Chaperone activity of human small heat shock protein-GST fusion proteins

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Abstract Small heat shock proteins (sHsps) are a ubiquitous part of the machinery that maintains cellular protein homeostasis by acting as molecular chaperones. sHsps bind to and prevent the aggregation of partially folded substrate proteins in an ATP-independent manner. sHsps are dynamic, forming an ensemble of structures from dimers to large oligomers through concentration-dependent equilibrium dissociation. Based on structural studies and mutagenesis experiments, it is proposed that the dimer is the smallest active chaperone unit, while larger oligomers may act as storage depots for sHsps or play additional roles in chaperone function. The complexity and dynamic nature of their structural organization has made elucidation of their chaperone function challenging. HspB1 and HspB5 are two canonical human sHsps that vary in sequence and are expressed in a wide variety of tissues. In order to determine the role of the dimer in chaperone activity, glutathione-S-transferase (GST) was genetically linked as a fusion protein to the N-terminus regions of both HspB1 and HspB5 (also known as Hsp27 and α B-crystallin, respectively) proteins in order to constrain oligomer formation of HspB1 and HspB5, by using GST, since it readily forms a dimeric structure. We monitored the chaperone activity of these fusion proteins, which suggest they primarily form dimers and monomers and function as active molecular chaperones. Furthermore, the two different fusion proteins exhibit different chaperone activity for two model substrate proteins, citrate

synthase (CS) and malate dehydrogenase (MDH). GST-HspB1 prevents more aggregation of MDH compared to GST-HspB5 and wild type HspB1. However, when CS is the substrate, both GST-HspB1 and GST-HspB5 are equally effective chaperones. Furthermore, wild type proteins do not display equal activity toward the substrates, suggesting that each sHsp exhibits different substrate specificity. Thus, substrate specificity, as described here for full-length GST fusion proteins with MDH and CS, is modulated by both sHsp oligomeric conformation and by variations of sHsp sequences.

Keywords Chaperone · Fusion protein · Small heat shock protein (sHSP) · Protein aggregation · Protein-protein interaction · Light scattering assay · Glutathione-S-transferase (GST)

Introduction

Small heat shock proteins (sHsps) are molecular chaperones present across all kingdoms of life. During cellular stress, such as elevated temperature or oxidative damage, protein structure is compromised leading to the exposure of hydrophobic residues and nonnative folded states. sHsps are upregulated in response to stress as part of a general protective mechanism to maintain cellular protein homeostasis, particularly as ATP-dependent chaperones become overburdened (Bukau et al. 2006; Ecroyd and Carver 2009; Haslbeck and Vierling 2015).

sHsps are ATP-independent, nonenzymatic chaperones, whose activity prevents aberrant protein aggregation by interacting with nonnative protein substrates (Haslbeck et al. 2005) to prevent their nonspecific interaction and aggregation. sHsp subunits are comprised of a conserved alpha-crystallin domain (ACD, ~90 amino acids) with N- and C-terminal extensions of highly variable amino acid composition and length

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(Fig. 1a). Unlike the ACD, the N-terminal region (NTR) and C-terminal region (CTR) are highly hydrophobic, intrinsically disordered regions that lack significant structure (Seit-Nebi and Gusev 2012; Sudnitsyna et al. 2012), making their structure determination intractable. There have been X-ray structures of plant and bacterial sHsps for almost two decades (Kim et al. 1998; van Montfort et al. 2001); however, there remains little structural information on full-length vertebrate sHsps to date, although an increase in complementary methods, Cryo-EM, SAXS, NMR, and mass spectrometry have gained insight into sHsp assemblies (Hilton et al. 2012; Delbecq and Klevit 2013). X-ray crystallography and NMR structures of the truncated ACD from vertebrates identify a β -sandwich topology composed from up to nine β -strands, forming a dimeric building block with several different interface arrangements (Kim et al. 1998; van Montfort et al. 2001; Ghosh et al. 2005; Laganowsky et al. 2010; Jehle et al. 2010; Clark et al. 2011; Rajagopal et al. 2015a). Three-dimensional structures of truncated vertebrate sHsp assemblies have been determined, which suggest regions within all three domains, NTR, ACD,

and CTR, are involved in regulation of subunit activity, assembly, and substrate specificity (Ghosh et al. 2005; Jehle et al. 2010; Takeda et al. 2011; Clark et al. 2011; Skouri-Panet et al. 2012; Peschek et al. 2013a, b).

Underlying sHsp function is a dynamic oligomerization process contributing to the higher-order organization of the chaperones. sHsps form functional homo- or heterooligomeric complexes, comprised of 2–40 subunits of 12–43 kDa. Importantly, sHsp oligomeric complexes are polydisperse, with wide variations in subunit number and composition (Giese 2002; Haslbeck et al. 2005; Peschek et al. 2013a, b; Delbecq and Klevit 2013). Dynamic subunit exchange, which can maintain or alter the heterogeneous protein composition, is a unique feature of sHsps. Structural reorganization, best characterized in eye lens sHsps (α -crystallins), is influenced by stress conditions and other cellular factors (Skouri-Panet et al. 2006; Clark et al. 2012). Structural dynamics and oligomeric organization appear to be dependent on both NTR and CTR (Haslbeck et al. 2004; Peschek et al. 2013a; Delbecq and Klevit 2013; Delbecq et al. 2015). Mutations within all

