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Structure and mechanism of protein stability sensors: The chaperone activity of small heat-shock proteins

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Abstract

Small heat shock proteins (sHSP) are a remarkably diverse group of molecular chaperones possessing a degree of structural plasticity unparalleled in other protein superfamilies. In the absence of chemical energy input, these stability sensors can sensitively recognize and bind destabilized proteins, even in the absence of gross misfolding. Cellular conditions regulate affinity towards client proteins allowing tightly controlled switching and tuning of sHSP chaperone capacity. Perturbations of this regulation, through chemical modification or mutation, directly lead to a variety of diseased states. This review explores the structural basis of sHSP oligomeric flexibility and the corresponding functional consequences in the context of a model describing sHSP activity with a set of three coupled thermodynamic equilibria. As current research illuminates many novel physiological roles for sHSP outside of their traditional duties as molecular chaperones, such a conceptual framework provides a sound foundation to describe these emerging functions in physiological and pathological processes.

Following their emergence from the ribosomes, newly synthesized proteins navigate an energy hypersurface replete with conformational dead ends and punctuated by kinetic detours to reach the native biologically active conformation (1,2). Along this journey, hydrophobic stretches of residues are progressively shielded from solvent, generally through packing in the protein core or at interfaces. Long transit times through local energy minima leave partially folded proteins with exposed hydrophobic surfaces vulnerable to the dead-end pitfalls of aggregation (3). Successful arrival to the global energy minimum does not eliminate the danger of aggregation. The marginal stability of the folded states implies that proteins constantly sample partially unfolded conformations exposing hydrophobic residues in the crowded cellular environment (4,5). The rates of these equilibrium transitions increase under stress conditions which may lead to loss of protein solubility. Aggregation wastes cell resources and protein aggregation diseases can be infectious, dementing and even fatal (6). Nature has devised multiple and redundant responses to inhibit protein aggregation or dispose of protein aggregates. One such response involves expression of multiple families of heat shock proteins that function as molecular chaperones each specialized for dealing with a different aspect of protein folding and stability (7–15). Some chaperones guide proteins along their journey to the native state avoiding local energy minima or rescuing them from such detours. Other chaperones buffer the rapid increase in aggregation-prone, misfolded proteins that inevitably follows environmental perturbations.

Although heat shock proteins have broad specificity for non native proteins, their functions vary in mechanistic complexity, ranging from passive binding to prevent aggregation to energy-dependent facilitated folding of specific classes of proteins (7–15). The small heat-

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shock proteins (sHSP) are a ubiquitous family of passive, energy-independent chaperones (16–19). This superfamily consists of heat-inducible proteins with a molecular mass between 12 and 40 kDa per subunit possessing a conserved stretch of ~100 residues referred to as the α -crystallin domain (Figure 1A). This domain is flanked at the N-terminal side by a hydrophobic region that varies in sequence and length and at the C-terminal side by a short tail or extension with a conserved sequence motif. Invariably, sHSP subunits assemble into oligomeric structures with a broad spectrum of sizes, symmetries, and structural order, which relate to their function and modes of interaction with unfolded or destabilized proteins (18–20). Interest in sHSP has been stimulated by the identification of their roles in critical cellular processes such as aging (21) and apoptosis (22–24), as well as by the discovery of mutants associated with human diseases including cataracts (25,26) and cardiomyopathies (27). Over the past two decades, genetic, biochemical and structural studies have provided a wealth of information on the physiological roles and functional mechanisms of sHSP. In *C. elegans*, sHSP promote longevity and couple the normal aging process to age-related diseases such as polyglutamine-expansion protein aggregation (21). sHSP may provide a therapeutic advantage for neurodegenerative diseases, such as amyotrophic lateral sclerosis and multiple sclerosis (28–30), with which they are associated. Studies using intact vascular smooth muscle demonstrate key roles for sHSP in regulation of smooth muscle tone and active participation in kinase signaling cascades (31). Discovery and characterization of additional physiological contributions continue to expand the importance of sHSP beyond their traditional chaperone duties. This review focuses on structural and mechanistic aspects of sHSP, including the determinants of their oligomeric assembly, their mechanism of sensing non native proteins, and the structural basis of regulation of sHSP chaperone activity.

