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An unusual dimeric small heat shock protein provides insight into the mechanism of this class of chaperones

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Abstract

Small heat shock proteins (sHSPs) are virtually ubiquitous stress proteins that are also found in many normal tissues and accumulate in diseases of protein folding. They generally act as ATP-independent chaperones to bind and stabilize denaturing proteins that can be later reactivated by ATP-dependent Hsp70/DnaK, but the mechanism of substrate capture by sHSPs remains poorly understood. A majority of sHSPs form large oligomers, a property that has been linked to their effective chaperone action. We describe AtHsp18.5 from *Arabidopsis thaliana*, demonstrating it is dimeric and exhibits robust chaperone activity, adding support to the model that suboligomeric sHSP forms are a substrate binding species. Notably, like oligomeric sHSPs, when bound to substrate AtHsp18.5 assembles into large complexes, indicating reformation of sHSP oligomeric contacts are not required for assembly of sHSP-substrate complexes. Monomers of AtHsp18.5 freely exchange between dimers, but fail to coassemble *in vitro* with dodecameric plant cytosolic sHSPs, suggesting AtHsp18.5 does not interact by coassembly with these other sHSPs *in vivo*. Data from controlled proteolysis and hydrogen-deuterium exchange coupled with mass spectrometry show that the N- and C-termini of AtHsp18.5 are highly accessible and lack stable secondary structure, most likely a requirement for substrate interaction. Chaperone activity of a series of AtHsp18.5 truncation mutants confirm that the N-terminal arm is required for substrate protection and that different substrates interact differently with the N-terminal arm. In total, these data imply that the core α -crystallin domain of the sHSPs is a platform for flexible arms that capture substrates to maintain their solubility.

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Keywords

chaperone; α -crystallin domain; protein flexibility; subunit exchange; hydrogen deuterium exchange; mass spectrometry of protein complexes

Introduction

The small heat shock proteins (sHSPs) and related α -crystallins are a virtually ubiquitous protein family, the majority of which have chaperone activity. sHSPs have a high capacity for ATP-independent binding of nonnative protein substrates, thereby preventing irreversible substrate aggregation and facilitating subsequent substrate refolding (or possibly degradation) by other ATP-utilizing components of the protein quality control network³. In addition to accumulating during heat, oxidative and other stresses, a number of sHSPs are major components of normal cells, and expression and/or mutation of sHSPs are linked to multiple diseases of protein misfolding, including neurodegenerative diseases, myopathies and cataract. Understanding the mechanism of sHSP action is, therefore, critical to many aspects of cell function in normal as well as stressed or diseased states.

sHSPs are defined by a conserved α -crystallin domain of ~90 amino acids flanked by a non-conserved N-terminal arm and short C-terminal extension. A majority of these small proteins (12–42 kDa) assemble into oligomers of 12 subunits. Crystal structures of two oligomeric sHSPs, *Methanococcus jannaschii* (a 24-mer) and *Triticum aestivum* (a dodecamer) showed that the α -crystallin domain is a seven-stranded IgG-like β -sandwich with topology identical to the Hsp90 cochaperone p23. Through strand exchange of a loop containing β 6, the monomers of these sHSPs form dimers, the basic building block of the oligomers. The α -crystallin domain fold is conserved in vertebrate sHSPs, but dimers are formed by antiparallel interactions of β 7, due the absence of the β 6 loop. Oligomers are assembled from dimers via interactions requiring both the N-terminal arm and C-terminal extension.

The current model for sHSP chaperone action involves a heat-induced structural change (also facilitated by phosphorylation in mammalian sHSPs), which exposes hydrophobic binding sites for denaturing substrate. Unlike GroEL and Hsp70, sHSPs can bind up to an equal mass of substrate, and the resulting sHSP-substrate complexes are large and heterogeneous. Release and refolding of substrate requires Hsp70/DnaK and ATP, and can be enhanced by Hsp100/ClpB or GroEL. The structural change required for substrate binding is unclear, with data supporting enhanced subunit exchange between the highly dynamic sHSP oligomers¹⁶, stable oligomer dissociation to suboligomeric species, or conformational alterations in tertiary structure not involving oligomer disruption. Sites of sHSP-substrate interaction are also poorly defined, as these sites appear to include those required for oligomerization, making it difficult to separate these functions. However, increasing evidence indicates that the N-terminal arm is a major mediator of substrate binding.

Genomic data have expanded our knowledge of the sHSP family, with bioinformatics identifying over 8,000 sHSP sequences from bacteria, archaea and all phyla of higher organisms. We have focused on sHSPs in higher plants, where 12 distinct gene families have been identified³³. By studying diverse plant sHSPs, we have sought to define fundamental features of the sHSP chaperone mechanism. Here we describe novel structural properties and chaperone activity of AtHsp 18.5, a nuclear/cytosolic sHSP from *Arabidopsis thaliana*. Remarkably, this sHSP is a stable dimer, rather than an oligomer, but exhibits robust chaperone activity. Unlike other plant sHSPs, AtHsp18.5 lacks the β 6 loop involved

in dimerization of other plant and bacterial sHSPs, indicating it must have a distinct dimer interface. We find properties and activity of AtHsp18.5 to provide new insights into the mechanism of this ubiquitous chaperone family.

