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Mechanistic Differences between Two Conserved Classes of Small Heat Shock Proteins Found in the Plant Cytosol*[S]

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Eman Basha, Christopher Jones[1], Vicki Wysocki, and Elizabeth Vierling[2]

From the Department of Chemistry and Biochemistry, University of Arizona, Tucson, Arizona 85721

The small heat shock proteins (sHSPs) and α-crystallins are highly effective, ATP-independent chaperones that can bind denaturing client proteins to prevent their irreversible aggregation. One model of sHSP function suggests that the oligomeric sHSPs are activated to the client-binding form by dissociation at elevated temperatures to dimers or other sub-oligomeric species. Here we examine this model in a comparison of the oligomeric structure and chaperone activity of two conserved classes of cytosolic sHSPs in plants, the class I (CI) and class II (CII) proteins. Like the CI sHSPs, recombinant CII sHSPs from three divergent plant species, pea, wheat, and *Arabidopsis*, are dodecamers as determined by nano-electrospray mass spectrometry. While at 35 to 45 °C, all three CI sHSPs reversibly dissociate to dimers, the CII sHSPs retain oligomeric structure at high temperature. The CII dodecamers are, however, dynamic and rapidly exchange subunits, but unlike CI sHSPs, the exchange unit appears larger than a dimer. Differences in dodecameric structure are also reflected in the fact that the CII proteins do not hetero-oligomerize with CI sHSPs. Binding of the hydrophobic probe bis-ANS and limited proteolysis demonstrate CII proteins undergo significant, reversible structural changes at high temperature. All three recombinant CII proteins more efficiently protect firefly luciferase from insolubilization during heating than do the CI proteins. The CI and CII proteins behave strictly additively in client protection. In total, the results demonstrate that different sHSPs can achieve effective protection of client proteins by varied mechanisms.

Small heat shock proteins (sHSPs)[3] and the related α-crystallins are a virtually ubiquitous family of stress proteins that can act as chaperones to prevent irreversible protein aggregation (1). sHSPs share a signature α-crystallin domain of ~90 amino acids, which has a conserved β-sandwich structure (1). Outside this domain the sHSPs are not well conserved, with the exception of an IXI/V motif toward the C terminus found in many family members. In their native state these small proteins (12–42 kDa) typically form large oligomers of anywhere from ~12 to >30 subunits. The unusual ability of sHSPs to bind up to an equal weight of denaturing clients is believed to require heat-induced conformational changes that expose hydrophobic client-binding sites (2, 3). For some sHSPs, high temperatures result in a shift in the oligomeric state to a much smaller subspecies, documented to be a dimer in several cases (4, 5). Heat-induced oligomer dissociation, observed in plant, bacterial and yeast sHSPs, has been proposed as a major mechanism by which sHSPs can expose normally inaccessible, hydrophobic client-binding surfaces. Other conformational changes that do not require oligomer dissociation are also suggested to result in presentation of client-binding surfaces (6).

The potential for diversity in sHSP mechanisms is particularly obvious in plants, in which ten separate families of sHSPs have been recognized to be conserved in both monocots and dicots (>200 million years) (7, 8). Four of these conserved gene families encode proteins that localize to the cytoplasm and shuttle in and out of the nucleus, while the other six have typical targeting sequences that localize them to cellular organelles (nucleus, chloroplasts, mitochondria, endoplasmic reticulum, and peroxisomes). A large body of data defining the current model for sHSP chaperone action has been derived from studies of one family of plant cytosolic sHSPs, the "class I" (CI) proteins, represented by wheat TaHsp16.9-CI, for which the crystal structure of the native dodecamer has been solved (5), and pea PsHsp18.1-CI, a close homolog (65% identical/86% similar), which has been used extensively in biochemical experiments (2, 3, 5, 9–12). Both of these plant CI sHSPs readily dissociate at high temperature to a dimeric form that is proposed to be the client-binding species (5). Although other classes of plant sHSPs have been demonstrated to act as chaperones, as defined by their ability to bind denaturing clients, little information is available regarding mechanistic details of this activity (2, 3, 6, 10, 11).

In this study we have focused on the other major class of sHSPs found in the plant cytosol, the "class II" (CII) proteins, which can accumulate to over 0.25% of total cell protein under heat stress conditions. CI and CII proteins are on average only 33% percent identical within a species (10), and evolutionary analyses indicates that CI and II sHSPs diverged over 400 million years ago, as both families are found in the moss *Funaria hygrometrica* (13). Recombinant CII oligomers of wheat TaHsp17.8-CII and pea PsHsp17.7-CII were estimated to have 9–10 or 12 subunits, respectively (2, 10), a size consistent with *in vivo* data (14). These CII sHSPs have chaperone activity as measured by suppression of protein aggregation (assayed by

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[1] Recipient of a graduate research fellowship in analytical chemistry from Pfizer.

[2] To whom correspondence should be addressed: Dept. of Chemistry & Biochemistry, University of Arizona, 1007 E. Lowell St., Tucson, AZ 85721. Tel.: 520-621-1601; Fax: 520-621-9903 or 3709; E-mail: vierling@u.arizona.edu.

[3] The abbreviations used are: sHSP, small heat shock protein; bis-ANS, (1,19-bi(4-anilino)naphthalene-5,5′-disulfonic acid; CI, Class I; CII, Class II.

light scattering) or ability to form complexes during heating with MDH (2, 10), Luc (10) and citrate synthase (15). mRNA expression profiling indicates CI and II proteins in *Arabidopsis thaliana* are expressed under similar stress and developmental conditions, suggesting that these proteins act closely together. In transformed protoplasts, CI and CII proteins localize to similar large cellular structures during heat stress (heat shock granules), and the presence of CII proteins was reported to be required for CI localization (16), further linking their activities. However, unlike diverse sHSPs in the mammalian cytosol, which coassemble into hetero-oligomeric structures, CI and II proteins from pea do not coimmunoprecipitate from cell lysates, and the recombinant proteins do not coassemble *in vitro* (16). These data, along with the evolutionary conservation of both classes, indicate CI and CII proteins have distinct functions in the plant cytosol and may well have different mechanisms of action.