Nowhere is protein folding and stability more readily associated with disease than in the ocular lens (32). The “wear and tear” of lens aging is recorded at the molecular level through accumulated modifications to the crystallins, the major protein components of fiber cells (33–35). In the absence of protein turnover in the lens, these modifications lead to a progressive loss of crystallin stability and solubility, as well as altered protein-protein interactions, all of which compromise lens transparency and refractivity and may lead to cataracts (33,36). Roughly 35% of a lens fiber cell by weight consists of α A- and α B-crystallin, a sHSP presumed to inhibit the aggregation of damaged and destabilized proteins (37,38). α -crystallin-deficient mice have smaller lenses that develop opacity shortly after birth (39). Furthermore, a number of point mutations in human α -crystallin genes have been associated with hereditary cataracts (25,26,40,41), confirming the critical role of this sHSP in maintaining lens transparency. α -Crystallins are the most studied sHSP and the lens provides a unique model system to link *in vitro* mechanistic analysis to phenotypic models (42,43).

Structural framework

sHSP assemble into remarkably versatile oligomers with a level of divergence unparalleled in other protein superfamilies. Not only do sHSP oligomers display wide variations in the number of subunits and their packing symmetry, but they also show substantially different degrees of order and dispersity (20). The spectrum ranges from highly symmetric and monodisperse assemblies (44) to polydisperse oligomers actively exchanging subunits (45,46). Although dynamics and heterogeneity hinder high resolution structural analysis of a class of sHSP, the determinants of variable architecture and its mechanistic roles have emerged from visualization of oligomers (20,44,47), characterization of their dynamics by spectroscopic approaches (48, 49) and the correlation between oligomer dynamics and substrate binding (50–54). On a more general level, the analysis of sHSP assemblies is providing unique insights into the role of dynamics in protein function and identifying new motifs of structural plasticity.

The α -crystallin domain building block

Consistent with its conserved sequence, the α -crystallin domain is a common structural module that plays a central role in sHSP oligomeric structure across the evolutionary spectrum. α -Crystallin domain dimers are the building block for the two ordered sHSP oligomers with known high resolution structures, *Methanocaldococcus jannashii* (MJ) Hsp16.5 (44) and *Triticum aestivum* (Ta) Hsp16.9 (47) (Figure 1B). Each α -crystallin domain monomer has an immunoglobulin core fold consisting of seven β -strands arranged in two antiparallel sheets (Figure 1C). The two monomers interact via an extended loop and the dimer is stabilized by swapping of a peripheral β -strand (β_6) which interacts with the edge of the β -sandwich in the symmetry-related subunit. While this seven-stranded core fold is conserved in the mammalian sHSP α A-crystallin (55), sequence modifications, including deletion of the swapped strand, lead to a different dimerization interface (56–58). In α A-crystallin and Hsp27, the two monomers are related by a two-fold symmetry axis located near the N-terminal end of strand 7 (Figure 1C). A similar geometry for the α B-crystallin dimer was deduced from small angle x-ray scattering (59) suggesting that the altered monomer/monomer packing is conserved among mammalian sHSP and thus may have important consequences for their oligomeric structures. Recent NMR analysis of a truncated construct of α B-crystallin consisting of its α -crystallin domain identified six β -strands (60). The sequence corresponding to β_2 of α A-crystallin appeared unstructured, possibly reflecting an artifact of truncation or more interestingly structural differences between α A- and α B-crystallin. Although the authors suggest their data in the region of strand 7 differ from the EPR model, close inspection of their sequence-specific secondary structure assignment suggests the perceived deviation of one residue (F113) is simply an artifact of sequence alignment.