Fig. 1 Domain organization and structure of sHsps. **a** All sHsps have a variable N-terminal region (NTR), a highly conserved α -crystallin domain (ACD), followed by the C-terminal region (CTR). Wild type HspB1 and HspB5 have an N-terminal 6xHis tag. Glutathione-S-transferase (GST) fusion proteins were constructed with GST fused to the N-terminal region of human HspB1 and HspB5. Corresponding

molecular weights of each protein are as indicated. **b** The sequence alignments for human HspB1 and HspB5 (alternatively known as CRYAB) demonstrate the conserved ACD is highly homologous while the NTR and CTR are notably unconserved, varying in length and sequence between these human sHsps. Sequence alignments were performed using T-Coffee (Notredame et al. 2000) (HspB1: P04792 and HspB1: P02511)

regions of the sHsps, the NTR, ACD, and CTR, that shift oligomeric dynamics are directly implicated in several inherited diseases (Bova et al. 1999; Evgrafov et al. 2004; Houlden et al. 2008; Raju et al. 2011; Clark et al. 2012), suggesting that oligomer dynamics and sHsp quaternary structure are important for chaperone activity. Additionally, phosphorylation and mutations to mimic phosphorylation of specific residues in the NTR have produced varying results. In some reports, there is an increased propensity for sHsps to form smaller oligomers, which illustrates an increase in chaperone activity for both HspB1 and HspB5 (Shashidharamurthy et al. 2005; Ecroyd et al. 2007; Jovcevski et al. 2015). In other studies, phosphorylation of a single NTR serine resulted in a lack of a dimeric structure and produced a broader oligomeric distribution (Aquilina et al. 2004). These results suggest that dimer formation may directly underlie chaperone activity; therefore, understanding this structure-function relationship has broad implications for understanding the broader mechanism of sHsp chaperone-like behavior.

sHsps are intrinsically promiscuous, as they sit at the nexus of protein homeostasis with the purpose of interacting with a wide-ranging diversity of substrates under various cellular conditions. This feature allows sHsps to be malleable to ever-changing cellular conditions, while making it difficult to identify substrate interacting sites. Far less is known about the mechanism of sHsp substrate binding and chaperone function compared to the ATP-dependent HSPs, Hsp70, Hsp90, and GroEL (Jaya et al. 2009; Haslbeck and Vierling 2015). Mutational studies combined with mass spectrometry analysis suggest that NTRs as well as the ACD (α -crystallin domain) play a crucial role in both substrate binding and oligomer assembly. These studies propose that some mutations can stabilize large oligomeric assemblies, preventing monomer or small oligomer disassembly, which reduces chaperone activity (Clark et al. 2012; Datskevich et al. 2013; Muranova et al. 2015; Makley et al. 2015).

This dynamic property of sHsps is fundamental to their chaperone function, yet little is known about the role of oligomerization in substrate binding and specificity, the function of individual oligomeric states, and the chemical features of sHsps involved in substrate binding (McDonald et al. 2012). Additionally, current studies suggest that a dimer is the smallest naturally occurring structural unit (Haslbeck et al. 2005; Basha et al. 2011); however, the smallest physiologically relevant unit with functional activity remains to be determined.

HspB1 and HspB5 (commonly referred to as Hsp27 and α B-crystallin, respectively) are two of the ten human sHsps and are ubiquitous in most human tissues as stress responders to prevent client protein aggregation and, in some cases, promote disaggregation. In order to identify chaperone activity of dimers and small oligomers, glutathione-S-transferase (GST) was genetically linked as

a fusion protein to the N-terminus regions of both proteins, to constrain HspB1 and HspB5 to small oligomers, dimers, or monomers. Our studies suggest that the fusion proteins primarily form dimers and monomers and they function as active molecular chaperones. Furthermore, the two different fusion proteins, GST-HspB1 and GST-HspB5, demonstrate different chaperone activity for two model substrates, MDH and CS, and exhibit altered chaperone activity relative to wild type HspB1 and HspB5. Malate dehydrogenase (MDH) and citrate synthase (CS) are both enzymes involved in the citric acid cycle and exist as dimers or tetramers in solution (Ech-Cherif el-Kettani et al. 1993; Goward and Nicholls 1994). Under gentle thermal stress, both proteins undergo partial unfolding, aggregation, and precipitation. Specifically, GST-HspB5 is a significantly poorer chaperone than wild type HspB5 for both substrates. These results suggest that small oligomers of sHsps are likely not the most “active” species for all substrates and the diverse oligomeric architecture of sHsps contributes to overall chaperone activity and substrate specificity.

Many changes in oligomeric propensity due to mutation, stress, and other cellular alterations have been illustrated (Clark et al. 2011; Rajagopal et al. 2015b; Jovcevski et al. 2015). Furthermore, alterations in oligomeric dynamics have specifically illustrated dimer formation, suggesting that altering the oligomeric structure and forming dimers may alter the chaperone capability of sHsps. Specifically, monomer and dimer formations have been identified in an inherited genetic mutation in HspB1, underlying the Charcot-Marie-Tooth disease (Almeida-Souza et al. 2010), yet mutations in HspB1 and HspB5 that favor dimer formation have illustrated both increases and decreases in chaperone activity, depending on the substrate (Hochberg et al. 2014; Jovcevski et al. 2015). These reports reinforce the complicated nature of sHsp chaperone activity and suggest that a complete understanding of how oligomerization correlates to chaperone activity remains unknown. These studies motivated our investigation of full-length sHsp fusion protein dimers.