RESULTS

AtHsp18.5 is heat-induced and behaves as a dimer

Sequences of AtHsp18.5 and homologous proteins from other plant species are aligned in Figure 1 compared to representative sequences of three other classes of cytosolic sHSPs from plants. These sHSPs share seven predicted β -strands that characterize the α -crystallin domain (ACD) and the characteristic C-terminal I/V-x-I/V motif, but notably, compared to other plant, bacterial and yeast sHSPs, AtHsp18.5 and homologues have a deletion of ~20 residues comprising a loop containing β 6. In dodecameric wheat Hsp16.9 (a CI plant sHSP) the β 6 loop incorporates into a β -sheet of another monomer to form the fundamental dimeric unit of the oligomer. Vertebrate sHSPs also lack the β 6 loop and dimerize through antiparallel interaction of extended β 7 strands. These sequence and structural data suggest that quaternary assembly of AtHsp18.5 and homologues is potentially different than either the vertebrate or plant sHSPs.

To investigate the quaternary structure of AtHsp18.5, we analyzed purified recombinant AtHsp18.5 compared to the well-characterized dodecameric, cytosolic class I sHSP, PsHsp18.1, from *Pisum sativum*. Non-denaturing PAGE (Fig. 2A) reveals that AtHsp18.5 migrates much faster than the 217 kDa PsHsp18.1 dodecamer and at a position consistent with the MW of a dimer. Glutaraldehyde crosslinking (Fig. 2B) produced primarily a doublet indicative of dimeric structure, and no higher MW forms were observed, even at a 3000 molar excess of glutaraldehyde (not shown). We attribute the crosslinked doublet of AtHsp18.5 to varying structures of a dimeric crosslinked species. A minor species above 56 kDa is likely a contaminant, as it does not increase with increasing glutaraldehyde concentrations. This pattern of crosslinking is in stark contrast to the dimeric and higher MW species observed for dodecameric PsHsp18.1.

For direct determination of molecular mass, AtHsp18.5 was analyzed by nano-electrospray ionization mass spectrometry (nano-ESIMS) (Fig. 2C) and found to be dimeric. Note that the nano-ESIMS method maintains noncovalent protein structure and has been used to characterize multiple dodecameric plant sHSPs. The dimeric structure of AtHsp18.5 was also confirmed in solution by sedimentation velocity analytical ultracentrifugation. Sedimentation of between 2.5 to 30 μ M protein at 20 °C yielded a single species with an estimated mass of 81–92% that of a dimer. No higher mass forms were detected and no distinct monomeric form was observed, although the lower mass estimate indicates some degree of dissociation, which is consistent with subsequent monomer exchange measurements (see below). The absence of a discrete monomeric species in the profiles indicates that the K_d is in the low μ M range.

We further determined that AtHsp18.5 dimeric structure is not dependent on disulfide linkage between subunits, as substitution of the single Cys residue (Cys98) with Ala did not alter the dimeric behavior of the protein in any of the above assays (not shown). We conclude that AtHsp18.5 is a non-covalent dimeric sHSP under all conditions tested.

Although AtHsp18.5 has an atypical quaternary structure, it is heat-induced at the mRNA and protein levels, as are other plant sHSPs. Data from the public data base show AtHsp18.5 mRNA increases dramatically at high temperature, and western analysis shows that the protein is not detected under control conditions, but increases in parallel to mRNA

accumulation to ~0.005 % of total protein under the conditions tested (Supplemental Fig. 1). Thus, AtHsp18.5 may function with other plant cytosolic sHSPs during stress.

Dimeric AtHsp18.5 is an effective chaperone

As the majority of highly active sHSP chaperones are oligomers, it was of significant interest to investigate chaperone activity of the AtHsp18.5 dimer. In a survey of several plant sHSPs we obtained initial evidence that AtHsp18.5 could protect firefly luciferase (Luc)³³. To extend this work, we tested the ability of AtHsp18.5 to protect Luc and two additional model substrates (malate dehydrogenase, MDH; and citrate synthase, CS) from heat-induced insolubility (Fig. 3A, Supplemental Fig. 2). AtHsp18.5 protected MDH as effectively as PsHsp18.1, with full protection achieved at a molar ratio, sHSP: substrate, of 1:1. Full protection of Luc required AtHsp18.5 at a molar ratio of 12:1 (sHSP:substrate), about twice the amount of PsHsp18.1 required. The dimeric sHSP was also six times less efficient in protecting CS, requiring a sHSP:substrate ratio of 18:1 compared to 3:1 for PsHsp18.1 (Supplemental Fig. 2). In all assays the sHSPs remained fully soluble (data not shown). These data indicate that sHSP dimers are effective chaperones with specific substrates, and furthermore, the differences in efficiency of protection of different substrates indicate AtHsp18.5 has unique interactions with each substrate.