Packing of the α -crystallin dimers

In the native Hsp16.5 and Hsp16.9 oligomers, α -crystallin domain dimers are held together by the C-terminal tail of one sHSP monomer binding in a hydrophobic groove at the C-terminal edge of the β -sandwich contributed by another dimer (Figure 1B). The sequence elements involved in this interaction are conserved across the superfamily suggesting this dimer-dimer connection is a core structural motif (16, 17). Indeed, EM analysis of Acr1, one of two *Mycobacterium tuberculosis* sHSP, suggests a similar role for this sequence in the assembly of a dodecameric oligomer (61). A hinge linking the C-terminal tail to the α -crystallin domain provides the flexibility necessary to generate oligomer diversity while preserving the dimer-dimer interface. The importance of this motif is highlighted by comparison of Hsp16.5 and Hsp16.9 oligomeric structures (44, 47). Despite possessing the same conserved α -crystallin domain fold and a similar dimeric building block, Hsp16.5 assembles into a spherical oligomer of 24 subunits with octahedral symmetry while Hsp16.9 subunits assemble into two stacked hexameric disks. Formation of these distinct assemblies is facilitated by a 30° change in the orientation of the C-terminal extensions relative to the α -crystallin domain allowing two different dimer packing arrangements within an Hsp16.9 oligomer. The Hsp16.5 and Hsp16.9 oligomers are also differentially stabilized by their respective N-terminal regions (47). The N-termini of Hsp16.9 monomers interact through an unusual helix knotting that intertwines monomers from the two rings (Figure 1B). In contrast, truncation of Hsp16.5 N-termini does not change the size or symmetry of the oligomer (62) (Figure 2A).

Oligomeric structure: from ordered symmetric to polydisperse

Mammalian sHSP, in contrast, form dynamic polydisperse oligomers rather than symmetrical assemblies (46,50,63–65). CryoEM analysis of α -crystallins and Hsp27 reveals particles of different sizes with little if any evidence of symmetry. This heterogeneity hinders high resolution analyses of mammalian sHSP, although some of their structural features have been deduced from systematic application of spin labeling and EPR spectroscopy (57). While the

α -crystallin domain fold appears to be preserved in the α -crystallins and Hsp27, the N-terminal region evolved to control the global packing arrangement, oligomer dynamics and order (45, 57,66). Mammalian sHSP exchange subunits in a process that involves equilibrium dissociation and reassociation of dimers and/or monomers. In a classic FRET experiment, Bova et al. measured the rate constant of subunit exchange between oligomers of α -crystallin and demonstrated that these rates are orders of magnitude faster than expected based on unfolding kinetics (45,64). Truncation of the N-terminal region leads to α A-crystallin dissociation into dimers and tetramers that do not exchange subunits (45,57,66,67). Similarly, *yeast* Hsp26 oligomerization depends on its N-terminal domain (68). Subunit exchange is not unique to polydisperse sHSP; the ordered TaHsp16.9 exchanges subunits with PsHsp18.1 under native conditions (47,69). Compelling evidence suggest that equilibrium dissociation is a mechanism of sHSP activation (51,54).

Determinants of sHSP oligomer plasticity

The interplay between the three sequence modules (N-terminal region, α -crystallin domain, and C-terminal extension) in the transition from ordered symmetrical to polydisperse assemblies was experimentally tested through structural and functional characterization of engineered Hsp16.5 oligomers (70). The transposition of an N-terminal sequence element from the polydisperse human Hsp27 into the monodisperse Hsp16.5 results either in a symmetric expansion or transformation to polydisperse assemblies (Figure 2). A hybrid approach combining spin labeling EPR and cryoEM revealed that the expanded symmetric oligomer, hereafter referred to as Hsp16.5-P1, consists of 48 subunits compared to 24 for the WT (Figure 3). In the Hsp16.5-P1 structure, the two monomers in the dimeric building block are not equivalent, primarily due to a change in the orientation of the C-terminal extension similar to the hinge mechanism observed in Hsp16.9 (Figure 3C). This rearrangement leads to a novel 4-fold window of about 45Å in diameter, which is not present in the WT oligomer. The four dimers around the 4-fold window are tethered by C-terminal extensions in the modified orientation. A polydisperse form of Hsp16.5, referred to as Hsp16.5-P1N, is created when the same Hsp27 sequence element is inserted at a different site within the Hsp16.5 N-terminal region (Figure 2C) (70).