Methods

Materials

MDH, insulin, and protease inhibitor cocktail were obtained from Thermo Fisher and CS was obtained from Sigma. Monoclonal antibodies Anti-HspB1 (G3.1, Thermo Fisher Scientific), Anti-HspB5 (Abcam), a polyclonal antibody for glutathione-S-transferase (Abcam), and secondary antibodies (HRP, Abcam) were obtained and used without further purification.

Recombinant protein preparation

Recombinant human HspB5 and HspB1 (both containing N-terminal His tags) were expressed in *Escherichia coli* BL21(DE3) cells at 30 °C for 3 h after induction at mid-log phase by 500 mM isopropyl β -D-thiogalactopyranoside (IPTG) using plasmids kindly gifted by Dr. Jason Gestwicki (UCSF). Recombinant GST-HspB1 and GST-HspB5 fusion proteins were expressed in *E. coli* BL21(DE3) cells at 25 °C for 5 h after induction at mid-log phase by 500 mM IPTG from the PGex-6P-1 (GE Life Sciences) plasmid, in which HspB1 or HspB5 was synthesized and cloned into the *Bam*HI and *Eco*RI restriction sites by GenScript (Piscataway, NJ, USA).

Recombinant human HspB1 and HspB5 protein purification protocols are similar to the ones described previously (Makley et al. 2015). Briefly, cells were lysed by sonication in lysis buffer (20 mM Tris pH 8.0/100 mM NaCl, 6 M urea, 5 mM β -mercaptoethanol, 15 mM imidazole) and HALT protease cocktail (Thermo Fisher). The insoluble material was removed by centrifugation and the supernatant was loaded onto Ni-NTA resin and washed with 10 column volumes of wash buffer (lysis buffer containing 30 mM imidazole). The proteins were eluted using an elution buffer (lysis buffer containing 150 mM imidazole) and the fractions containing protein were pooled, followed by an addition of 5 mM EDTA. The proteins were refolded by injecting 1-mL samples of concentrated protein onto a Superdex 200 HR 10/30 column equilibrated with Buffer A (20 mM sodium phosphate, pH 7.2, 100 mM NaCl) at 4 °C. Refolded protein eluted as an oligomer with a diameter of $\sim$ 165 Å, consistent with previous literature values. GST-HspB1 and GST-HspB5 were lysed by sonication in lysis buffer with HALT protease cocktail. Crude protein was dialyzed using 10,000 MW SnakeSkin tubing into 1 $\times$ PBS. Crude supernatant was collected and loaded onto glutathione beads (GE Life Sciences) and eluted using elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0). Proteins were loaded onto a Superdex 200 HR 10/30 column equilibrated with Buffer A (20 mM sodium phosphate, pH 7.2, 100 mM NaCl) at 4 °C for refolding. This step was sufficient as checked by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to obtain pure proteins. His tags were not removed from wild type proteins.

SDS-PAGE and native-PAGE

For SDS-PAGE, discontinuous polyacrylamide gels using a 4% (v/v) stacking and 10% (v/v) 4–20% (v/v) resolving gel or 8–16% (v/v) resolving gel (as indicated) were used as per manufacturer's instructions (Bio-Rad). For native-PAGE, samples were loaded onto discontinuous polyacrylamide gels using a 4–20% (v/v) resolving gel (Bio-Rad). Coomassie or silver staining (Pierce Kit) was performed as indicated.

Analytical size-exclusion chromatography

The oligomeric sizes of HspB1 and HspB5 were determined by size-exclusion chromatography (SEC). sHsp samples (2 or 12 μ M where indicated) and specified ratios of substrates (MDH and CS) were loaded onto a Superdex 20 10/300 size-exclusion column (GE Healthcare), which had been equilibrated in 50 mM phosphate and 100 mM NaCl (pH 7.4), at a flow rate of 0.3 mL/min at room temperature. The size-exclusion columns were calibrated using standards (Bio-Rad): bovine thyroglobulin (670 kDa), bovine γ -globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), and vitamin B12 (1.35 kDa).

Western blotting

Protein samples of 5 μ M were subjected to SDS-PAGE or native-PAGE, immunoblotted onto PVDF membranes, and washed. Respective membranes were probed with mouse anti-GST (ThermoFisher Scientific), mouse anti-HspB5 (Abcam), or anti-HspB1 (Abcam). The sHsps were visualized following development with the Fast ECL kit (Thermo Scientific).

In vitro chaperone assay

CS and MDH aggregation was measured by solution absorption, similar to previously described methods (Ghosh et al. 2005). 1 $\times$ PBS buffer (pH 7.3), containing 1.2 μ M of respective substrate, was heated to 45 °C in a quartz cuvette then indicated chaperone proteins were added to final concentrations of 1.2 μ M. The final volume of each sample was 1 mL. Absorbance was measured at 340 nm in a Cary-100 UV-Vis spectrophotometer equipped with an automatic temperature controller every 3 min for 60 min at 45 °C with continuous stirring.

Tryptophan fluorescence

The tryptophan fluorescence of 1.2 μ M of sHsp or 1:1 ratios of sHsp:substrate (1.2 μ M) was measured in 3 mL of 1 $\times$ PBS. The excitation wavelength of 280 nm and an emission wavelength ranging from 310 to 400 nm were recorded with a 5-nm band pass on a fluorometer. Measurements were taken at 25 °C (room temperature) and at 45 °C (heated) after sample equilibration.

