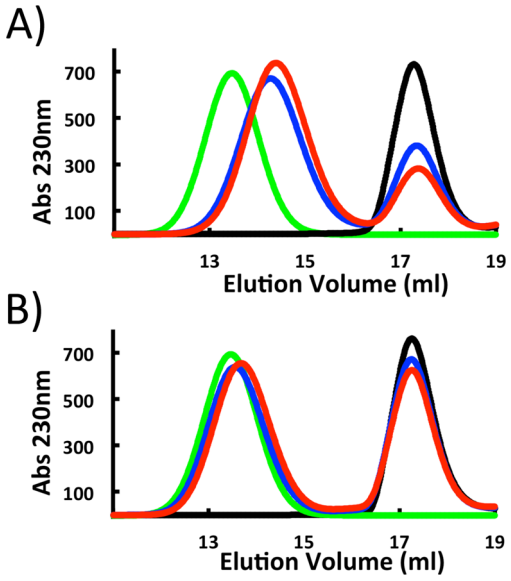

Figure 5. Formation of HSPB5/HSPB6 heterooligomers. (A) aSEC traces of HSPB5 oligomers (green) and HSPB6 (black) alone, and as an equimolar mixture incubated at 37 °C for 1 h (blue) and 2 h (red) prior to aSEC. (B) aSEC traces of HSPB5 oligomers (green) and S134Q-HSPB6 (black) alone, and as an equimolar mixture incubated at 37 °C for 1 h (blue) and 2 h (red) prior to aSEC.

time course of 120 min at 37 °C. The peak that corresponds to the HSPB6 homooligomer decreased over time, and a peak that contains both HSPB5 and HSPB6 subunits appears at an intermediate elution time.

The ability to observe formation of HSPB5/HSPB6 heterooligomers by aSEC provides a platform for assessing the importance of the ACD−CTR interaction in the formation of a native oligomeric complex, where all subunits contain their native NTRs and CTRs. To address whether binding of the HSPB5-CTR by the ACD $\beta4/\beta8$ groove of HSPB6 plays a determinant role in the formation of the heterooligomer, the S134Q mutation was introduced into HSPB6 to block the binding of HSPB5 CTRs (analogous to S135Q-HSPB5). S134Q-HSPB6 is dramatically inhibited in its ability to form HSPB5/HSPB6 heterooligomers: there is only a very small decrease in the magnitude of the HSPB6 peak, and the HSPB5/HSPB6 peak observed when wt subunits are incubated is not readily formed (Figure 5B). Thus, as observed with the simplified ACD-only mixing experiments, the ability of an incoming HSPB6 subunit to bind an HSPB5-CTR is a strong determinant for heterooligomer assembly.

To further test this conclusion, we performed mixing experiments with wt HSPB6 and S135Q-HSPB5 oligomers, the mutation that increases the accessibility of HSPB5 CTRs (Figure 6). Comparison of a 30 min incubation of wt HSPB5 with wt HSPB6 and S135Q-HSPB5 with wt HSPB6 shows the latter to incorporate HSPB6 subunits to a greater extent. Importantly, both heterooligomers compared in this experiment are equivalent in their hydrodynamic radii. This is consistent with the notion that the accessibility of oligomer CTRs (i.e., the relative population of CTRs in the free vs bound state) dictates both the rate and extent of subunit recruitment in the formation of both homo- and heterooligomers.

Role of the NTR in Oligomer Formation. Because of its enigmatic and heterogeneous structure, understanding the role of the NTR in oligomer formation remains a formidable

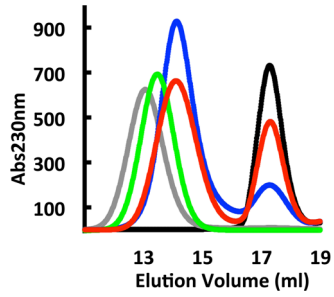

Figure 6. Interactions of S135Q-HSPB5 with HSPB6. aSEC traces of HSPB5 oligomers (green) and HSPB6 alone (black), and as an equimolar mixture incubated at 37 °C for 30 min (red) prior to aSEC compared to an equimolar mixture of S135Q-HSPB5 and HSPB6 incubated at 37 °C for 30 min (blue) prior to aSEC. The aSEC trace for S135Q-HSPB5 is colored gray.

challenge. The HSPB6 NTR drives neither formation of large HSPB6 homomeric species nor the incorporation of HSPB6 into preexisting HSPB5 oligomers. However, once incorporated, an HSPB6 NTR may contribute to the global oligomer distribution and/or stability of the heterooligomer. To further assess a contribution of the HSPB6-NTR to the final oligomeric distribution of HSPB5/HSPB6 heterooligomers, we compared the incorporation of HSPB6 with the incorporation of the NTR deletion ΔN-HSPB6. Over a 2 h time course, ∼60% of FL HSPB6 was incorporated into HSPB5/HSPB6 heterooligomers based on the decrease in the aSEC peak volume observed for HSPB6 (Figure 7). In contrast, only ∼25% of ΔN-HSPB6 was