The results of this protein engineering experiment are consistent with the notion that modification of the N-terminal region, a distinctive hallmark in sHSP evolution, affects the overall oligomeric order and symmetry of the assembly. Changes in the N-terminal region are propagated to the dimer-dimer interface and can lead to expanded symmetrical assemblies or to irregular and polydisperse assemblies. Creation of the polydisperse Hsp16.5-P1N also resolves the conundrum of how an irregular assembly can form from a folded and stable structural building block, in this case the conserved α -crystallin domain dimer. A dimer-dimer interface with minimal contacts and a flexible C-terminal hinge provides this plasticity by adopting different angles to accommodate multiple higher order oligomers.

Both the expanded and polydisperse engineered forms of Hsp16.5 have increased affinities to destabilized substrates relative to WT Hsp16.5, supporting the direct coupling between oligomeric structure and chaperone activity (70). In contrast, deletion of the N-terminal region significantly reduces substrate affinity, suggesting this buried sequence is likely critical for substrate binding.

Mechanism of sHSP chaperone activity

While all molecular chaperones bind proteins in non-native states, they differ in their mechanism of substrate recognition, as well as in the conformation and fate of the bound substrate (11). Despite a critical role in conferring thermotolerance (18), sHSP involvement in cellular protein refolding is less clear. One current model postulates that under conditions of

extreme stress sHSP provide an energy-independent mechanism to buffer the increase in non-native proteins (71,72). sHSP chaperone activity does not require the input of ATP energy and the binding capacity can reach one substrate protein per sHSP subunit of equal molecular mass. Thus, sHSP can be a major contributor to the chaperone capacity of a cell. Although sHSP/substrate complexes are stable (71,72), release and refolding of the bound substrate can be accomplished *in vitro* through interactions with Hsp70. Cooperative cellular chaperone networks have been proposed to play a central role in protein folding and in stress response (73).

Initial characterization of sHSP chaperone activity relied primarily on observing the suppression of light scattering by aggregating proteins. In brief, substrate proteins are exposed to conditions that promote unfolding, subsequent aggregation and precipitation. Chaperone efficiency is defined empirically as the reduction in light scattering by the substrate in the presence of the chaperone. These convenient and experimentally simple assays confirmed the broad specificity of sHSP and defined the basic characteristics of their interactions with substrate proteins (18). However, determination of binding parameters and structural analysis of the interaction complex are hindered by the non-equilibrium nature of these aggregation-based assays. The experimental observable, change in light scattering, reflects both the kinetic competition between self-association of the substrate and binding to the chaperone (49). Furthermore, these assays employ strongly denaturing conditions resulting in a conformationally heterogeneous ensemble of bound substrates that is not readily amenable to structural analysis. These extreme conditions may also compromise the structural and functional integrity of the chaperone. From a physiological perspective, mutations or post-translational modifications can shift the folding equilibrium towards non native states but often do not lead to a $\Delta G_{\text{unf}} > 0$ characteristic of most aggregation assays. Thus, such assays do not recapitulate the predominant interactions of chaperones within the cellular environment that must precede nucleation of aggregation.

Mchaourab *et al.* demonstrated that sHSP detect the increased excursions of destabilized proteins towards aggregation-prone non-native states under conditions that favor the native state in the absence of substrate aggregation (74). Progressive reduction in the folding equilibrium constant of T4 lysozyme (T4L) via site-directed mutagenesis triggers the formation of a stable complex with sHSP although T4L folded state is favored by 3–7 kcal/mol as calculated from the experimentally determined ΔG_{unf} . These studies took advantage of the thorough analysis of T4L folding and stability that yielded a library of thermodynamically destabilized, site-directed mutants with known crystal structures (75,76). Complex formation between substrate and sHSP was initially detected by altered spectroscopic signatures of spin or fluorescence labels (74,77,78) attached at a unique, solvent-exposed cysteine in the substrate. Recently, a new label-free approach has been introduced to simultaneously determine the kinetics and thermodynamics of chaperone/substrate interactions (79).