Statistical analysis

KaleidaGraph 4.5.2 software was employed for data analysis and graphing. Data represent the mean ($\pm$) standard error of the mean (SEM) for $\geq$ 3 independent replicates. Normalization

was performed for chaperone activity data. Data were normalized to the maximal aggregation of each substrate protein.

Results

The sHsps generally form large, polydisperse oligomeric complexes. Data in the literature suggest that dimers are the smallest functional sHsp unit, although monomers have been shown to undergo subunit exchange from some oligomeric complexes (Shashidharamurthy et al. 2005; Ghosh et al. 2007; Carra et al. 2013). Additionally, different chaperone activities have been identified across the ten different human sHsps, suggesting there is a substrate specificity aligned with particular sHsps, likely arising from their specific expression patterns, tissue specificity, and differences in primary sequences (Fig. 1b) (Treweek et al. 2015). Furthermore, alterations in oligomeric distributions are linked to several diseases, suggesting oligomer dynamics and structure contribute to chaperone function (Meehan et al. 2007; Almeida-Souza et al. 2010; Muranova et al. 2015). Specifically, mutations that shift oligomeric propensity toward smaller species and dimers have shown both increases and decreases in chaperone activity depending on the substrate. We hypothesized that this would be the case for full-length dimers; however, we were interested in investigating the changes in chaperone activity by restricting sHsp oligomeric conformation to forming dimers through the use of GST-fusion protein and by comparing the interactions of the sHsp fusion proteins with two different substrates.

Synthesis and structural characteristics of GST-sHsp fusion proteins

To investigate the function of sHsps that are only capable of forming small oligomers (primarily dimers) and monomers, we prepared GST-sHsp fusion proteins using two human sHsps, HspB1 and HspB5. GST was used for dimer formation due to the propensity of GST to form homodimers in solution and its well-characterized structure. This method of using GST fusion proteins to construct dimeric proteins has been applied in many cases for evaluating the structure/function relationship of many proteins (Niedziela-Majka et al. 1998; Masino et al. 2002; Oakley 2005). The GST-tag was appended to the N-terminal region of each respective sHsp. Our motivation for appending GST to the N-terminus of each sHsp was two-fold. The first rationale was due to availability of GST cloning vectors, which place the tag on the N-terminus of the fusion protein. We were additionally motivated due to evidence that the sHsp NTR facilitates sHsp oligomerization (Delbecq et al. 2015). We reasoned that disrupting potential NTR interactions may facilitate dimer formation by limiting

Fig. 2 Native-PAGE electrophoresis, size-exclusion chromatography, and western blot analysis of GST-sHsps. **a** Native-PAGE electrophoresis of *GST-HspB1* and *GST-HspB5* ran on a 4–20% gradient gel. Lane 1 and 3 signify *GST-HspB5* and *GST-HspB1*, respectively, indicating monomer and dimer formations observed under native conditions. *GST* only (lane 2) indicates the observed dimer structure. **b** SEC analysis performed on a Sephadex 200 10/300 GL column at 4 °C. *GST-HspB1* and *GST-HspB5* were evaluated at 2 μ M concentrations in phosphate buffer at pH 7.4. *GST-HspB1* eluted in two primary peaks, ~16 and ~18 mL, which correspond to the *dimer* and *monomer*, respectively. *GST-HspB5* eluted in two major peaks, ~17 and ~18.5 mL. For reference, the molecular weight standards for the size-exclusion chromatograms are assigned as follows: 1. aggregate peaks (>670 kD), 8.4 mL; 2. thyroglobulin (669 kDa), 10.5 mL; 3. γ -globulin (158 kDa), 14 mL; 4. ovalbumin (44 kDa), 16.5 mL; 5. myoglobin (17 kDa) 18.25 mL; 6. vitamin B₁₂ (1.35 kDa), 21 mL

interactions between sHsp NTRs. Additionally, to ensure that quaternary structure for both GST and HspB1 were not further disrupted, all experiments were performed under nonreducing conditions. GST and HspB1 are known to form disulfide bonds that contribute to quaternary structure. Performing the experiments under nonreducing conditions may contribute to the results reported below. Evaluation of chaperone activity was monitored and compared using two model substrates that undergo heat-denaturation, MDH and CS. MDH and CS have

been extensively used as model substrates for sHsps as well as other chaperones (Cheng et al. 2008; Rajagopal et al. 2015b; Cha et al. 2016).

To ensure production of full-length fusion proteins, both native and denaturing protein gels were evaluated (Fig. 2a, SI. Fig. 4) demonstrating the production of monomeric and dimeric GST-HspB1 and GST-HspB5. GST alone was a control in the gel. Western blot analysis of GST-HspB5 confirmed the presence of the fusion protein by labeling with both anti-GST and anti-HspB5 antibodies in separate blots (SI. Fig. 4). For western blot analysis, native-PAGE gels confirmed the size of GST (~26 kDa), GST-HspB1 (~51 kDa), and GST-HspB5 (~49 kDa). GST-HspB1, GST, and HspB1 were also confirmed by western blotting after native-PAGE gel electrophoresis (SI. Fig. 1). The western blots confirm that GST is fused to the sHsps and that monomers and dimers are the primary products observed in native-PAGE electrophoresis.