To investigate basic mechanistic features of CII sHSP chaperone activity, here we examine structural properties and chaperone activity of three CII proteins compared with three CI proteins from pea, *Arabidopsis* and wheat. As isolated, all of these proteins, both CI and CII, are dodecamers, but despite having the same native stoichiometry, none of the CII sHSPs hetero-oligomerize with CI sHSPs *in vitro*. Significantly, unlike CI oligomers, which readily dissociate to dimers during heating, CII oligomers are remarkably stable, although they still expose hydrophobic surfaces during heating and act as very effective chaperones. The CII proteins exemplify how similar or better efficiency in client binding can be achieved in different ways by different sHSPs.

MATERIALS AND METHODS

Protein Purification—The CI sHSPs, PsHsp18.1-CI (P19243), AtHsp17.6-CI (AAG21467), and TaHsp16.9-CI (CAA45902; PDB 1GME) were purified after expression in *Escherichia coli* as described (17), except without the addition of urea during DEAE chromatography. The CII sHSPs, PsHsp17.7-CII (AAA33670), AtHsp17.7-CII (CAB87676), and TaHsp17.8-CII (AF350423) were purified the same way except that the ammonium sulfate precipitation step was removed. Proteins were quantified using the extinction coefficient of each protein (ϵ_{280} = 16,500 for both PsHsp18.1-CI and TaHsp16.9-CI; ϵ_{280} = 11,125 for AtHsp17.6-CI; ϵ_{280} = 4,485 for PsHsp17.7-CII and TaHsp17.8-CII; and ϵ_{280} = 3,115 for AtHsp17.7-CII). Masses of the purified proteins were confirmed by mass spectrometry. All concentrations are based on monomer molecular weight.

Non-denaturing PAGE—Non-denaturing PAGE was performed using 4–15% acrylamide gels (18). Running conditions were 30 min at 20 mA at room temperature, then 12 h at 10 mA at 4 °C. Standards were thyroglobulin 669 kDa, ferritin 440 kDa, catalase 232 kDa, lactate dehydrogenase 140 kDa, and bovine serum albumin 67 kDa (Amersham Biosciences, Piscataway, NJ). Proteins were mixed together in 25 mM Tris buffer, pH 7.5, as described in the figure legend.

Blue-native PAGE—Proteins were loaded on 4–20% acrylamide blue-native gels (19) and run at the indicted temperatures. For heated gels, buffer was pre-incubated at the indicated temperature to maintain temperature during electrophoresis. Protein samples were loaded with no pre-heat treatment. Running conditions were 30 min at 100 V, followed by 10 mA until the dye front approached the bottom of the gel.

Aggregation Protection Assays—1 μM Luc or 3 μM MDH was incubated in low salt buffer (25 mM sodium phosphate, 25 mM KCl, and pH 7.5) at 42 °C for 8.5 min or 2 h at 45 °C, respectively, with different sHSPs at molar ratios indicated in the figures and legends (9). After centrifugation at 13,000 rpm for 15 min the soluble and pellet fractions were analyzed by SDS-PAGE and Coomassie Blue staining.

Size-exclusion Chromatography (SEC)—Heat stability of the sHSP oligomers was examined by injecting 100 μl of 24 μM sHSPs onto a heat-jacketed TSKgel G4000PWXL column (Tosoh Corp., Tokyo, Japan, separation range 2,000–300,000 Da) equilibrated with low salt buffer at the corresponding temperature. Proteins were eluted at flow rate of 1 ml/min. For analysis of sHSP-client complex formation, sHSPs were incubated at different molar ratios with 3 μM MDH or 1 μM Luc in low salt buffer at the temperatures and times described in the figure legends. After incubation, samples were cooled on ice for 2 min and centrifuged at 13,000 rpm for 15 min. The supernatant was analyzed by SEC on a TSKgel G5000 PWXL column (4,000–1,000,000 Da) at room temperature in low salt buffer as for oligomer stability.

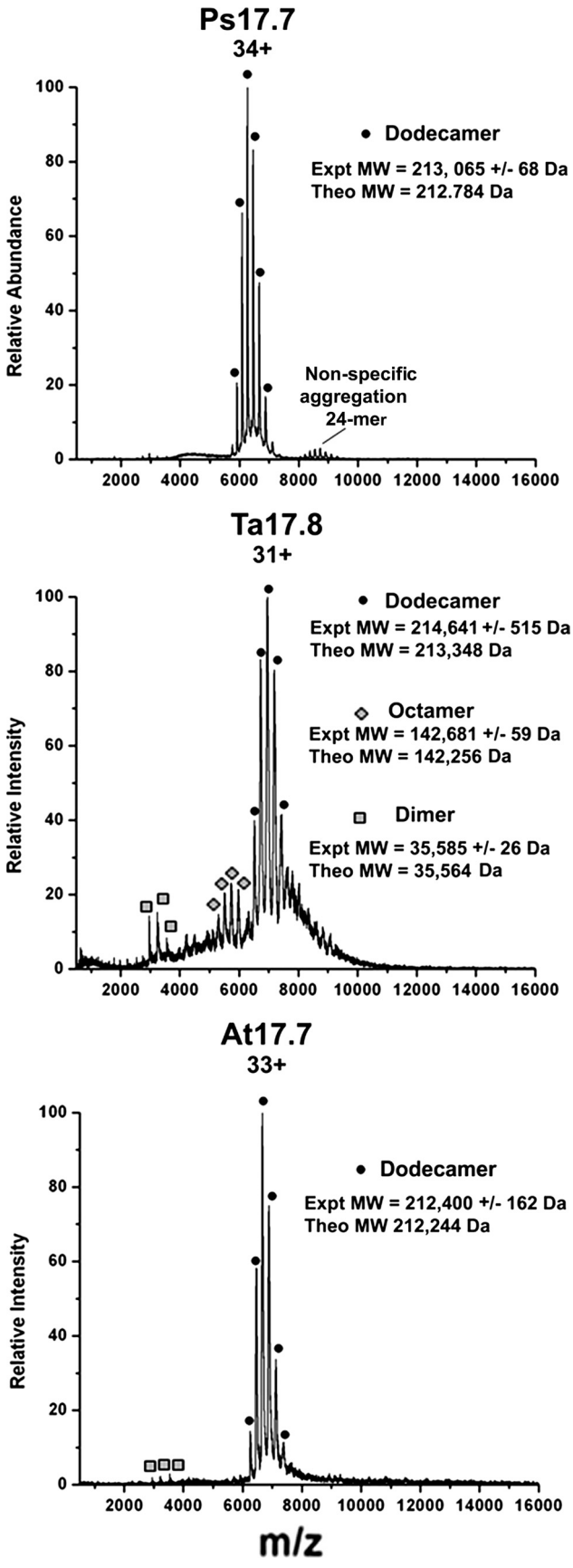
Bis-ANS Labeling—sHSPs (12 μM) were incubated at the indicated temperatures for 1 h with bis-ANS at a 10-fold molar ratio to sHSP, as described previously (3), except bis-ANS was added and UV cross-linking performed during the last 10 min of incubation. Proteins were separated by SDS-PAGE, and protein fluorescence was visualized by UV illumination of the gel.