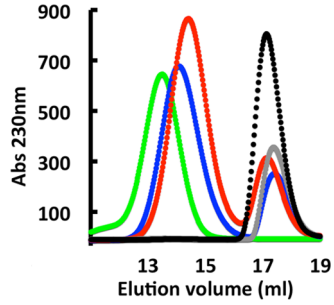

Figure 7. Role of the NTR in HSPB5−HSPB6 interactions. aSEC traces of HSPB5 oligomers (green) and HSPB6 (black) alone, and as an equimolar mixture incubated at 37 °C for 2 h (red) prior to aSEC compared to an equimolar mixture of the HSPB5 oligomer and ΔN-HSPB6 incubated at 37 °C for 2 h (blue) prior to aSEC. The aSEC profile for ΔN-HSPB6 alone is colored gray.

incorporated, based on peak volume. Additionally, when FL-HSPB6 subunits are incorporated, the resulting HSPB5/HSPB6 heterooligomer elutes later than when the smaller ΔN-HSPB6 subunits are incorporated, demonstrating the presence of the HSPB6 NTR has a detectable impact on the heterooligomer that is formed. Altogether, the results confirm that while the HSPB6 NTR does not drive oligomerization *per se*, it makes significant contributions to the stability of the heterooligomer and the energetics and extent of incorporation.

DISCUSSION

All sHSPs are composed of an NTR, an ACD, and a CTR. Despite their heterogeneity, three types of interactions have been observed or inferred in sHSP oligomeric structures: an ACD−ACD dimer interface, an ACD−CTR interaction, and ill-

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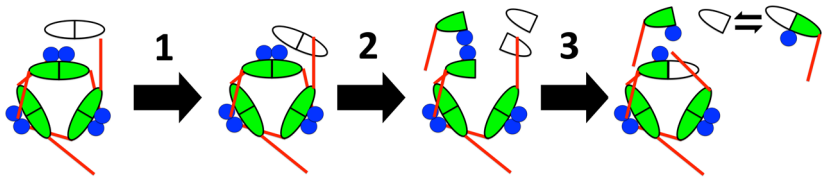

Figure 8. Model for subunit recruitment and incorporation. (1) Through an ACD−CTR interaction, an ACD-only (white) is recruited to the oligomer though interactions with CTRS that are "free" in the oligomer [shown as a hexamer; NTR (blue), ACD (green), and CTR (red)]. (2 and 3) Association through the CTR permits mixing at the ACD−ACD interface through monomer exchange and results in the subsequent dissociation of a subunit previously bound to the oligomer.

defined interactions involving NTRs. We used an ACD-only construct that forms a dimer on its own to parse out the importance and interplay of these interactions in subunit recruitment and oligomer assembly. We detected two classes of interactions between an ACD-only and an HSPB5 oligomer (Figure 2): (1) a weak oligomer−ACD complex that manifests as broadening of the ACD elution peak toward shorter elution times without a detectable change in the elution of the oligomer peak (Figure 2C), which we could attribute to ACD−CTR interactions, and (2) incorporation of ACD-only subunits into an oligomer to form a "mixed" oligomer that elutes later than the original oligomer (Figure 2A). This incorporation into the oligomer is dependent on the formation of ACD−ACD interactions between ACD-only and the full-length HSPB5 oligomer. The fact that "locked" ACD-only dimers are recruited but not incorporated into the oligomer is most consistent with exchange occurring at the dimer interface (Figure 2C). Thus, incorporation of ACD-only into an HSPB5 oligomer proceeds through exchange of monomeric subunits. Importantly, the ACD−CTR interaction is a prerequisite for exchange at the ACD−ACD interface. This implies that subunit exchange occurs with subunits recruited to the oligomer and that the interactions within an oligomer are interdependent. Our observations with HSPB5/HSPB6 heterooligomers are consistent with the observations of interactions of ACD-only with HSPB5 oligomers and those made by others (Figures 5 and 6).[21]

We propose that the accessibility of CTRs (i.e., CTR_{free}:CTR_{bound}) dictates both the extent and rate of recruitment of a subunit to the HSPB5 oligomer. The fact that we can both enhance and diminish the extent of both ACD-only and HSPB6 heterooligomer formation with HSPB5 by modulating the ACD−CTR interaction underscores the importance of this interaction in sHSP subunit recruitment and incorporation (Figures 2 and 4−6). Thus, conditions known to increase CTR_{free} such as heat shock or a small decrease in pH will enhance subunit exchange.[32,33] Others have proposed the ACD−CTR interaction is relevant for subunit dissociation and may destabilize the ACD−ACD interface to promote subunit exchange.[25,34] Whether CTR−ACD binding *per se* has a direct effect on the intrinsic rate of exchange at the dimer interface can be neither confirmed nor disputed by the results presented here. However, by bringing a subunit to the oligomer, ACD−CTR binding increases the effective local concentration of nonoligomeric subunits, which will manifest in an observed increase in the extent of dimer exchange. Though the affinity of ACD−CTR binding ($K_d \sim 100\ \mu M$) is lower than that of ACD−ACD binding ($K_d \sim 2\ \mu M$), the ∼1000 s^{-1} rate of exchange at the dimer interface is substantially higher than the rate of < 60 s^{-1} for ACD−CTR binding.[26,34,35] Thus, once bound by a CTR, ACDs of incoming and oligomer-bound subunits can rapidly exchange. The values quoted are for HSPB5; if other human sHSPs have appreciably different affinities and/or exchange rates, they could impact these steps in subunit recruitment and/or exchange.