To identify potential oligomerization of the fusion proteins, GST-HspB1 and GST-HspB5 (2 μ M) were evaluated by size-exclusion chromatography (Fig. 2b) and compared to standards (SI. Fig. 2). GST-HspB1 and GST-HspB5 samples identified two primary peaks each (Fig. 2b), which were eluted from the column corresponding to a dimer (~16, 16.5 mL elution volume, respectively) and monomer (~18, 18.5 mL elution volume, respectively). In addition, both fusion proteins exhibited a peak to initially come off the column (~8.5 mL), which we suggest may be large oligomers or some misfolded protein. There are some small peaks corresponding to late elution volumes (>20 mL), which we attribute to some residual GST from the purification or small impurities in the protein mixture. The chromatograms indicate that GST-HspB5 may form more monomers relative to GST-HspB1. This may be due to the different conformations HspB1 and HspB5 can adopt or it may be due to the disulfide crosslinking, which occurs in HspB1 but not HspB5. There is a single cysteine in HspB1 found in the ACD and contributes to an intersubunit disulfide bond at the ACD dimer interface. The lack of this potential crosslink could lead to more monomerization of GST-HspB5 (Diaz-Latoud et al. 2005; Baranova et al. 2011) and therefore result in more GST-HspB1 dimers.

The model substrates, CS and MDH, both heat-denaturing substrates, were evaluated with GST-HspB1 and GST-HspB5 to identify chaperone activity from a wide array of sHsps and other chaperones, such as GroEL (Basha et al. 2006; Elad et al. 2007). These substrate:sHsp complexes were made in a 1:1 M ratio to identify whether the chaperone was capable of protecting each substrate from aggregation and to identify structural changes upon mixing. Intrinsic tryptophan fluorescence was used to assess changes in protein tertiary structure after mixture with the substrates (Ghisaidoobe and Chung 2014). There are only two tryptophan residues in HspB5. GST, CS, and MDH each have several Trp residues, 4, 9,

and 4, respectively, which are spread throughout the structure. Therefore, specific insights into changes in the protein environment are difficult to determine; however, general observations are outlined. Generally, sHsp chaperone function is attributed to hydrophobic regions of the sHsp interacting with nonnative substrates (Nakamoto and Vigh 2006). When CS and MDH were individually mixed with GST-HspB5 (1:1 ratio), opposite effects were observed relative to the

◀ **Fig. 3** Effect of substrates and temperature on intrinsic tryptophan fluorescence. Tryptophan excitation occurred at 280 nm and emission was recorded between 310 and 400 nm. **a** Samples of 1.2 μ M GST-HspB5, GST-HspB5, and CS (1:1 M ratio) or CS (1.2 μ M) were measured at 25 °C. Relative fluorescence intensity of the GST-HspB5:CS mixture elicited a large increase in fluorescence relative to the chaperone or substrate alone. **b** Samples of 1.2 μ M GST-HspB5, GST-HspB5, and MDH (1:1 M ratio) or MDH (1.2 μ M) were measured at 25 °C. Upon mixing of GST-HspB5 and MDH, less relative fluorescence was observed compared to the GST-HspB5:CS mixture and to the MDH only sample. **c** All samples above GST-HspB5, GST-HspB5+CS, CS, MDH, and GST-HspB5+MDH were heated to 45 °C and equilibrated for 10 min prior to recording fluorescence. Relative fluorescence intensities of heated (45 °C)/RT (25 °C) were plotted for each sample. All samples, except for CS, exhibited a smaller fluorescence signal upon heating to a similar degree except for GSTHspB5+MDH, with an ~80% decrease in fluorescence

fluorescence of each substrate protein alone (Fig. 3a, b) at 25 °C. However, an increase in Trp fluorescence was observed for both CS and MDH upon mixing with GST-HspB5 relative to the sample containing only GST-HspB5. These results suggest that upon mixing, some interactions are occurring between the Trp-containing protein sequences, that are generally hydrophobic protein regions.

Chaperone activity of GST-HspB1 and GST-HspB5

In order to determine whether or not GST-sHsps would exhibit chaperone activity, GST-HspB1 and GST-HspB5 were mixed with substrates, CS and MDH, in 1:1 M ratios and subjected to heating. This ratio was evaluated to determine whether sHsps in dimer or small oligomer form would exhibit chaperone activity and to prevent possible sHsp oligomerization by increasing the relative concentration of sHsp in the mixture. Increasing the relative concentration of GST-sHsps (12 μ M) resulted in a shift to more higher-ordered oligomers (shorter retention time) during size-exclusion chromatography (SI Fig. 3). At lower molar concentrations (2 mM), the larger oligomers are reduced.

The chaperone function of HspB1, HspB5, GST-HspB1, and GST-HspB5 was compared to determine whether the GST-sHsps could protect model substrates, MDH and CS, from heat-induced aggregation. For all experiments, samples were mixed together, heated to 45 °C and stirred, during which light scattering was measured for 60–90 min. In the absence of chaperone, both MDH and CS formed visible aggregates, which resulted in light scattering at 340 nm. The ratios used for the chaperone anti-aggregation assays was 1:1 for all experiments and demonstrated that each of the four chaperone exhibited chaperone activity (Fig. 4). Specifically, for the substrate MDH, the most effective chaperones were HspB5 and GST-HspB1, demonstrating an ~80% decrease in aggregation (Fig. 4a). The least effective chaperone was GST-HspB5, with ~25% decrease in aggregation. HspB1

exhibited a ~50% decrease in aggregation. For both HspB1 and HspB5, the corresponding GST-fusion sHsp maintained significantly different chaperone activity relative to the wild type counterpart when MDH was the model substrate (Fig. 4a). These results indicate that both sequence and oligomerization dynamics influence substrate binding and chaperone activity. While all the sHsps demonstrate chaperone activity, both GST-HspB5 and HspB1 exhibit altered kinetics with a rapid initial association (increase in light scatter) relative to HspB5 and GST-HspB1 (Fig. 4a).