Partial Proteolysis—Protein samples (24 μM) were incubated with trypsin at a 400:1 (w/w) ratio (sHSP: trypsin) in 25 mM sodium phosphate, 25 mM KCl, pH 7.5. Samples were incubated either at room temperature or 37 °C for the indicated times. The reaction was stopped by the addition of SDS sample buffer followed by boiling for 5 min. Samples were separated on 10–17% gradient acrylamide SDS-PAGE and stained with Coomassie Blue.

Nano-electrospray Ionization Mass Spectrometry—Native size of sHSP oligomers was determined by nano-electrospray ionization mass spectrometry on a Q-ToF 2 (Waters Corp., Millford, MA) as described previously (20). Briefly, samples were buffer exchanged into ammonium acetate, pH 7, using microconcentrators (Bio-Rad). Each sample was loaded into a tapered glass capillary pulled in-house (Sutter Instruments P-97 micropipette puller, Novato, CA). A platinum wire was inserted into the capillary and a voltage of 1.5–2.0 kV was applied to generate ions. The cone voltage was varied between 50 and 200 V until optimum ion transmission and protein desolvation were achieved. Pressure in the source region was raised by partially restricting the vacuum line to the rotary pump to optimize ion transmission.

RESULTS

CII sHSPs Are Dodecamers at Room Temperature—To define common biochemical properties of CII sHSPs, we worked with proteins from three diverse plant species, the previously stud-



ied proteins PsHsp17.7-CII from pea (2) and TaHsp17.8-CII from wheat (10), and in addition, AtHsp17.7-CII from *A. thaliana*, which had not been characterized before. In all experiments these CII proteins were directly compared with CI sHSPs from the same plant species, including the well-characterized wheat TaHsp16.9-CI and pea PsHsp18.1-CI (3, 9), along with AtHsp17.6-CI from *Arabidopsis* (21). These different CII proteins are ~48% identical/81% similar, and the CI sHSPs are ~58% identical/83% similar, when the non-conserved N-terminal arm is not considered in the comparison (supplemental Fig. S1). When CI and CII proteins are compared within a species, they are only ~33% identical/72% similar (10). All of these proteins contain, or can be predicted to contain, all seven β-strands that characterize the sHSP α-crystallin domain (supplemental Fig. S1).

We first determined the native oligomeric state of these three recombinant CII sHSPs using nano-electrospray ionization mass spectrometry. The CI proteins have all been shown by this technique to be dodecamers, consistent with the crystal structure of TaHsp16.9-CI and analytical ultracentrifugation (2, 5, 21). Results with all three recombinant CII proteins reveal that they are also dodecamers (Fig. 1). Previous analytical ultracentrifugation measurements had estimated that wheat TaHsp17.8-CII might have as few as 9 subunits and that the pea PsHsp17.7-CII oligomer was 11–12 subunits (2, 10). We attribute this difference in measured oligomeric size to the use of urea in the chromatography step of previous purifications. We have since found that urea destabilizes the CII oligomers (supplemental Fig. S2), although urea does not appear to have a similar effect on CI oligomers (not shown). However, for studies reported here, urea was eliminated from the purification procedure for all proteins, both CI and CII. We conclude that the native oligomeric state of the recombinant CII proteins is dodecameric.

It is relevant to note that the TaHsp17.8-CII nanospray mass spectrum shows a low abundance of octamers and, along with the AtHsp17.7-CII spectrum, even shows some dimer (Fig. 1). The same sub-oligomeric species are even more prominent when these two proteins are purified using urea (supplemental Fig. S2). PsHsp17.7-CII is much less affected by urea and is more stable in the nano-electrospray mass determination experiment. Altogether, these observations indicate that the basic substructure of CII dodecamers comprises an even number of subunits.

CII sHSP Dodecamers Do Not Dissociate to Stable Dimers at High Temperatures—TaHsp16.9-CI, as well as yeast Hsp26 and *Synechocystis sp.* PCC 6803 Hsp16.6 are documented to dissociate to dimers at elevated temperature (4, 5), a step which would expose hydrophobic sites with the potential for client binding. To determine if this dissociation is common to all CI proteins and to CII proteins as well, we analyzed the oligomeric state of the six plant sHSPs using non-denaturing blue-native

FIGURE 1. **Class II sHSPs are dodecameric.** Nano-electrospray ionization mass spectra of the class II sHSPs showing the charge species envelope. Calculated experimental and theoretical masses are listed. Deviation of the experimental mass from the calculated mass is due to retention of buffer and water solvent molecules under the gentle nano-electrospray ionization conditions used, which are required to retain oligomeric structure.

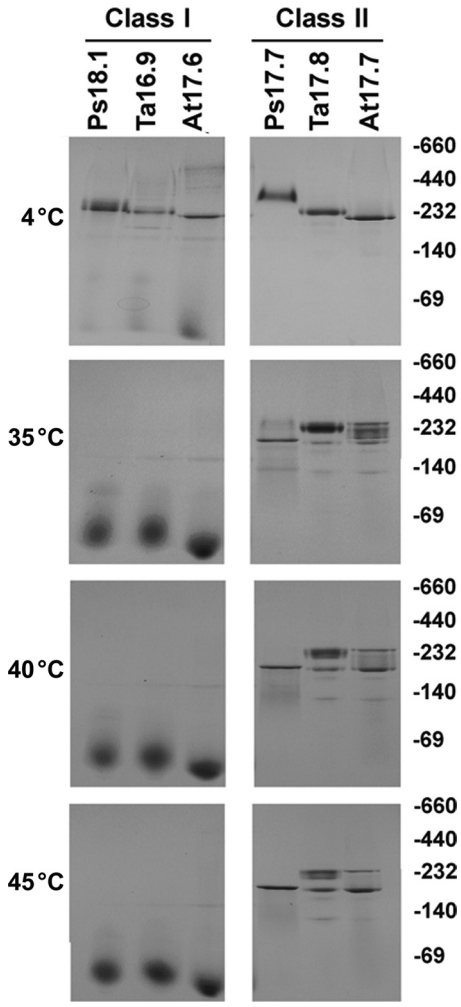


FIGURE 2. **Class II sHSP oligomers do not dissociate to stable dimers.** The indicated sHSPs (15 μl of 24 μM) were separated on 4–20% acrylamide blue-native gels maintained at the indicated temperatures. Gels were stained with Coomassie Blue. Positions of protein molecular weight markers in kDa are shown on the *right*.