The precise nature of the role of the NTR in oligomer recruitment and assembly remains enigmatic. Although we did not set out explicitly to study the NTR, our results do shed light on some possible roles. Specifically, our results show that the NTR plays a deterministic role in the stability and distribution of HSPB5/HSPB6 heterooligomers (Figures 5 and 7). By extrapolation, we conclude that the NTR plays a thermodynamic role in oligomer assembly. This conclusion is consistent with studies of phosphorylated states of HSPB1 and HSPB5, in which phosphorylation of NTR residues promotes disassembly of large oligomers into smaller species, and studies of nonmammalian sHSPs, which also demonstrate that alterations in NTRs result in changes in the size and/or stability of their respective oligomers.[36−39]

Altogether, observations reported here support a stepwise model by which subunits are incorporated into HSPB5 oligomers (Figure 8). First, a subunit is recruited via its $\beta4/\beta8$ groove to an oligomer CTR through an ACD−CTR interaction. A mutation or conditions such as increased temperature or decreased pH that increase the fraction of CTRs in the "free" state will therefore have a direct effect on both the rate and extent of subunit incorporation.[29,30] Next, exchange at the ACD dimer interface occurs to form a new dimer composed of one original subunit and one newly recruited subunit. A key implication of this step is that subunit exchange in and out of an oligomer occurs (predominantly) via monomeric subunits, even though sHSP dimers are presumed to be the structural building blocks of oligomers. Likely, displaced monomers from this step are able to form new dimeric species off the oligomer, based on reported exchange rates and dimer affinities for the ACD−ACD interaction.[35] Finally, the NTR of an incoming subunit is likely sequestered in the interior of the oligomer where it contributes to the thermodynamic stability and size of the newly formed heterooligomer.

The model presented here implies there are two requirements for the recruitment of sHSP subunits into an oligomer. First, it must have sufficient sequence complementarity to bind a CTR displayed by an oligomer through an ACD−CTR interaction. The ability to bind a CTR's IXI motif is defined by four residues in the $\beta4/\beta8$ groove of the ACD, which are conserved in all human sHSPs except ODF1 (Figure S4 of the Supporting Information). Second, a recruited subunit must be capable of forming a heteromeric ACD−ACD dimer interface to be fully incorporated into an oligomer. The long β-strand that makes the dimer interface is also remarkably well conserved (Figure S4 of the Supporting Information), implying

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that a majority of human sHSPs may be able to form heterodimers through their ACDs. Therefore, formation of the heterooligomers observed to date (e.g., HSPB1/HSPB5, HSPB4/HSPB5, HSPB1/HSPB6, and HSPB5/HSPB6) likely proceeds via a mechanism similar to the one proposed here, though the explicit role(s) of the highly variable NTRs remains to be defined.[19−21,24,40]

Our observations here suggest a dominant route in subunit recruitment. Less kinetically favorable modes of subunit recruitment that do not involve the CTR may also exist. The fact that S135Q-HSPB5, which cannot bind IXI motifs, is able to form oligomers and the fact that S134Q-HSPB6, though at a rate much slower than that of wt HSPB6, forms hetero-oligomers with HSPB5 suggest that alternate interaction modes do exist. Identifying and characterizing other modes of recruitment and their role(s) in processes such as the initial steps in the assembly of an oligomer will require further study and new approaches.

In summary, we have assigned roles for the interactions observed to exist in sHSP oligomers in the process of assembly and subunit exchange. Given the conservation in the relevant sequences, we imagine that most human sHSPs will assemble using these rules, albeit with inevitable variations that remain to be defined. We note that the insights obtained here stem from simple biochemical approaches using versions of sHSPs designed based on our structural understandings of them. Similar approaches applied to other sHSPs should make them similarly tractable and forthcoming, allowing for a fuller understanding of the mechanism(s) and diversity of sHSP oligomer assembly.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

aSEC elution volumes for molecular weight standards and relevant proteins, a representative SDS−PAGE gel from ACD interface exchange experiments, HSQC spectra of ΔN-HSPB6 with HSPB5-CTR peptide bound, and a sequence alignment of the ACD regions of all human sHSPs are available. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00490.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

sHSP, small heat shock protein; ACD, α-crystallin domain; aSEC, analytical size exclusion chromatography; NTR, N-terminal region; CTR, C-terminal region; NMR, nuclear magnetic resonance; HSQC, heteronuclear single-quantum coherence spectroscopy; TROSY, transverse relaxation-optimized spectroscopy.

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