Oligomeric sHSPs protect substrates by formation of large sHSP-substrate complexes, which has been assumed to involve reformation within the complex of some of the native sHSP oligomeric contacts¹. To examine the complexes formed between AtHsp18.5 and substrate we first performed SEC analysis of the sHSPs with MDH, Luc or CS before heating. The absence of interaction between sHSP and substrate before heating is obvious (left-hand panels in Fig. 3B and Supplemental Fig. 2B); the substrate and sHSP elute independently at their predicted molecular mass. Also, as the amount of sHSP was increased, the sHSP peak increases, while the substrate peak remains the same. Because of the similar mass of the dimeric AtHsp18.5 and substrate, there is overlap in the sHSP and substrate peaks, and substrate appears as a shoulder in the AtHsp18.5 peak (Supplemental Fig. 3).

To examine interaction of the sHSP and substrate after heating, SEC analysis was then performed on the soluble fractions from the substrate protection assays shown in Fig. 3A, at all concentrations at which the sHSPs fully protected substrate. MDH, Luc and CS heated alone do not enter the column (Supplemental Fig. 3), and the sHSPs themselves show no change in migration after heating. However, when substrate and sHSP are heated together new, higher MW peaks are observed that represent aggregated substrate in complex with sHSP (Fig. 3B, right hand panels; Supplemental Fig. 3). The native substrate peak disappears completely, and the height of the sHSP peak declines with the appearance of the complex peak. The apparent size of the complexes also decreased with the increase in sHSP:substrate ratio, as seen in previous experiments. Surprisingly, the AtHsp18.5-substrate complexes span a higher apparent mass range and show more heterogeneity than PsHsp18.1-substrate complexes. This result indicates that although AtHsp18.5 is dimeric, like oligomeric sHSPs, substrate protection involves assembly of complexes containing multiple substrate molecules protected by multiple sHSP subunits³⁹. Ability of the AtHsp18.5 dimer to protect substrates demonstrates that oligomeric structure itself or reformation of oligomeric contacts is not required for substrate protection, and that sHSP dimers can effectively interact with substrate.

AtHsp18.5 is a dynamic dimer but does not coassemble with other major cytosolic sHSPs

Oligomeric plant sHSPs display quaternary dynamics, typically involving rapid exchange of dimers and slower equilibration of monomers. To investigate whether AtHsp18.5 dimers are

dynamic, we added an a Strep II tag (WSHPQFEK) to either the N or C-terminus of AtHsp18.5, mixed either purified tagged protein with wild type, and examined the resulting species on non-denaturing PAGE (Fig 4A). Note that addition of the tag did not negatively affect AtHsp18.5 chaperone activity (see Fig. 7). In all mixtures three bands are observed that correspond in migration to a dimer of wild-type protein, a dimer of N or C-Strep-tagged protein, or to the heterodimer of wild-type and Strep-tagged monomers. Therefore this dimeric sHSP is undergoing monomer exchange. The relative abundance of the three species was the same after different times or temperatures of incubation prior to electrophoresis, and dissociation of the proteins in urea followed by dialysis also resulted in three species (Fig 4A).

PAGE analysis did not allow an estimate of subunit exchange rate, which might be related to chaperone activity. For rate estimates we used nano-ESIMS to measure the appearance of heterodimers over time when wild-type or N- or C-Strep-tagged AtHsp18.5 were incubated together. Fig. 4B shows an example of how the mass of the heterodimer comprising wild-type plus C-terminal strep-tagged sHSP is easily identified and increases with time of incubation. Rates of exchange were then estimated from nano-ESIMS of incubations performed at 25, 35 or 45 °C (Fig. 4C). Exchange data fit to first order rate constants that increased ~6-fold from 25 to 45 °C. However, even the rate at 45 °C was 10-fold lower than exchange rates measured for exchange of dimers in dodecameric plant sHSPs. These results suggest that the rate of monomer exchange is unlikely to control interaction with substrate.

Because AtHsp18.5 dimers are dynamic we sought to determine if AtHsp18.5 might function in coassemblies with other sHSPs, as is seen for certain sHSPs in mammals⁶. AtHsp18.5 is found in the plant cytosol³³ and is most closely related to Class I cytosolic plant sHSPs (Fig. S4A). We therefore examined the ability of AtHsp18.5 to coassemble with each of six class-I sHSPs, as well as a more distantly related class-II sHSP from Arabidopsis. When incubated together at room temperature for 5 hrs AtHsp18.5 did not coassemble with any of these sHSPs (Fig. S4B). Incubation for up to 24 hrs at room temperature or heating for 1 hr at 35°C followed by 5 hrs at room temperature also failed to promote coassembly (not shown). In contrast, coassembly between different Class I sHSPs can be readily observed by this approach.