PAGE (Fig. 2) and SEC run at different temperatures (supplemental Fig. S3). On blue-native PAGE the three CI sHSPs showed dramatic conformational changes at high temperatures (35, 40, and 45 °C). The dodecameric form disappeared completely and a new single band migrating at a dimeric molecular weight appeared even at the lowest heating temperature tested (35 °C). However, the three CII sHSP dodecamers showed significant heat stability, with none of the proteins migrating below the 140-kDa marker even at 45 °C. In this analysis, the most heat-stable sHSP was TaHsp17.8-CII, with the majority of the protein migrating above the 232-kDa marker at all temperatures. No protein was seen at a position expected for a dimer or monomer (which runs at the dye front) for any of the CII sHSPs. It is possible that the faster migrating CII forms seen at higher temperatures result from new conformations or rearrangement of the dodecamer rather than subunit loss. The stability of the CII sHSP oligomers is not due to disulfide bonds, because the same behavior was observed when the proteins were incubated in the presence of 60 mM dithiothreitol (not shown), and PsHsp17.7-CII has no Cys residues. The temperature-induced changes of both the CI and CII proteins were also fully reversible. When the sHSPs were heated to 45 °C for 1 h and then cooled, they migrated identically to unheated proteins (not shown).

As an independent examination of the heat stability of the CI and CII oligomers, we performed SEC at increasing temperatures (supplemental Fig. S3). At room temperature, all of the proteins eluted at 7.2 min, which is the position of a dodecamer. The three CI sHSPs showed a significant loss of the dodecamer peak at temperatures as low as 35 °C, even during the 12 min SEC run. At 40 °C the PsHsp18.1-CI dodecamer was no longer resolved on the column, and by 45 °C TaHsp16.9-CI and AtHsp17.6-CI oligomers were similarly absent. The majority of the heat-dissociated form eluted as a broad peak centered at the position expected for a dimer, ~9.4 min. In contrast, the CII sHSP dodecamers showed greater heat stability, as seen in the blue-native PAGE. Even after heating to 45 °C, the peaks of absorbance for PsHsp17.7-CII, TaHsp17.8-CII, and AtHsp17.7-CII eluted at 8.2, 8.4, and 8.6 min, respectively, which is a relatively minor shift, similar to that observed on blue-native PAGE. AtHsp17.7-CII is particularly stable, with considerable protein eluting as a dodecamer, even at 45 °C. In contrast to what was observed with the CI sHSPs, no temperature-induced appearance of species at the position of a dimer is seen (supplemental Fig. S3). In summary, the CI and CII dodecamers exhibit very different structural changes at high temperatures, and there is no evidence that CII proteins stably dissociate to dimers.

Different CII sHSPs Can Coassemble, but CII and CI sHSPs Do Not Hetero-oligomerize—Dissociation to a stable sub-oligomeric species is only one way sHSPs could expose normally unavailable hydrophobic sites for client binding. It has also been noted that many sHSPs rapidly exchange subunits between oligomers and that the rate of exchange increases at elevated temperatures, although the oligomeric state may remain the dominant species (21). This temperature-enhanced subunit exchange could also expose client binding sites and has been correlated with chaperone activity in mammalian sHSPs (22). To test if the CII proteins, despite the apparent stability of the dodecamer, also undergo rapid subunit exchange, we analyzed the proteins alone or after mixing using non-denaturing PAGE. This assay has been used previously to examine sHSP subunit mixing (5, 14), and the composition of hetero-oligomers confirmed by mass spectrometry (21). Equal volumes of 24 μM sHSPs (final concentration 12 μM each) were mixed for 1 h or 5 h at room temperature before analysis. Mixing PsHsp17.7-CII with either TaHsp17.8-CII or AtHsp17.7-CII resulted in a unique pattern of bands representing multiple hetero-oligomeric species (Fig. 3*A*). It is difficult to visualize hetero-oligomers formed between TaHsp17.8-CII and AtHsp17.7-CII due to the close migration of these two sHSPs, but subunit mixing is apparent. Mixing the CII sHSPs for only 15 min at different temperatures showed that the co-assembly process is not only fast, but also temperature dependent (Fig. 3*B*). These data show that the ability of the CII sHSPs to exchange subunits is not prohibited by heat stability of the oligomer, but rather that it is promoted at high temperatures (Fig. 3*B*). Consistent with previous observations (14), mixing any of the CII sHSPs with CI sHSPs did not result in any co-assembly products either