A conceptual framework emerged from studies of T4L binding to sHSP that also incorporates previously reported mechanistic elements (54,74,80). It is grounded in experiments that identify the conformational states recognized by sHSP, define the nature of the chaperone structural changes that are required for or accompany recognition and binding, and determine binding affinity and stoichiometry. The resulting minimalist model is summarized by three coupled equilibria. The substrate folding equilibrium (eq.1) describes the transition between the substrate native, partially unfolded and the unfolded states. Equation 2 reflects the equilibrium associated with the transition of sHSP to a high affinity and/or high capacity state ((sHSP)_a). This phenomenological description captures reported experimental characteristics of sHSP chaperone activity, but simplifies the underlying distribution of oligomeric states and their relative affinities to non native proteins. A more complete description should allow for low affinity substrate binding by inactivated sHSP. Equations 1 and 2 are coupled by the

binding of (sHSP)_a to partially (I) or globally unfolded (U) states of the substrate (eq.3). Under steady-state conditions, the binding reflects an energetic preference of the non-native substrate states to associate with chaperones versus refolding to the native state N. The model predicts mutations or age-related substrate modifications that shift the equilibrium of eq. 1 towards non native states should induce sHSP complex formation. Similarly, the coupled equations predict that changes in the equilibrium of eq. 2 favoring the activated state of the chaperone, lead to increased binding. The interpretive and predictive power of this model was tested in series of studies.

sHSP as stability sensors

Experiments confirm that manipulation of equation 1 affects the level of bound substrate (equation 3) as predicted. The affinity of α A-, α B-crystallin, Hsp27 (54, 74, 81) and Hsp16.5 binding to T4L destabilized mutants correlates with the mutant's ΔG_{unf} . This is a remarkable result considering that these mutants have similar structures in the folded state with no local static unfolding, as demonstrated by x-ray crystallography (Figure 4A). Binding occurs in two modes with different affinities and stoichiometries (Figure 4C). The most destabilized T4L mutants activate a low affinity but high capacity mode whereby each sHSP subunit binds a monomer of T4L. By comparison, in the high affinity mode an average of four sHSP subunits bind a monomer of T4L. The two-state nature of the T4L folding equilibrium implies sHSP recognize the unfolded state, U, and/or partially unfolded intermediates whose populations increase with a decrease in ΔG_{unf} . Because binding occurs under conditions that strongly favor the folded state, the threshold for stable binding reflects the free energy balance between association with the sHSP and refolding to the native state. Consequently, in a set of mutants of similar native-state structures, sHSP sense the reduction in the stability of the native state.

The structure of bound substrate

The reported spectrum of bound conformations appears to be substrate-specific and ranges from unfolded to loosely collapsed or molten globular (82–88). For instance, β - and γ -crystallins bound to α -crystallin were reported to have significant secondary structure while an early report on α -lactalbumin binding suggests extensive unfolding. Although it is conceivable these differences may reflect the substrate-specific folding pathways, another contributing factor may be the intrinsic difficulty in the structural analysis of heterogeneous chaperone/substrate complexes. Heterogeneity is accentuated in aggregation-based assays where binding is induced by global substrate unfolding and determined by kinetics as discussed above. The possibility that the substrate intermediate recognized by sHSP differs substantially from the stably bound conformation due to subsequent rearrangements further confounds comparison of these studies. To overcome some of these issues, Claxton *et al.* carried out a systematic analysis of T4L structure when bound to α -crystallin under steady-state conditions in the absence of T4L aggregation (84). They monitored proximities of residue pairs that fingerprint the tertiary fold and secondary structures of T4L (Figure 5A). Binding to α -crystallin increases inter-residue distances within each domain as well as across the active site interface suggesting a loss of T4L native structure. More importantly, an increase in the characteristic $i, i+4$ helical distances implies extensive unfolding of bound T4L. Similar extensive unfolding was deduced for β B1-crystallin where binding to α -crystallin disrupts the dimer interface (89,90). In the context of the thermodynamic stability sensor model discussed above, a compelling interpretation of these results is that α -crystallin binds unfolded T4L. In contrast, a systematic study monitoring hydrogen deuterium exchange, report limited protection of malate dehydrogenase (MDH) bound to Hsp16.9 (87). This suggests that either the bound conformation is partially folded or specific regions of unfolded MDH are protected in the chaperone complex. The lack of any significant changes in the Hsp16.9 protection by MDH binding led the authors to favor the second interpretation. Understanding the origin of

the differences in bound-substrate structures is critical for refining a mechanistic model of sHSP chaperone activity.