We also investigated whether AtHsp18.5 might be present in large complexes in vivo, due to assembling either with other sHSPs or unrelated proteins. Non-denaturing electrophoresis followed by western blotting was performed with purified protein, purified protein added to extracts from control plants, or extracts from control or heat stressed plants. Results revealed AtHsp18.5 migrating only at positions corresponding to dimeric or monomeric species, consistent with the conclusion that the protein does not stably assemble with other cellular components, including dodecameric sHSPs (Supplemental Fig. 5).

Flexibility and core structure of AtHsp18.5

Despite its small size and dimeric structure, we have been unable to obtain diffraction quality crystals of AtHsp18.5. We suspected this might reflect significant flexibility of the protein. We previously used HDXMS to demonstrate that amide hydrogens in the N-terminal arm and C-terminal extension of dodecameric PsHsp18.1 and TaHsp16.9 are in rapid equilibrium with solvent (>70% exchanged in 5 sec) and, thus, are not involved in highly stable secondary structures. The same technique was applied to AtHsp18.5, and, remarkably, the pattern of HDX across AtHsp18.5 was essentially parallel to that of the dodecameric sHSPs (Fig. 5A). The majority of the protein outside the ACD fully exchanged >80% of amide hydrogens within 5 sec, indicating that these regions are not involved in stable secondary structures. In contrast, amide hydrogens in the AtHsp18.5 ACD are highly

protected, and this domain is not interrupted by the more readily exchanged $\beta 6$ loop that is present in PsHsp18.1 and TaHsp16.9.

To study further the AtHsp18.5 structure compared to dodecameric PsHsp18.1, we performed partial proteolysis coupled with MS. AtHsp18.5 and PsHsp18.1 were incubated with trypsin (sHSP/trypsin, w/w = 400/1) in the absence or presence of 150 mM KCl at room temperature for 0 to 24 hr (Fig. 5B). In the absence of KCl, we observed a stable fragment above 11 kDa in the AtHsp18.5 sample after 24 hrs of trypsin digestion; even doubling the trypsin had no effect on stability of this AtHsp18.5 fragment (not shown). In contrast, PsHsp18.1 is much more susceptible to trypsin, and after 24 hrs of digestion in the absence of KCl only minor amounts of two fragments over 11 kDa remain (Fig. 5B, left panel). Trypsin digestion in the presence of 150 mM KCl significantly slowed the rate of proteolysis. Under these conditions intermediate digestion products accumulated to higher levels at the earlier time points, but did not otherwise alter the overall pattern of digestion, and there was no evidence for a stable fragment corresponding to the ACD in PsHsp18.1.

We used MS to determine the identity of the ~11 kDa, trypsin-resistant fragment from AtHsp18.5. As determined by MS, this fragment corresponds to a combination of residues 48–150 and 48–153 at 11,688 and 12,058 Da, respectively (full length AtHsp18.5 = 162 residues, 18,528 Da) (Supplemental Table 1). These fragments result from cleavage at those trypsin sites closest to, but outside the ACD. Thermolysin and chymotrypsin digests gave similar results, with no cleavage within the AtHsp18.5 ACD and absence of a similar protected fragment in PsHsp18.1 (not shown). Proteolysis of TaHsp16.9 yielded results comparable to PsHsp18.1 (not shown). Therefore, the AtHsp18.5 ACD appears to be folded and assembled in a uniquely protease-protected form compared to these dodecameric sHSPs. Considered with the HDXMS data we conclude that AtHsp18.5 comprises a compact ACD flanked by relatively unstructured “arms”.

Both the N- and C-termini contribute to dimer stability

To obtain insight into the dimer structure, nano-ESIMS was performed on AtHsp18.5 digested with trypsin for 4 hr in the presence of KCl (sample with asterisk in Fig. 5B). Figure 5C shows that distinct mass species comprising different protein fragments are resolved. The 48–150/153 fragments are found in multiple monomer peaks (peaks A and B), while the 11–150, and 11/12–153 fragments (C, D and E) are found essentially only in dimer peaks. Interestingly, the 48–153, but not the 48–150 fragment, was also found in complex with 11/12–153 and 11–150. These data indicate that part of both the N-terminal arm and C-terminal extension of at least one subunit contribute to stability of the AtHsp18.5 dimer.