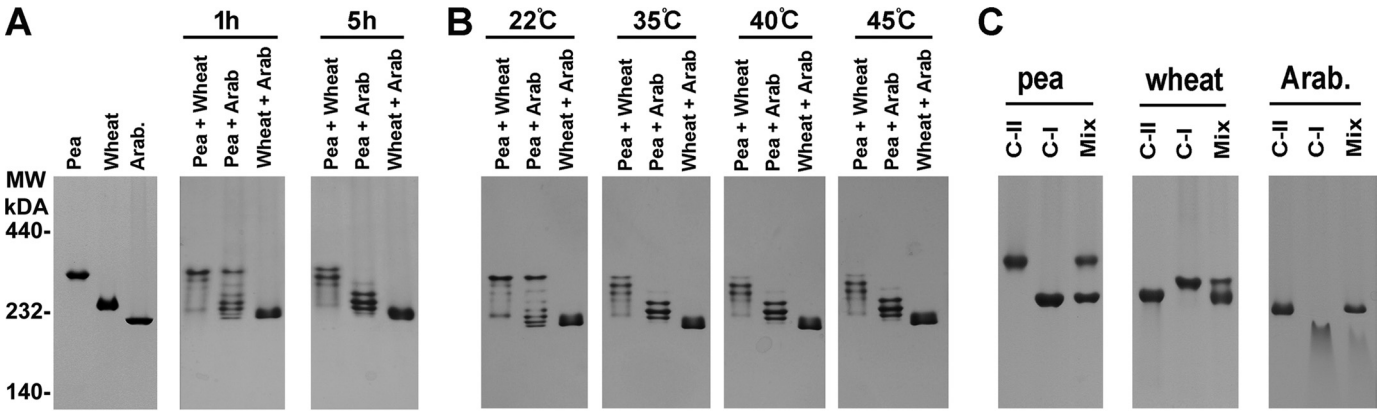


FIGURE 3. **Class II sHSPs form unique hetero-oligomers.** Non-denaturing PAGE was used to examine oligomeric species of purified sHSPs alone and after mixing for different times or at different temperatures. sHSPs (24 μM) were mixed together to a final concentration of 12 μM each in 25 mM Tris pH 7.5 and 15 μl were loaded on the gel. Gels were stained with Coomassie Blue. *A*, *left panel*, each class II sHSP analyzed alone. *Middle* and *right panels*, mixed proteins were incubated at room temperature for 1 or 5 h as indicated, before electrophoresis. *B*, mixed proteins were incubated at the indicated temperatures for 15 min before electrophoresis. *C*, indicated CI and CII sHSPs were separated alone or after mixing at room temperature. Separation of all samples was on 4–15% PAGE.

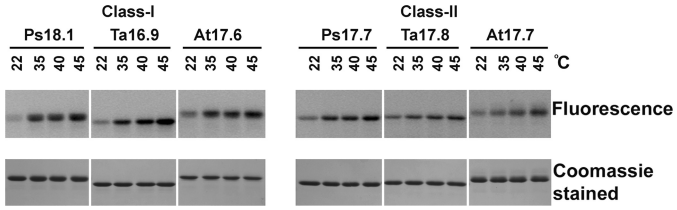


FIGURE 4. **CI and CII sHSPs bind bis-ANS at elevated temperatures.** 100 μl of 12 μM sHSPs were incubated for 60 min at the indicated temperature. The fluorescent probe bis-ANS was added at a 10-fold molar excess for the last 10 min of treatment. The samples were subjected to UV cross-linking by irradiation for 10 min at 256 nm. Following cross-linking 20 μl of each sample was run on a 10–17% gradient SDS-PAGE gel. Bis-ANS binding was visualized by fluorescence under UV illumination, and the gel was stained with Coomassie Blue to show equivalent amount of protein.

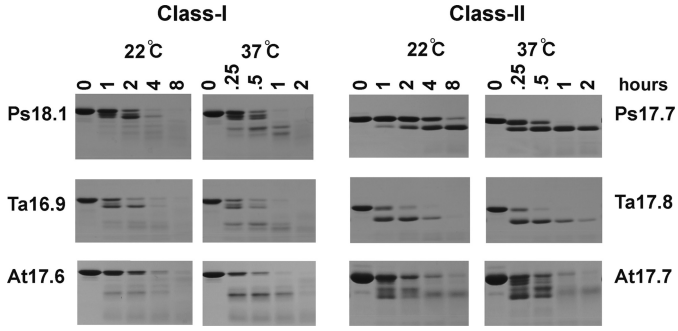


FIGURE 5. **CI and CII proteins exhibit altered conformation at 37 °C as revealed by partial proteolysis.** 24 μM protein was incubated with trypsin at a 1:400 w/w ratio (trypsin:sHSP) in low salt buffer at either room temperature (22 °C) or 37 °C for the indicated times (*hr*). Treatments were stopped by boiling in SDS sample buffer. Samples (20 μl) were loaded on 10–17% acrylamide gradient SDS gels and stained with Coomassie Blue. Note that shorter times were used for the 37 °C digestions to visualize comparable fragmentation.

at room temperature (Fig. 3*C*) or at higher temperatures (data not shown), even though CI proteins readily exchange subunits with other CI proteins (Refs. 5,21 and supplemental Fig. S4).

CII sHSPs Undergo Conformational Changes at High Temperature—It is clear that the CII sHSPs do not dissociate to stable dimers at high temperature as do the CI proteins, although they are capable of subunit exchange. To test for other heat-induced structural changes that might facilitate interaction of CII proteins with denaturing clients, we examined bis-ANS binding at different temperatures. Bis-ANS is a fluorescent probe that binds to hydrophobic regions in proteins and that has been used it to visualize exposure of hydrophobic surfaces on sHSPs and other proteins (3). Our previous studies showed heat-dependent interaction of bis-ANS with PsHsp18.1-CI (3). The six sHSPs were incubated at 22, 35, 40, or 45 °C in the presence of bis-ANS and exposed to UV light to cross-link the probe to the sHSP. At 22 °C all the sHSPs bound to a similar low level of bis-ANS (Fig. 4). However, labeling increased dramatically with heating for all six sHSPs, consistent with increasing exposure of hydrophobic surfaces at elevated temperatures. The increased bis-ANS binding at high temperature is not observed when the same experiment is performed with IgG (Ref. 3 and data not shown). Thus, the CII proteins exhibit temperature-dependent exposure of hydrophobic sites similar to the CI proteins, but must do so by a mechanism that does not involve stable dissociation to a dimeric species.