In the complex with α -crystallin, the hydrophobic C-terminal of T4L is predominantly buried in a low dielectric/low accessibility region while regions of the N-terminal domain have increased backbone dynamics and are water exposed (Figure 5B) (84). Long stretches of residues in the N-terminal domain possess flexibility characteristics of unfolded proteins in a sterically unhindered environment. The asymmetric pattern of contact suggests a preferential interaction of the sHSP with hydrophobic residues, considered a universal motif in substrate recognition by chaperones.

Role of oligomeric assembly in recognition and binding: Regulation of substrate binding

Substrate contact regions have been identified in the N-terminal segment as well as in the α -crystallin domain of sHSP (68,72,91–94). Neither region is readily accessible in the native oligomer suggesting that substrate binding requires substantial structural rearrangement. The conserved architecture of sHSP places the N-terminal domain in the core of the oligomer surrounded by an α -crystallin shell. Substrate access can be achieved either by expansion of the oligomer as observed for Hsp16.5-P1 (70) or through equilibrium dissociation (47,51,54). The notion of sHSP activation by dissociation was originally invoked to explain the increase in chaperone efficiency at higher temperatures (49,53,95–98). A dynamic threshold for function, mediated by increased rate constant of dissociation, was postulated to describe chaperone efficiency enhancement of α -crystallin mutants (49). Together, these observations suggest that the oligomer architecture serves as a switch that could be activated by stress signals. By regulating access to the substrate binding regions, the oligomer, with its complex symmetry and order, controls the sHSP apparent affinity and capacity and protects from aberrant interactions with folded proteins.

In the context of this model, equilibrium dissociation allows sHSP to sense the presence of non native proteins and dynamically respond to shifts in cellular folding equilibria. Modulation of the equilibrium appears to be central for the physiological roles of mammalian sHSP (99–101). α B-crystallin and Hsp27 are phosphorylated in the context of cellular transduction pathways linked to growth, differentiation and programmed cell death. Hsp27 phosphorylation at three serines leads to complete dissociation into a small multimer (101–103), presumably a dimer. Alterations in the equilibrium between various sub-oligomers were also reported as a consequence of α B-crystallin phosphorylation (104).

The role of equilibrium dissociation in regulating sHSP affinity was systematically tested by correlation of Hsp27 oligomeric state to T4L binding (54). In contrast to α A- and α B-crystallin, Hsp27 dissociation into discrete small multimers (dimers) can be conveniently monitored by size-exclusion chromatography (Figure 6A and 6B). Perturbation of Hsp27 oligomer equilibrium by phosphorylation mimicking mutations that alter the relative stability of the two major oligomeric states profoundly affects the affinity and level of T4L binding. The mutations substantially increase T4L binding affinity and activate the high capacity mode (Figure 6C). Conversely, mutations of Hsp27 or its phosphorylation mimic that reverse the dissociation, i.e. stabilize the native large oligomer, reduce but do not eliminate T4L binding levels suggesting that the small multimer is the binding-competent species. T4L binding induces reassembly into Hsp27/T4L complexes with different hydrodynamic properties: those arising from low affinity binding are larger consistent with the higher capacity of this mode (54). In addition to establishing a direct link between dissociation and affinity, these results demonstrate that phosphorylation is an activation mechanism for Hsp27 and α B-crystallin.

There is evidence that the activation of some sHSP may occur without dissociation (18). Such a mechanism may be particularly relevant for monodisperse and symmetric sHSP where there is no definitive evidence of equilibrium dissociation. Evidence exists suggesting mechanistic decoupling between subunit exchange and the chaperone activity of yeast Hsp26 (105,106). In the absence of directly measuring binding affinities, it is impossible to unequivocally evaluate the contribution of native oligomer binding. Hsp16.5-P1 provides a structural framework for dissociation-independent activation where an increase in the size of the outer shell opening allows increased access to substrate binding regions (70). The extent to which sHSP from lower organisms utilize this proposed mechanism is yet to be fully explored.