To further investigate the chaperone function of GST-HspB1 and GST-HspB5, the model substrate CS was used to compare the anti-aggregation chaperone function on the fusion proteins relative to wild type HspB1 and HspB5. Interestingly, HspB5 was the most effective at preventing aggregation with ~80% reduction in aggregation. HspB1, GST-HspB1, and GST-HspB5 all had similar effectiveness with ~60% reduction in aggregation (Fig. 4b). Both fusion proteins exhibited a more rapid onset of aggregation with chaperone compared to wild type protein HspB1 and HspB5 (Fig. 4b); however, attenuation was observed within the first 5 min of aggregation. Controls were completed with GST alone, GST+CS, GST+MDH, GST-HspB1 only, and GST-HspB5 only (SI Fig. 4). In these samples, the presence of GST with each substrate leads to visible aggregation and an increase in light scatter. Neither GST-HspB1, GST-HspB5, nor GST aggregated in the absence of a substrate (SI Fig. 4). Overall, each chaperone effectively suppressed aggregation of CS and MDH in each assay.

In order to further evaluate the chaperone activity for both substrates, we compared light scatter (aggregation) at 340 nm after 30 min of heating with stirring at 45 °C. Wild type proteins, HspB1 and HspB5, displayed similar activity for each substrate (within error), and wild type HspB5 was more active (prevented more aggregation) than all other chaperones tested (Fig. 5). The fusion proteins displayed varying activities for each substrate. GST-HspB1 prevented more aggregation of MDH relative to CS after 30 min and exhibited more chaperone activity relative to HspB1 (Fig. 5). When GST-HspB5 was the chaperone, CS exhibited less aggregation relative to MDH, which is the opposite of what was observed for GST-HspB1. Importantly, HspB5 prevented more aggregation of both substrates relative to GST-HspB5. GST was a control protein during the experiment and elicited large light scatter in the presence of both substrates with visible aggregation observed (Fig. 5).

Chaperone-substrate complexes

All four chaperones, HspB1, HspB5, GST-HspB1, and GST-HspB5, were evaluated by SEC alone or after complexation with either CS or MDH (1:1 M ratio) after heating to 45 °C for

Fig. 4 Anti-aggregation chaperone activity with heat-denatured substrates, CS and MDH. **a** HspB1, HspB5, GST-HspB1, and GST-HspB5 all protect MDH from heat-denatured aggregation at 45 °C. More rapid aggregation kinetics between 1 and 10 min were observed for less active chaperones, HspB1 and GST-HspB5. **b** HspB1, HspB5, GST-HspB1, and GST-HspB5 all protect CS from heat-denatured

aggregation at 45 °C. Faster aggregation kinetics between 1 and 10 min were observed for both fusion proteins, GST-HspB1 and GST-HspB5. Aggregation was determined by light scattering at 340 nm. All protein samples were 1.2 μ M in concentration and chaperone-substrate mixtures were performed at 1:1 M ratios

30 min. Fractions were collected and evaluated by SDS-PAGE on 8–16% gradient gels under nonreducing conditions. When samples were analyzed, mixtures of HspB5 and CS or MDH were eluted in the same collected fractions, indicating

Fig. 5 Chaperone activity after 30 min of heating at 45 °C. Each chaperone or control (GST) was evaluated for amount of aggregation, measured by light scatter at 340 nm, after 30 min of heating at 45 °C with each heat-denatured substrate, CS or MDH. The error bars are $\pm$ SEM for the mean of ≥ 3 replicates. Data were normalized to the amount of aggregation observed for each substrate in the absence of chaperone or control

that a complex was between the chaperone and substrate formed and eluted together (Fig. 6a). Similar complexes were observed for GST-HspB5 and upon mixing with CS and MDH (Fig. 6b). Small shifts in SEC peaks were observed when CS and MDH were mixed with each chaperone (Fig. 6c, d). HspB5 and GST-HspB5 peaks were identified for large complexes (>670 kD) and two smaller complexes, ~ 100 and ~ 25 kD, respectively (Fig. 6c), suggesting that some of the chaperone, in the absence of a substrate, form large complexes after heating. HspB5 forms more of these larger complexes relative to GST-HspB5 (Fig. 6a, b). A 1:1 M ratio was chosen for these experiments to promote chaperone activity by providing sufficient chaperone in the mixture to interact with the denaturing substrate. However, we did not want to increase the chaperone ratio, which may have promoted higher-order oligomerization of the sHsp.

HspB1 and GST-HspB1 were evaluated and peaks for both chaperones were observed early in SEC analysis, which correspond to large complexes (<670 kD); however, only a small relative amount of each protein exhibited this quaternary form (Fig. 7). Upon mixing HspB1 with either MDH or CS, small shifts in the SEC elution were observed and both protein products eluted from the column at the same time, suggesting formation of an interaction between the proteins (Fig. 7a, c). Similar results were observed when GST-HspB1 was mixed with CS and MDH and further analysis by SDS-PAGE demonstrates that several different-sized complexes were eluted, highlighted in fractions between 15 and 17 mL (Fig. 7b, d).