In order to test the importance of different domains in chaperone activity of AtHsp18.5, based on the trypsin cleavage data we designed and purified several different truncation mutants of AtHsp18.5 comprising residues 13–153, 25–153, 48–153 and 39–162. The 13–153 preparation was found to have ~50% of a fragment comprising residues 25–153, due to use of an alternative start Met (see Fig. 1), and we were not able to obtain pure 13–153. We first estimated the relative dimer stability of the purified proteins using Blue Native PAGE and nano-ESIMS. The full length (WT) and N-strep and C-strep constructs migrated at positions consistent with dimeric forms (Fig. 6A), as also seen in Fig. 2 and Supplemental Fig. 5. Based on the relative migration of the truncations, we conclude that the 48–153, 39–162 and 48–150 truncations migrate as monomers. Heating the proteins and separating them at 45 °C did not result in a significant change in the migration of any of the proteins; dimeric forms were stable where present.

Nano-ESIMS provides further information about the relative stability of the dimeric forms of the truncation mutants. The MS conditions used resulted in some dissociation of the

native WT dimer, which is typical in experiments investigating non-covalent protein complexes. However, for the full length proteins, WT, N-strep and C-strep, the predominant form observed is the dimer, although somewhat more dissociated species appear in the N-strep sample. All of the truncation mutants show considerably more monomeric species, consistent with decreased stability of their dimeric forms. Estimating the dimer to monomer ratio from the area under the ion peaks at a series of protein concentrations further confirms that the 37–162 and 48–153 truncations form the least stable dimers, and that the 25–153 truncation has intermediate stability (Supplemental Fig. 6). While these data do not provide quantitative measures of dimerization constants, they clearly demonstrate that the full length N-terminal arm and C-terminal extension contribute to dimer stability.

AtHsp18.5 chaperone activity is dependent on the N-terminal arm

We used the truncated AtHsp18.1 mutants to investigate which parts of the protein were required for optimal chaperone activity in protection of MDH and Luc. The ratio of sHSP to MDH that is required to maintain MDH solubility after heating was used as a measure of relative efficiency of the proteins. The N- and C-terminal Strep-tagged proteins actually showed slightly better protection of MDH, and the 13/25–153 preparation showed slightly reduced effectiveness (Fig. 7A). The three proteins missing 25 or more amino acids from the N-terminus (25–153, 48–153 and 39–162) exhibited no chaperone activity in protecting MDH. Using Luc as the heatsensitive substrate yielded the same results with the exception of the 25–153 truncation and the 13/25–153 preparation (Fig. 7B). While 25–153 had no activity with MDH, it was about half as effective as wild type with Luc, and the mixed preparation, 13/25–153 appeared roughly twice as effective as wild type. In total the results show that the ACD alone lacks activity, and presence of the C-terminus does not compensate for removal of most of the N-terminal arm. Furthermore, the N-terminal arm interacts differently with different substrates and is essential for AtHsp18.5 chaperone activity.

DISCUSSION

The dimeric structure, as well as the ability of the AtHsp18.5 dimer to act as an effective chaperone, is atypical of sHSPs and provides new information relative to how these proteins can achieve substrate protection. The majority of plant, animal and bacterial sHSPs are oligomeric³, and only a limited number of other dimeric and even monomeric recombinant sHSPs have been tested for chaperone activity. Both recombinant human HspB6 (Hsp20) and HspB8 (Hsp22) have apparent dimeric structure and can suppress aggregation of some substrates (as shown by light scattering), although dimeric rat HspB6 was reported to exhibit no chaperone activity. The apparent dimeric structure of these sHSPs may be limited to the recombinant form, as they assemble into hetero-oligomers with sHSPs and in some cases with other proteins in the mammalian cytosol. Tetrameric and monomeric sHSPs from *C. elegans* have no detectable chaperone activity. The unusual dimer of Tsp36, which has two α -crystallin domains per monomer, can prevent aggregation of insulin and citrate synthase⁴⁶, but further investigation of its mechanism of action has not been performed. Multiple studies have also tested the activity of sHSP dimers created from native oligomers by N- or C-terminal truncations. Results from these experiments are mixed, with the truncated sHSPs supporting anywhere from good to no protection of specific substrates. These disparate results with different sHSPs have led to opposing conclusions about the requirement of the oligomeric state for sHSP chaperone activity, as well as the importance of the termini in substrate interactions. The chaperone activity of the AtHsp18.5 dimer clearly demonstrates that substrate binding and formation of sHSP-substrate complexes does not require an oligomeric sHSP to be populated to a significant extent at equilibrium. This result also lends support to the model in which dimers that are released from oligomeric

sHSPs, either by subunit exchange or temperature-induced dissociation, can act as the substrate binding species. It should be recognized, however, that the availability of dimers may not be the rate-limiting activation step required for sHSP-substrate interaction for all sHSPs. Continued study of AtHsp18.5 should provide further insight into sHSP-activating structural changes distinct from changes in oligomeric structure.