Implications of the model for understanding the role of sHSP in health and disease

This review presents an integrated structural and functional model of sHSP. Its central elements link sHSP affinity to oligomer structural dynamics and the free energy of substrate unfolding. Given the substantial binding capacity of sHSP, dynamic regulation of their affinity is critical for cellular response to stress and aging. A static, low affinity may allow damaged proteins with marginal stability to “hang around” long enough to aggregate and precipitate. The exhaustion of α -crystallin from the water soluble fraction of lens cells is a marker of age-related nuclear cataracts (107). On the other hand, a static high affinity may promote substrate unfolding based on the coupled thermodynamic model (Scheme 1). The buffering capacity of sHSP will be titrated out clogging the chaperone network and the degradation machinery. This extreme case of a chaperone “gone awry” may be at the origin of the deleterious effects of some congenital cataracts mutations in α A- and α B-crystallin (25,27). The α A-crystallin mutants, α A-R49C and α A-R116C, linked to hereditary cataracts have been shown to alter affinity to T4L by orders of magnitude and increase the effective number of binding sites compared to WT (80). The enhanced binding by the α A-crystallin mutants shifts the substrate folding equilibrium towards non-native intermediates, thereby promoting unfolding of otherwise fully functional proteins (eqs. 1–3). Given the high concentration of α A-crystallin in the lens, the implied molecular basis of cataracts is a gain of function that leads to the binding of undamaged proteins and subsequent precipitation of the saturated α -crystallin complexes in the developing lens of affected individuals.

Despite remarkable advances in understanding the mechanism of sHSP chaperone activity, much remains to be discovered regarding their physiological and biochemical functions. The eleven human sHSP appear to have important roles in signaling pathways and we are just beginning to frame their contributions to health and disease. Emerging animal models, ex-vivo tools and cell culture systems promise to bridge the gap between the test tube and the organism to understand the contribution of sHSP to physiological and pathological states.

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ABBREVIATIONS

sHSP, Small heat shock proteins; MJ, *Methanocaldococcus jannashii*; Ta, *Triticum aestivum*; FRET, Förster resonance energy transfer; EPR, Electron paramagnetic resonance; CryoEM, Cryo electron microscopy; ATP, adenosine triphosphate; T4L, T4 Lysozyme; MDH, malate dehydrogenase.

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Figure 1. Small heat shock protein architecture

(A) Schematic representation of Hsp16.5, Hsp16.9, and α A-crystallin drawn to scale depicting the α -crystallin domain (gray) flanked by N (blue) and C (red) terminal extensions with β strands as black arrows. (B) Quaternary structures of *M. jannaschii* Hsp 16.5 spherical 24-mer (left) and the dodecameric double disk of wheat Hsp16.9 (right) with N and C terminal extensions colored as (A) and the asymmetric α -crystallin domains of each dimer shaded in light and dark gray. (C) Hsp dimers with strands numbered as in (A). α A-Crystallin depicted as modeled from EPR distance analysis (55). Note the domain swapped strand 6 of Hsp16.5 and 16.9 and its absence in α A-crystallin.

Figure 2. CryoEM structural information for Hsp16.5 wild type and engineered variants
 (A) Three-dimensional cryoEM reconstructions of WT Hsp16.5, Hsp16.5-TR, and Hsp16.5-P1 (left to right) shown cropped in half and radially color-coded (radius 10 Å = blue; radius 90 Å = red) with a 50 Å scale bar. (B) Three selected cryoEM class sum images of Hsp16.5-P1 displaying nearly perfect 2-, 3-, and 4-fold symmetry (left to right). (C) Three cryoEM class sum images of Hsp16.5-P1N selected to represent the diversity and lack of symmetry in the majority of the class sum images. Adapted with permission from (70). © 2009 The American Society for Biochemistry and Molecular Biology . All rights reserved.