Fig. 6 Chaperone function of wild type *HspB5* and *GST-HspB5* fusion proteins with substrate proteins *CS* and *MDH*. **a** *HspB5*, *HspB5*+*CS*, and *HspB5*+*MDH* were heated to 45 °C for 30 min and evaluated by SEC and nonreducing SDS-PAGE electrophoresis. **b** *GST-HspB5*, *GST-HspB5*+*CS*, and *GST-HspB5*+*MDH* were heated to 45 °C for 30 min and evaluated by SEC and nonreducing SDS-PAGE electrophoresis. All chaperone-substrate mixtures were evaluated using 1:1 M ratios. Fractions listed in **a** and **b** correspond to milliliters obtained from SEC fractions collected in **c** and **d**, respectively. **c** SEC evaluation of *HspB5*, *HspB5*+*CS*, and *HspB5*+*MDH* on a Superdex 200 10/300 GL column at

4 °C. **d** SEC evaluation of *GST-HspB5*, *GST-HspB5*+*CS*, and *GST-HspB5*+*MDH* on a Superdex 200 10/300 GL column at 4 °C. Approximately three peaks are observed for each sample. Molecular weight markers are indicated by lines adjacent to each gel image. For reference, the molecular weight standards for the size-exclusion chromatograms are assigned as follows: 1. aggregate peaks (>670 kDa), 8.4 mL; 2. thyroglobulin (669 kDa), 10.5 mL; 3. γ -globulin (158 kDa), 14 mL; 4. ovalbumin (44 kDa), 16.5 mL; 5. myoglobin (17 kDa) 18.25 mL; 6. vitamin B₁₂ (1.35 kDa), 21 mL

The peaks observed when *GST-HspB1* and *MDH* were mixed are shifted slightly to the left, suggesting a larger complex is forming. This is compared to the sample containing *GST-HspB1* and *CS*, where one large peak is observed at 15 mL and a smaller one at ~17 mL is observed, which do not appear to be shifted (Fig. 7d). Overall, these results suggest that *GST-HspB1* monomers and dimers interact with each substrate.

Discussion

sHsps are polydisperse in size and undergo rapid, dynamic equilibrium changes between oligomeric states. The different chaperone quaternary states are suggested to be essential for allowing sHsps to prevent denaturing substrate protein aggregation, one form of sHsp chaperone activity. This dynamic

nature of sHsps obscures the study of the chaperone and prevents a better understanding of how different oligomeric states contribute to chaperone activity in general and specifically of how oligomeric states interact with different substrate proteins. Moreover, differences observed between the structural studies of homo- and heterooligomers and mutational studies suggest there may be unobserved interactions, particularly when substrates are mixed with sHsps (Giese 2002; Giese and Vierling 2004). There is evidence that a dimer is the smallest “functional” unit for sHsps; however, this remains unknown. We have approached these questions by restricting the quaternary oligomerization of sHsps by creating N-terminal fusion proteins between glutathione-S-transferase (GST) and two human sHsps, *HspB1* and *HspB5*. GST was chosen for its well-known solubility and propensity to form dimers (Tudyka and Skerra 1997; Oakley 2005). The fusion

Fig. 7 Chaperone function of wild type HspB1 and GST-HspB1 fusion proteins with substrate proteins CS and MDH. **a** *HspB1*, *HspB1*+CS, and *HspB1*+MDH, MDH, and CS were heated to 45 °C for 30 min and evaluated by SEC and nonreducing SDS-PAGE electrophoresis. **b** *GST-HspB1*, *GST-HspB1*+CS, and *GST-HspB1*+MDH were heated to 45 °C for 30 min and evaluated by SEC and nonreducing SDS-PAGE electrophoresis. All chaperone-substrate mixtures were evaluated using 1:1 M ratios. Fractions listed in **a** and **b** correspond to milliliters obtained from SEC fractions collected in **c** and **d**, respectively. **c** SEC evaluation of *HspB1*, *HspB1*+CS, *HspB1*+MDH, CS, and MDH on a Superdex 200

10/300 GL column at 4 °C. **d** SEC evaluation of *GST-HspB1*, *GST-HspB1*+CS, and *GST-HspB1*+MDH on a Superdex 200 10/300 GL column at 4 °C. Approximately three peaks are observed for each sample. Molecular weight markers are indicated by lines adjacent to each gel image. For reference, the molecular weight standards are assigned as follows: 1. aggregate peaks (>670 kDa), 8.4 mL; 2. thyroglobulin (669 kDa), 10.5 mL; 3. γ -globulin (158 kDa), 14 mL; 4. ovalbumin (44 kDa), 16.5 mL; 5. myoglobin (17 kDa) 18.25 mL; 6. vitamin B₁₂ (1.35 kDa), 21 mL

proteins, GST-HspB1 and GST-HspB5, appear to primarily form monomers and dimers in solution. These chaperones were evaluated for chaperone activity with two model substrates, MDH and CS. Heat-induced aggregation of both substrates was attenuated in the presence of both fusion proteins; however, GST-HspB1 and GST-HspB5 exhibited varying levels of chaperone activity toward each substrate. Furthermore, comparison to wild type proteins, HspB1 and HspB5, provided insight into the role of oligomeric dynamics and chaperone function.