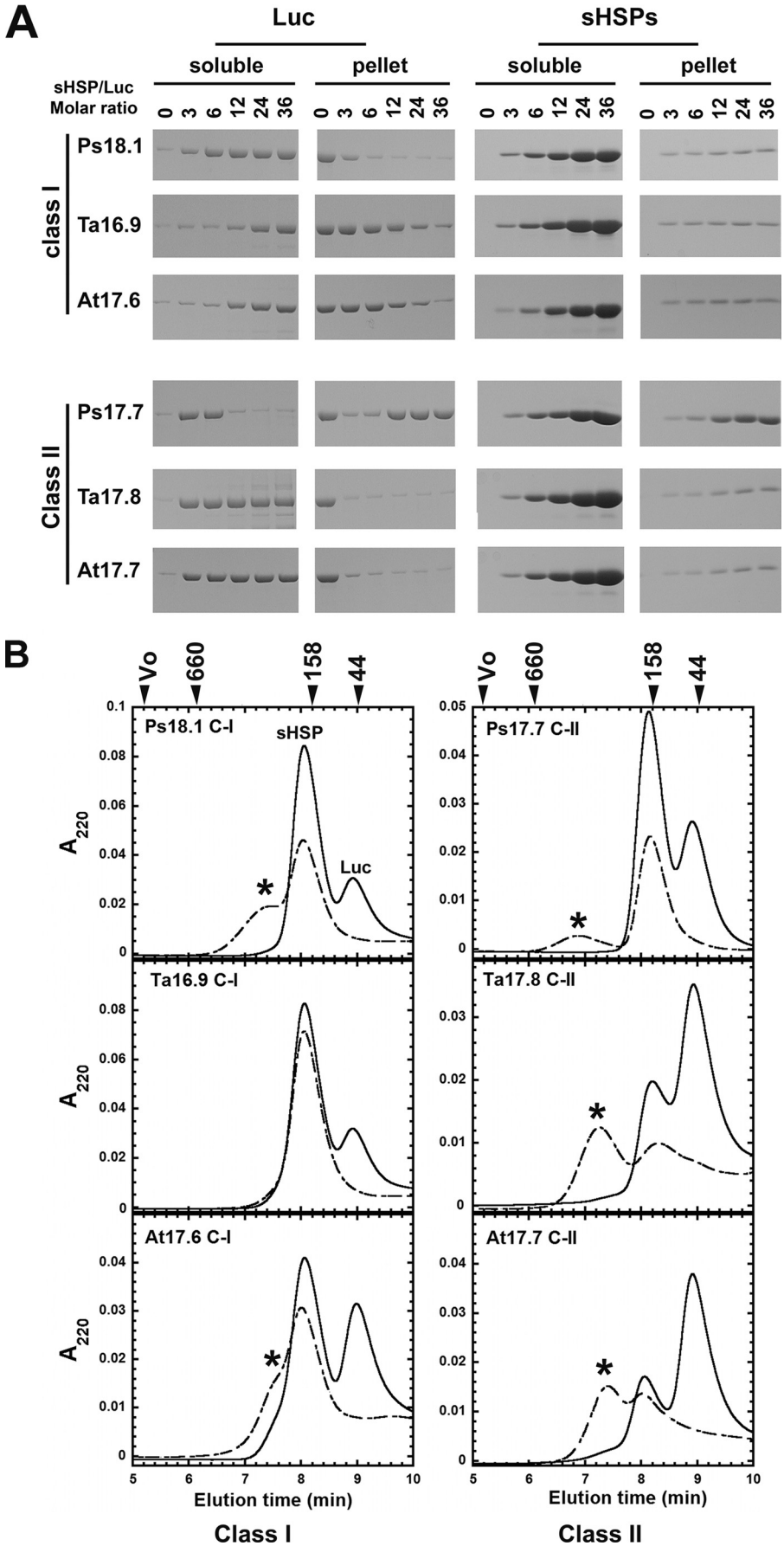
As an additional approach to detect conformational changes at high temperature, we performed limited proteolysis with trypsin. Each protein (24 μM) was incubated with trypsin at a 1:400 (w/w) ratio (trypsin:sHSP) at either room temperature or 37 °C (Fig. 5). Digestion of the three CI sHSPs showed that elevated temperature resulted in faster disappearance of the full-length proteins; the extent of cleavage after 0.5 h at 37 °C was significantly greater than cleavage after 2 h at 22 °C. This >4-fold increase in the rate of trypsin cleavage was not simply a result of an increase in trypsin activity at 37 °C. When denatured PsHsp18.1-CI was digested with trypsin at 22 °C or 37 °C the apparent rate of digestion was the same (supplemental Fig. S5*A*). In addition, less than a 2-fold increase in the rate of trypsin digestion was observed at 37 °C when a peptide with a single trypsin cleavage site was used as a substrate (supplemental Fig. S5*B*). Therefore, we conclude that the increased rate of trypsin digestion of the sHSP at 37 °C results from conformational changes that allow increased accessibility of protease to the sHSP.

Similar results were obtained with trypsin digestion of the CII proteins at 22 °C *versus* 37 °C. The rate of appearance of

digestion fragments was 8-fold faster for the pea CII and 4-fold faster for the wheat and *Arabidopsis* CII proteins at 37 °C (Fig. 5). Interestingly, digestion of PsHsp17.7-CII revealed a relatively large trypsin-resistant fragment, which was not seen for the other two proteins. The location of trypsin sites is very similar for all three proteins (supplemental Fig. S1), although PsHsp17.7-CII lacks a trypsin site found in the other two proteins in the α-crystallin domain between predicted β7 and β8. Whether the absence of this site is responsible for stability of this fragment remains to be determined. In total, the bis-ANS binding and trypsin digestion results indicate that the CII proteins undergo significant structural rearrangement at elevated temperatures.

CII sHSPs Form Stable Complexes with Denaturing Clients—We have previously shown that the CII protein from wheat and the CI protein from pea can protect Luc during heating, while the CI protein from wheat cannot (10). To determine whether the CII proteins show differential ability to protect Luc we directly compared the six different sHSPs. Luc (1 μM) was incubated with different molar ratios of sHSPs at 42 °C for 8 min. Samples were processed into a soluble and pellet fraction and visualized by SDS-PAGE (Fig. 6*A*). All three CII proteins were very effective in protection of Luc from insolubilization, with virtually full protection at a molar ratio of 3:1 (sHSP:Luc). Surprisingly, with PsHsp17.7-CII at the higher molar ratios of 12:1 and 24:1 Luc became less soluble. In these samples PsHsp17.7-CII was also found in the pellet (Fig. 6*A*), although PsHsp17.7-CII remained soluble when incubated under the same conditions in the absence of Luc (not shown).

In contrast to the low molar ratio of CII sHSPs required to protect Luc, only the CI protein from pea was similarly effective, showing complete Luc protection between a 3 and 6 molar ratio of sHSP:Luc.



The *Arabidopsis* CI protein required 12–24 molar sHSP:Luc, and wheat CI, as previously shown (8), was unable to achieve the same level of protection, even at a molar ratio of 24:1. Using MDH as a client showed that the *Arabidopsis* and wheat CII sHSPs were also able to protect MDH as efficiently as the CI sHSPs (supplemental Fig. S6). PsHsp17.7-CII precipitated with MDH at higher ratios as seen for Luc (not shown).