Figure 3. CryoEM structure and pseudoatomic model of Hsp16.5-P1

(A) CryoEM structure at 10 Å resolution aligned along the 3- and 4-fold symmetry axes, left to right. The radial color coding is the same as in Figure 2. (B) Pseudoatomic model of Hsp16.5-P1 containing 48 α -crystallin domain monomers as viewed along the 3- and 4-fold symmetry axes. The independent monomer positions are colored either gold with magenta C terminal tails (chain A) or green with blue C terminal tails (chain B). (C) An α -crystallin domain dimer from the pseudoatomic model showing the two different C-terminal tail conformations (left). Superimposition of the two monomers within the dimer (right) shows the deviation in the angle of C-terminal tail relative to the α -crystallin domain. This angle deviation allows the dimer to accommodate packing around either the 3- or 4-fold openings of the assembly. Adapted with

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Figure 4. Destabilized model substrate for Hsp binding analysis

(A) Structural superimposition of cysteineless pseudo wild type T4 Lysozyme (blue) and destabilized mutants L99A (green) and L46A (red) demonstrating preservation of tertiary structure. (B) Chemical denaturant unfolding curves of L99A and L46A as monitored by intrinsic tryptophan fluorescence depicting the relative destabilization of the two substrate mutations. (T4L WT $\Delta G = 11.3$ kcal/mol). (C) Binding isotherms demonstrating that the affinity of α -crystallin to destabilized T4L mutants is reflective of relative destabilization. Left shift of L99A curve demonstrates higher affinity binding. K_D of both low and high affinity substrate binding modes are reported adjacent to features of the binding isotherm corresponding to each mode.

Figure 5. Structure of bound substrate and binding modes

(A) Crystal structure of T4L-L99A depicting representative residue pairs within the C terminal domain (red) labeled to probe the tertiary fold and in the N terminal region (blue) monitoring helical secondary structure proximities at $i, i+4$ residues shown in equilibrium with a schematic representation of an unfolded form. (B) Model depicting structural aspects of the low and high affinity binding modes. In each case there is extensive substrate unfolding with loss of proximities in labeled pairs tracking both secondary and tertiary structure. Labeled substrates report a net orientation with C termini located in a more conformationally restrictive, solvent inaccessible environment and N termini experiencing greater conformational mobility and solvent accessibility. The hydrodynamic radius of the high affinity binding mode is comparable

to chaperone in absence of substrate and this radius increases in conditions favoring low affinity binding.

Figure 6. Hsp dissociation promotes activation and substrate binding

(A) Size exclusion chromatography elution profiles of wild type Hsp27 and a phosphorylation mimic in which the three serines phosphorylated by MAPK2 are replaced by aspartic acids to resemble this triply phosphorylated form (S15D/S78D/S82D). This phosphomimic shifts the oligomerization equilibrium in a manner favoring the complete disassembly of higher order oligomers observed in WT Hsp27 to smaller species. (B) The equation represents the dissociation of Hsp27 from a large oligomer (L) to a multimer (M) where p is an integer that accounts for the difference in the number of subunits between the two oligomeric states. Below, this equilibrium is depicted graphically in colors corresponding to the predominant SEC peaks in (A) as well as the binding isotherms in (C). (C) Binding is detected as quenching of a

fluorescently labeled T4 lysozyme substrate. Phosphorylation induced dissociation of Hsp27-D3 drastically increases the affinity of Hsp27 for substrate compared to WT Hsp27. Combined with panel (A), this data reinforces a model of chaperone activation and regulation through phosphorylation induced changes in oligomeric state.

$$N \rightleftharpoons I_1 \rightleftharpoons \cdots \rightleftharpoons I_i \rightleftharpoons U \quad [1]$$

$$sHSP \rightleftharpoons (sHSP)_a \quad [2]$$

$$(sHSP)_a + \begin{pmatrix} I_1 \\ U \end{pmatrix} \rightleftharpoons \begin{pmatrix} C_1 \\ C_u \end{pmatrix} \quad [3]$$

Scheme 1.
Coupled Equilibria Describing Minimalist Model