The ACD of AtHsp18.5 is unusually stable compared to the ACD of dodecameric plant sHSPs such as PsHsp18.1; the AtHsp18.5 ACD is resistant to proteolysis and shows maximum protection from amide hydrogen exchange throughout the domain (Fig. 5). The AtHsp18.5 ACD is also unique because it lacks the loop containing β -strand 6, which averages ~21 residues in plant sHSPs³², and through strand exchange forms the dimer interface. The same region between β 5 and β 7 is ~14 residues in animal sHSPs³² and also does not include the β 6 loop. The vertebrate proteins dimerize through antiparallel interactions of an extended β 7. In AtHsp18.5, sequence alignment and structural prediction indicate only ~4 residues separate β 5 and β 7, making the AtHsp18.5 ACD similar to the ACD of the structural homolog p23⁵³ and suggesting it may have yet a different mode of dimerization than previously characterized sHSPs. Although AtHsp18.5 monomers exchange, the rate of exchange is very slow compared to the time frame of substrate protection, consistent with the dimer, rather than the monomer, being the substrate binding species. Analysis of truncation mutants of AtHsp18.5 indicates that the conserved C-terminal I/V-X-I/V motif (aa 155–157), which is involved in oligomerization of other sHSPs³, is not required for dimerization. Removal of half or more of the 61 residue N-terminal arm results in destabilization of the dimer, but even the isolated ACD forms weak, but detectable dimers. Together with the HDXMS data, the picture emerges of a very stable dimeric ACD core with highly flexible N- and C-terminal arms. Thus, our studies of AtHsp18.5 support the model that the structural flexibility of the N-terminal arms of the dimer allows sHSPs to bind diverse substrates³.

The importance of the N-terminal arm in substrate interactions is consistent with activity measurements of AtHsp18.5 truncation mutants. Especially striking is the difference in protection of two substrates, MDH and Luc, by the different truncations. Under the conditions tested, Luc was more effectively protected by truncations that reduced protection of MDH, demonstrating differences in sHSP-substrate contacts for these two proteins. It is also significant that even wild type AtHsp18.5 showed very inefficient protection of citrate synthase in our assays. Along with previous data, these results demonstrate that there is no single substrate binding site on the sHSPs. In addition to removing substrate binding sites, truncations can potentially uncover hydrophobic sites that may otherwise not be primary sites for substrate interaction. This could explain how certain AtHsp18.5 truncations actually enhance protection of Luc (eg. 13/25–153), and how some substrates can still be protected even by truncated dimeric forms of vertebrate oligomeric sHSPs. Because sHSPs appear to bind exposed hydrophobic surfaces of denaturing proteins, the ability to protect diverse substrates must require varied configurations of available binding surfaces on the sHSP. We propose that the flexible termini of the sHSPs combined with exposed hydrophobic sites on the ACD can provide the necessary variation required to capture different unfolding proteins. The stability of the AtHsp18.5 ACD core may make this protein an excellent model for design new chaperones with N-varied and C-terminal extensions capable of highly efficient protection of diverse proteins from aggregation.

MATERIALS AND METHODS

Amino acid alignment

The AtHsp18.5 DNA sequence was used to query the complete TIGR Plant Transcript Assemblies database (<http://plantta.tigr.org/index.shtml>) with tBlastx, identifying *Corylus*

avellana (C.a., AF021807), *Populus trichocarpa* (P.t, Pt826045), *Gossypium hirsutum* (G.h., TA2993.3635), *Citrus aurantium* (C.au. TA1184166), *Brassica rapa* (B.r., TA34073711), and *Brassica napus* (B.n., DC818680) with e scores of 1.0×10^{-25} or lower. The *Corylus avellana* sequence came from a tBlastx search of the NCBI nonredundant database. *Populus trichocarpa* (JGI version 1.0; http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) and *Oryza sativa* (TIGR) genome sites were also searched. No homologues were detected in *O. sativa*. Cytosolic sHSPs of class I, II and III are represented by *Arabidopsis thaliana* Hsp17.4-CI (CAB90950), Hsp17.7-CII (CAB87675) and Hsp17.4-CIII (AAD25777). Sequences were downloaded into Bioedit⁵⁴ and aligned using default parameters of ClustalW⁵⁵.

Protein production and quantification

The N- or C-terminal Strep II tag (amino acid sequence: WSHPQFEK) was added to the wt AtHsp18.5 plasmid by polymerase chain reaction. The Hsp18.5 truncation mutants were also generated by polymerase chain reaction introducing a start and or stop codon at the necessary positions. Proteins were purified as described and quantified using calculated extinction coefficients (ϵ_{280} =16,500 for PsHsp18.1-CI; ϵ_{280} =19,615 for AtHsp18.5-WT; ϵ_{280} =16,865 for 13/25–153 truncation; and ϵ_{280} =14,115 for 25–153, 39–162 and 49–153 truncations). Monomeric masses of the purified proteins were confirmed as predicted by mass spectrometry (Supplemental Table 2).