To examine further the interaction between these different sHSPs and client, the sHSP:Luc mixtures were examined by SEC, using an sHSP:Luc ratio of 12:1, except PsHsp17.7-CII, where the ratio used was 6:1 (Fig. 6*B*). Before heating, sHSP and Luc elute at the positions expected for the dodecamer and monomer, respectively. After heating, the Luc peak is no longer detected, and the sHSP peak decreases, while a new peak representing a complex between the two proteins is observed (*asterisk*). Luc heated alone does not enter the column, and sHSP heated alone migrates as a dodecamer when returned to room temperature and analyzed as room temperature, as shown previously (3, 9, 10) (not shown). The amount of complexes observed and the apparent size of complexes varied for the different sHSPs. The highly effective CII proteins formed abundant, relatively large sHSP-client complexes, while the pea and *Arabidopsis* CI proteins formed less abundant, smaller complexes, and as expected no complexes were seen for the wheat CI protein which does not protect Luc (9). In total all the CII proteins are highly effective chaperones, capable of forming stable complexes with clients, although the dodecamers do not stably dissociate.

The Client Protection Activity of CI and CII Proteins Is Additive—The CI and CII proteins are both found in the plant cytosol after heat stress and are also expressed together under other conditions. It was therefore of interest to determine how combining CI and CII might enhance client protection. For this experiment the *Arabidopsis* CI and CII proteins were mixed at molar ratios between 4:0 and 0:4 and then used in the Luc protection assay at sHSP:Luc molar ratios of 0.75:1 to 12:1. Results show that protection was directly proportional to the amount of CII protein present; the presence of CI protein did not enhance or decrease the protective activity of CII (Fig. 7*A*). To further test if activity of the two proteins was additive, the *Arabidopsis* CI and CII proteins were again mixed at molar ratios between 4:0 and 0:4 and tested for protection of MDH at sHSP:MDH ratios of 0.25:1 to 2:1. Either protein alone can fully protect MDH at a 1:1 molar ratio. Results again show that protection was strictly additive, with no synergistic positive or negative effect resulting from using the two proteins together (Fig. 7*B*).

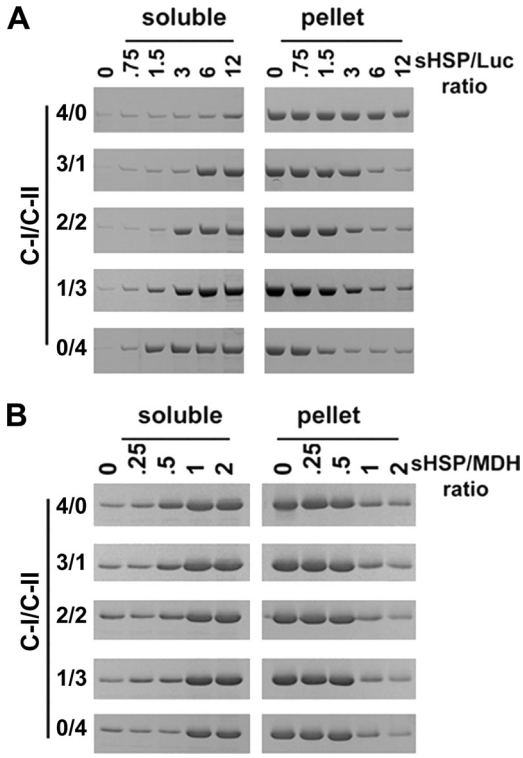


FIGURE 7. **Protection of client proteins by CI and CII proteins is additive.** SDS-PAGE and Coomassie Blue staining of the soluble and pellet fractions obtained after heating of Luc or MDH with sHSPs. *A*, 1 μM Luc was incubated for 8 min at 42 °C with the indicated ratio of sHSP, using a combination of *Arabidopsis* CI and CII sHSPs mixed at ratios of 4:0, 3:1, 2:2, 1:3, 0:4. *B*, aggregation-protection of MDH. 3 μM MDH was incubated with sHSPs at the indicated ratios for 1 h at 45 °C. After heating, the mixtures were separated into soluble and pellet fractions and equal fraction volumes were analyzed. Only Luc and MDH are shown. sHSPs in the samples were fully soluble under all conditions (not shown).

DISCUSSION

There is significant controversy concerning the mode of activation of sHSPs for client binding. Different mechanisms of heat-induced activation have been suggested, including dissociation of sHSP oligomers to a sub-oligomeric species, increased rates of subunit exchange, as well as conformational changes unlinked to oligomeric structure (4, 5). Here we compared structural and chaperone properties of six recombinant proteins representing two conserved classes of sHSPs found in the plant cytosol. The results indicate that not all sHSPs need operate by the same mechanism to effectively protect clients and that the different mechanisms proposed need not be mutually exclusive.

The recombinant plant cytosolic CII sHSPs from three divergent plant species, pea, wheat, and *Arabidopsis*, are all

FIGURE 6. **CII sHSP effectively protect Luc from heat-induced aggregation and form stable sHSP-Luc complexes.** *A*, 1 μM Luc was incubated for 8 min at 42 °C with sHSPs at the molar ratios as indicated. Samples were separated into soluble and pellet fractions and equal fraction volumes were analyzed by SDS-PAGE. Gels were stained with Coomassie Blue. Panels on the *left* show the amount of soluble or pelleted Luc, whereas panels on the *right* show soluble or pelleted sHSP. Protection of Luc is evident from the amount found in the soluble fraction compared with the control in the absence of sHSP. With the exception of PsHsp17.1, all the sHSP remains soluble with heating in the presence of substrate. *B*, sHSP-Luc complexes separated by size exclusion chromatography. 1 μM Luc was incubated with 12 μM sHSPs for 8 min at 22 °C (*solid line*) or 42 °C (*dashed line*), except PsHsp17.7 C-II, which was used at 6 μM. Insoluble material was removed by centrifugation, and the supernatant was loaded onto TSKgel G5000 PWXL column at room temperature and eluted in low salt buffer at a flow rate of 1 ml/min. *Asterisks* mark positions of the sHSP-Luc complexes. Note that in heated samples there is no longer a peak at the position of native Luc, because Luc is either associated with the sHSP, or insoluble and removed by centrifugation prior to loading on the column. *Arrows* indicate elution of MW standards, left to right: *Vo* (void volume), 660, 158, and 44 kDa.

dodecamers, as are the CI proteins from the same species. The apparent size of CI and CII proteins detected in leaf extracts using non-denaturing PAGE and sucrose gradients is consistent with these determinations using recombinant proteins (14). Unlike the CI proteins, however, recombinant CII sHSPs do not dissociate to stable dimers upon heating. Nonetheless, like the CI proteins, the CII sHSPs undergo significant and reversible, heat-induced structural changes that result in exposure of hydrophobic surfaces. On a molar basis the CII sHSPs are equally, or more effective chaperones than the CI proteins when tested with model substrates. While both CI and CII proteins operate in the plant cytosol, *in vitro* they do not hetero-oligomerize and their chaperone activity is additive. In total, these two classes of plant sHSPs demonstrate that it is unlikely a single model can explain how diverse sHSPs act to protect clients from irreversible denaturation.