We demonstrate that GST-HspB1 and GST-HspB5 primarily form monomers and dimers at relatively low molar concentrations (2 and 12 μ M) by SEC and native-PAGE electrophoresis. While some differences in oligomeric propensity are observed as the concentration of the fusion proteins increases, this is consistent with previous observations that sHsps form oligomers in a concentration-

dependent manner. However, the formation of primarily monomers and dimers of GST-sHsps contrasts with wild type HspB1 and HspB5, which form primarily large (>250 kDa) oligomeric complexes in solution. In addition, structural changes observed through tryptophan fluorescence experiments suggest conformational changes in the proteins are occurring after the proteins are mixed in solution (1:1 ratios) at 25 °C. All of the tryptophan residues in HspB1 and HspB5 are found in either the NTR or ACD, with the majority present in the ACD. Hydrophobic tryptophans are often buried, but can become accessible hydrophobic surfaces through which molecular chaperones can interact with substrates (Sheluho and Ackerman 2001; Sokołowska et al. 2015). The fluorescence experiments provide some evidence that the fusion proteins are interacting with the substrate proteins; however, since GST, HspB1, and HspB5 contain tryptophan residues,

these data do not identify specific interactions between the proteins.

We were primarily interested in determining whether the fusion protein maintained chaperone activity and how activity compared to that of each wild type sHsp. Chaperones were mixed in 1:1 ratios with substrate proteins to prevent large oligomeric sHsp complexes from forming. Often, an excess of chaperone is used in aggregation-prevention assays; however, we were interested in determining whether small oligomers, monomers, and dimers of GST-sHsp fusion protein would exhibit chaperone activity when not used in large excess. GST-HspB1 and GST-HspB5 were effective chaperones for MDH and CS; however, the activity levels varied greatly. GST-HspB1 prevented a much greater amount of MDH aggregation compared to GST-HspB5, ~80 and ~45%, respectively. Interestingly, HspB5 was a much more efficient chaperone overall. Importantly, when comparing HspB1 and GST-HspB5 (the least active chaperones), a rapid initial increase in aggregation/light scatter was observed, which then attenuated. This rapid increase in light scatter was not observed for the two more effective chaperones, GST-HspB1 and HspB5. The heat-denaturing conditions suggest that the disordered state of the substrate is not interacting as effectively with the chaperone in these cases, at least during initial heating. We suggest that thermal-induced reorganization of both the sHsp and substrate may occur upon initial heating, and for the less effective chaperones, this may initiate some less favorable interactions. Similar light scattering results were observed between HspB5 and MDH at 42 °C, illustrating a decrease in MDH light scatter over time in the presence of HspB5. These results also demonstrated a slow initial onset of aggregation for MDH, which was proposed to be due to slow partial denaturation of the substrate (Regini et al. 2010). Our results support a role for oligomerization and higher-order quaternary structure in optimal chaperone activity for both HspB1 and HspB5 when MDH is the substrate protein. It is possible that larger oligomeric states are required for HspB5 to interact efficiently with MDH.

Using another heat-denatured substrate, CS, we compared the chaperone activity of HspB1, HspB5, GST-HspB1, and GST-HspB5. In contrast to the previous substrate, there was less variability in chaperone activity between each chaperone. HspB1, GST-HspB1, and GST-HspB5 all exhibited similar activity, preventing ~60% of CS aggregation. HspB5 was the most active, preventing ~75% of CS aggregation. In this case, both fusion proteins displayed faster aggregation during the first 5 min of the assay relative to HspB1 and HspB5, which displayed much slower aggregation kinetics, by comparison. Interestingly, the chaperone activity of each GST-sHsp was not identical, which suggests a role for both oligomerization and sequence diversity in determining substrate-chaperone interactions.

The altered aggregation onset observed in the chaperone assays could be due to several factors. In the case of CS, where both GST-sHsps exhibited faster initial aggregation, the explanation could be due to the lack of oligomeric dynamics of the fusion proteins. An alternative possibility is that the N-terminal fusion protein could prevent favorable association between the NTR of each sHsp and the substrate, which is important for early contacts with the denaturing substrate, but less important for overall prevention of aggregation. We cannot rule out the possibility that the GST-sHsps become misfolded; however, if this were the case, we would likely expect little to no chaperone activity and be nonfunctional. For example, in the disease-related mutation HspB5R120G, the mutant chaperone increases relative amounts of aggregation due to a decrease in chaperone stability (Bova et al. 1999). However, in all the experiments, GST-HspB1 and GST-HspB5 exhibit anti-aggregation chaperone activity for both CS and MDH, indicating the protein is folded in a competent state. Future experiments will explore the role of the sHsp NTR, through construction of GST-sHsp NTRs, to probe the role of this region in substrate interactions and the implications of sequence variability in establishing chaperone-substrate interactions.

In summary, we have generated N-terminal fusion proteins between GST and two human sHsps, HspB1 and HspB5. These proteins exhibit reduced polydispersity compared to native sHsps, primarily forming monomers and dimers in solution. Using two model heat-denatured substrates, GST-HspB1 and GST-HspB5 displayed comparable chaperone activity, suggesting that large oligomers and dynamic polydisperse architectures are not a requirement for chaperone activity. Furthermore, substrate specificity was observed, such that GST-HspB1 was a more effective chaperone relative to native HspB1 in preventing aggregation of MDH. However, this specificity was not exhibited for all substrates, which indicates there is likely more than one mechanism guiding chaperone-substrate interactions. Therefore, we propose that sHsp oligomerization states may provide one mechanism contributing to substrate specificity, along with differences in the primary structure of each sHsp, and that using fusion proteins and additional chemical biology methods to restrict oligomeric conformations will provide useful tools toward understanding the importance and role of polydispersity and quaternary structure in sHsp chaperone activity.

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