Gel electrophoresis and crosslinking

Non-denaturing and SDS-PAGE were performed by standard techniques. For analysis of subunit exchange on SDS-PAGE, proteins were either mixed together in 25mM Tris, 1 mM EDTA pH 7.5 and incubated as described, or proteins were incubated in 6 M urea at room temp for 1 h then dialyzed against 5000 volumes 25mM Tris, 1 mM EDTA pH 7.5, overnight, prior to electrophoresis.

Blue native PAGE (5–22% acrylamide) was run at 40°C as described⁵⁶ using 12 μ L of 24 μ M sHSPs. For crosslinking, sHSPs (24 μ M) were incubated with glutaraldehyde at the molar ratios indicated for 1h at room temperature. The reaction was stopped with a 100-fold molar excess of glycine to glutaraldehyde. Samples were separated by SDS-PAGE (5–15% acrylamide) and stained with Coomassie Blue.

Analytical ultracentrifugation

Wild-type AtHsp18.5 was analyzed by sedimentation velocity centrifugation using a Beckman XL-I dual detection analytical ultracentrifuge with sapphire windows and UV detection at 280 or 230 nm. Five protein concentrations from 2.5–30 μ M in 10 mM sodium phosphate buffer, pH 7.5 were sedimented at 40,000 rpm, 20 °C, and data were analyzed using SEDFIT and the c(s) distribution method, with the Lamm equation parameters of partial specific volume = 0.729 mL/g, buffer density = 1.005 g/mL, and a confidence interval 0.68 (one sigma)⁵⁷.

Mass spectrometry of subunit exchange

Subunit exchange was observed by native mass spectrometry. 100 μ M solutions of individual proteins were first buffer exchanged into 10 mM, pH 7 ammonium acetate (Sigma-Aldrich, St. Louis, MO) using Micro Bio-Spin columns (Bio-Rad, Hercules, CA), and then placed in a water bath to equilibrate to the desired temperature. Once equilibrated, equimolar quantities of each protein were mixed and returned to the water bath. Aliquots were periodically removed for mass spectrometric analysis.

Mass spectrometric analysis (nano-ESI/MS) was performed on a QTOF 2 instrument (Waters Corporation, Milford, MA). 4–6 μ L of protein solution was loaded into borosilicate glass capillaries (Corning, Inc., Corning, NY)⁵⁸ pulled in-house with a P-97 micropipette puller (Sutter Instruments, Hercules, CA) and then electrosprayed using a home-built nanoESI source. Instrument settings were optimized to maintain intact protein complexes in the gas phase while obtained clearly resolved peaks⁵⁹. Typical experimental conditions were as follows: capillary voltage, 1.5–2.0 kV; sample cone 125 V; extractor cone 1 V; source pressure, $\sim 8.5 \times 10^{-3}$ mbar; analyzer region pressure 3.1×10^{-5} mbar. For subunit exchange experiments mass spectra were collected at various intervals between 90 sec and 24 hrs following protein mixing. The amount of heterodimer was quantified relative to the amount of the original homodimeric species to determine the rate of subunit exchange.

Mass spectrometry (MS) of intact protein and proteolytic fragments

Typically, 1–5 μ L of protein was analyzed essentially as described above with the following specific experimental parameters. Mass spectrometry of intact complexes requires a balance of conditions for successful ionization and preservation of noncovalent interactions. Optimal ion transmission and preservation of non-covalent interactions was achieved with the following settings: sample cone, 150 V; extractor 1 V; ion transfer stage pressure, 1×10^{-2} mbar; quadrupole analyzer pressure, 1×10^{-4} mbar; ToF pressure, 3×10^{-6} mbar. For fragment identification, proteolysis fragments were eluted from a STYROS R2 polymer HPLC column (OraChrom, Inc., Woburn, MA) using a gradient of 5–90% acetonitrile (50 μ L/min) and injected into a Q-ToF Micro mass spectrometer (Waters, Corp.). Nano-ESI/MS on the fragments was as described for the intact protein.

Hydrogen-deuterium exchange (HDX) and MS

Peptide mapping of AtHsp18.5 by HPLC-Tandem MS was performed as described previously²¹. HDX experiments were initiated by diluting protein ~ 20 -fold into labeling solution (D_2O , 10 mM sodium phosphate, pD 7.0) to a final concentration of 10 μ M. After a 5 sec labeling period, protein digestion, peptide identification, mass analysis and back exchange correction were performed²¹.

Aggregation protection assay

1 μ M firefly Luc (Promega, CA), 2 μ M CS or 3 μ M porcine mitochondrial MDH (both Roche, Germany) were incubated with sHSPs at the molar ratios, temperatures and times indicated, and samples were processed as described¹⁸. For analysis of sHSP-substrate complexes, supernatants from aggregation protection assays were applied to a TSKgel G5000PW_{XL} column (flow rate 1 mL/min) with a mobile phase of 25 mM Na phosphate, 150 mM KCl, pH 7.4.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

sHSP	small heat shock protein
ACD	α -crystallin domain
MS	mass spectrometry
nano-ESIMS	nano-electrospray ionization mass spectrometry
HDX	hydrogen deuterium exchange

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Highlights

- AtHsp18.5 is a dimeric small heat shock protein that acts as a robust chaperone
- The α -crystallin domain is protease resistant and flanked by flexible arms.
- AtHsp18.5 lacks the β 6 dimerization loop found in other plant and bacterial sHSPs
- AtHsp18.5 monomers readily exchange between dimers
- Substrate protection requires the N-terminal arm.