By comparing representative CI and CII sHSPs from three divergent plant species we can define properties of these proteins that are specific to each sHSP class. From our analysis we conclude that CII dodecamers are considerably more heat stable than CI sHSPs and do not dissociate to stable dimers at high temperatures. The same heat stability was also observed for purified CII sHSPs from tomato and the moss, *Funaria hygrometrica* (not shown). Thus, heat stability appears to be a general property of CII sHSPs, and the active, client-binding form of CII sHSPs may well not be a dimer, as is believed to be the case for CI sHSPs (12). It is of interest to mention that mammalian sHSP oligomers also do not stably dissociate at high temperature (23). However, the chaperone activity of mammalian sHSPs is activated by phosphorylation-dependent dissociation to a smaller, sub-oligomeric species (24, 25). The CI and CII proteins do not have canonical phosphorylation sites.

It is also notable that the pattern of coassembly products seen with the three CII proteins is significantly different than that observed for coassembly of the CI proteins. When the different CII proteins coassemble, multiple distinct bands of coassembled oligomers are observed (Fig. 3, *A* and *B*). In contrast, coassembled CI sHSPs appear to form a single compact band (Ref. 5 and supplemental Fig. S5). We suggest that this difference reflects a difference in the stability of the substructure of CI and CII sHSPs, which could also explain the failure of these different sHSP classes to coassemble (Fig. 3 and Ref. 14). A dimer is the most stable dissociation product and the major unit of exchange between CI dodecamers (21, 26). The distinct hetero-oligomeric species in the CII coassembly mixtures suggest that coassembly of different CII proteins does not occur at the level of monomer or dimer. Results of the nano-electrospray MS indicate that the stable substructure of CII proteins consists of an even number of subunits (Fig. 1 and supplemental Fig. S2). There is no evidence for presence of a hexameric form in the mass spectrum experiments. Thus, we speculate that subunit exchange between CII dodecamers is occurring at the level of a tetramer. Whether the oligomer or a transiently dissociated species acts as the client-binding form remains to be determined.

Despite the heat stability of the CII sHSPs, the dodecamers obviously undergo significant structural rearrangement at elevated temperatures as indicated by enhanced binding of the hydrophobic probe bis-ANS and enhanced susceptibility to trypsin digestion (Figs. 4 and 5). We suggest that changes in migration behavior on Blue-native gels at high temperature may also reflect conformational changes rather than dissociation of subunits, as no lower molecular weight species are ever observed on the gels. Like the CI sHSPs, no interaction with client is observed until after heating, when the sHSP and client form a high molecular complex. It is reasonable to conclude that the heat-induced structural changes in the CII sHSPs are important for client interaction, but this is not directly demonstrated by our data.

Although the CII proteins investigated here show many common properties, there are also properties unique to each protein. This is particularly evident in the client protection experiments. Complexes formed between each CII sHSP and heat-denatured Luc are of different sizes (Fig. 6*B*). Even more striking is the precipitation of PsHsp17.7-CII when high ratios of sHSP to Luc or MDH are used. Under the same heating conditions, the pea sHSP alone is stable and soluble, indicating interaction with denaturing client must drive sHSP precipitation. Why only the pea CII sHSP exhibits this behavior is unclear. As each plant species contains more than one gene encoding CII sHSPs, we cannot rule out that there is a CII sHSP in wheat or *Arabidopsis* with similar behavior, or a pea CII sHSP that would not precipitate. *In vivo* interactions could also change this behavior. These observations indicate it is important to limit generalizations derived from tests of the chaperone activity of a single protein.

The differences between the CI and CII sHSPs with regard to dodecamer stability, along with their inability to hetero-oligomerize, suggests that they may recognize denaturing clients in distinct ways. However, when CI and CII sHSPs were combined in aggregation-protection assays, we found their chaperone function was strictly additive. There was no evidence that the proteins either potentiated or interfered with each other's activity. How CI and CII sHSPs may interact *in vivo* is also unclear. During heat stress both CI and CII sHSPs localize to "heat shock granules" (HSGs), which are electron dense cytoplasmic particles formed after heat stress (27, 28). CII sHSPs have been reported to be required to recruit CI sHSPs to these cytoplasmic structures, but there is no evidence that recruitment involves direct interaction of the two classes of sHSPs. As we show here, CI and CII sHSPs do not coassemble *in vitro*, and two-hybrid experiments by others failed to detect CI-CII interactions (16). Further studies are needed to determine why these two distinct classes of sHSPs have been conserved over 400 million years of plant evolution and how their activities differ.

Whereas the chaperone activity of CI and CII proteins *in vitro* is very clear, information about the *in vivo* chaperone activity of plant sHSPs is limited. Studies using plant protoplasts showed that introduction of either CI or CII sHSPs reduced thermal inactivation of Luc in a cellular environment (29). Mutants with reduced expression of a subset of CI sHSPs show some reduced heat tolerance (30), and genetic analysis in *Arabidopsis* has linked CI sHSPs with the activity of the Hsp100/ClpB family of chaperones (31). Overproduction of AtHSP17.7 C-II in transgenic plants has been reported to increase salt and drought tolerance, but by an unknown mech-

anism (15). More recent experiments used RNAi to inhibit CI or CII sHSP expression in plant protoplasts and reported that CI sHSP expression was essential for thermal protection of Luc, but there was little effect of reducing CII sHSPs (32). The latter result is in direct contrast to our observations of the effectiveness of CII sHSPs in protecting Luc *in vitro*. Discovering natural clients for both the CI and CII sHSPs and defining any *in vivo* partner proteins are essential next steps in understanding how these proteins facilitate stress tolerance.

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