Figure 1. AtHsp18.5 and homologues are unusual cytosolic plant sHSPs lacking β -strand 6
Alignment of AtHsp18.5 (A.t.18.5) with homologs from *Corylus avellana* (C.a.), *Populus trichocarpa* (P.t.), *Gossypium hirsutum* (G.h.), *Citrus aurantium* (C.au.), *Brassica rapa* (B.r.), and *Brassica napus* (B.n.) compared to other cytosolic sHSPs from class I, II and III³³ represented by *Arabidopsis thaliana* Hsp17.4-CI, Hsp17.7-CII and Hsp17.4-CIII. Secondary structural elements are based on alignment with TaHsp16.9⁹. The ACD comprises β 2 through β 9 (delimited by arrowheads). Consensus sequences for AtHsp18.5 and homologues, or for all proteins are shown. Residues corresponding to the beginning and end of the truncated proteins as discussed in the text are underlined.

Figure 2. Native AtHsp18.5 is dimeric

A) Non-denaturing PAGE of 15 μ l of 24 μ M sHSP. B) 24 μ M sHSPs were crosslinked with glutaraldehyde at the indicated molar ratios and 15 μ l analyzed by SDS-PAGE. Asterisks indicate position of the dimeric crosslinked species. C) Nano-ESIMS of native AtHsp18.5. Inset shows dimer mass as determined from the deconvoluted spectrum.

Figure 3. Dimeric AtHsp18.5 protects substrates from aggregation in large sHSP-substrate complexes

3 μ M MDH or 1 μ M Luc were heated with either PsHsp18.1 or AtHsp18.5 at the indicated molar ratios. Heat treatments were 45°C for 1 h for MDH and 42°C for 8.5 min for Luc. A) SDS-PAGE of MDH and Luc from the soluble or pellet fractions after heating at the indicated molar ratio of sHSP to substrate. Gels were stained with Coomassie Blue. B) SEC analysis of the soluble fractions from Panel A. Asterisk indicates position of sHSP-substrate complexes.

Figure 4. AtHsp18.5 monomers exchange between dimers at a temperature-dependent rate

A) Nondenaturing -PAGE separation of wild type (WT) and N- or C-terminally Strep tagged AtHsp18.5 either alone, or after incubation together as indicated. B) Representative spectra of subunit exchange between WT (*) and AtHsp18.5 carrying a C-terminal Strep tag (C). Spectra from 90 sec, 45 min, and 18 hrs show an increase in the relative amount of heterodimer species (gray bars). C) Rate of appearance of the heterodimer between AtHsp18.5 and C-terminal Strep tag as a function of temperature. Inset: calculated rate constants.

Figure 5. The AtHsp18.5 ACD forms a stable core flanked by flexible N- and C-termini
A) Percent HDX for peptides of AtHsp18.5 compared to TaHsp16.9 and PsHsp18.1²¹ after a 5 sec pulse labeling at pD 7.5 at room temperature. Each peptide is represented by a colored bar, with the color indicating percentage of amide HDX as shown in the legend. B) 24 μ M AtHsp18.5 or PsHsp18.1 was incubated with trypsin at a 400:1 molar ratio at room temperature for times indicated with or without KCl. Products were analyzed by SDS-PAGE. C) Nano-ESIMS of the 4 hr trypsin digest in 150 mM KCl (asterisk Panel B). Inset shows expansion of the higher m/z data.

Figure 6. AtHsp18.5 truncated at both the N- and C-terminus retains the ability to dimerize
 A) Blue native PAGE of the indicated proteins separated at 4 °C (left) or at 45 °C (right).
 The 48–150 truncation was obtained by trypsin digestion of the WT protein in the absence
 of KCl as in Fig. 5B B) Nano-ESIMS of the indicated proteins at 100 μ M, showing detection
 of different amounts of dimer and monomer forms in all samples.

Figure 7. Truncations of the N- and C-termini alter substrate protection efficiency and the ACD alone is ineffective as a chaperone

3 μ M MDH or 1 μ M Luc were heat denatured with the indicated molar ratios of wild type or truncated AtHsp18.5 proteins. Denaturation of MDH was for 1 hr at 45 °C and 8.5 min at 42°C for Luc. A) SDS-PAGE of MDH from the soluble or pellet fractions. B) SDS-PAGE of Luc from the soluble or pellet fractions. Gels were stained with Coomassie Blue.