

Small Heat-Shock Proteins: Paramedics of the Cell

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Abstract The small heat-shock proteins (sHSPs) comprise a family of molecular chaperones which are widespread but poorly understood. Despite considerable effort, comparatively few high-resolution structures have been determined for the sHSPs, a likely consequence of their tendency to populate ensembles of inter-converting conformational and oligomeric states at equilibrium. This dynamic structure appears to underpin the sHSPs' ability to bind and sequester target proteins rapidly, and renders them the first line of defence against protein aggregation during disease and cellular stress. Here we describe recent studies on the sHSPs, with a particular focus on those which have provided insight into the structure and dynamics of these proteins. The combined literature reveals a picture of a remarkable family of molecular chaperones whose thermodynamic and kinetic properties are exquisitely balanced to allow functional regulation by subtle changes in cellular conditions.

Keywords α -Crystallin, Molecular chaperone, Polydispersity, Protein dynamics, Small heat-shock protein (sHSP)

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Abbreviations

<i>Af</i>	<i>Archaeoglobus fulgidus</i>
<i>At</i>	<i>Arabidopsis thaliana</i>
<i>Bt</i>	<i>Bos taurus</i> (cow)
<i>Dr</i>	<i>Danio rerio</i> (zebrafish)
<i>E. coli</i>	<i>Escherichia coli</i>
EM	Electron microscopy
EPR	Electron paramagnetic resonance spectroscopy
<i>Hs</i>	<i>Homo sapiens</i> (human)
IM	Ion mobility
<i>Mj</i>	<i>Methanocaldococcus jannaschii</i>
MS	Mass spectrometry
<i>Mt</i>	<i>Mycobacterium tuberculosis</i>
nES	Nanoelectrospray
NMR	Nuclear magnetic resonance spectroscopy
<i>Ps</i>	<i>Pisum sativum</i> (pea)
<i>Rn</i>	<i>Rattus norvegicus</i> (brown rat)
<i>Sc</i>	<i>Saccharomyces cerevisiae</i> (bakers' yeast)
sHSP	Small heat shock protein
<i>Ta</i>	<i>Triticum aestivum</i> (wheat)
<i>Tsp</i>	<i>Taenia saginata</i> (beef tapeworm)
<i>Xa</i>	<i>Xanthomonas axopondis</i>

1 Introduction

The small heat-shock proteins (sHSPs) are a family of almost ubiquitous stress proteins [1]. Most organisms encode multiple sHSP genes, an observation particularly clear in the case of higher organisms [2], with Californian Poplar having as many as 36 [3]. In humans the sHSPs number 10 [4] and are implicated in a range of cellular processes including modulation of cytoskeletal dynamics, stabilisation of membranes and apoptosis [5]. These diverse cellular roles appear to be linked by the ability of the majority of the sHSPs to interact with non-native states of proteins [6, 7]. This property is fundamental to their general behaviour as “molecular chaperones” [8], acting to prevent improper polypeptide associations and aggregation [9].

Molecular chaperones play a vital role in protein homeostasis [10], the mechanism through which the cell maintains proper function by balancing the influence of a multitude of biochemical pathways [11]. It has recently become apparent that the native state of proteins is in general less thermodynamically favoured than the amyloid aggregates they can form [12], revealing an underlying metastability of the proteome [13]. Consequently, the breakdown of “proteostasis” can lead to a variety of diseases [14], many of which are characterised by the aggregation and deposition of misfolded proteins [15]. sHSPs represent a central node in the proteostasis “network” [11], and in the main are dramatically up-regulated under conditions of cellular stress to being among the most abundant of all proteins [16, 17]. Furthermore, they are often found associated with protein aggregates obtained post mortem from victims of protein-misfolding disorders [18]. The sHSPs’ chaperone function is therefore crucial to the cell’s tolerance to stress, and their malfunction is implicated in a range of human pathologies [19–21]. Together these observations suggest that the sHSPs are on the front line of defence against the deleterious consequences of protein unfolding.

Despite their obvious importance, the sHSPs remain relatively poorly characterised on the molecular level. This is largely due to their tendency to populate a range of dynamic oligomeric states at equilibrium, rendering them refractory to many structural biology approaches [22]. As a consequence, high-resolution information exists only for very few members of the family [9]. Recent years have, however, seen considerable developments in the techniques available to structural biologists, and the means to combine data from multiple sources into “hybrid” approaches [23, 24]. Concomitantly there have been significant recent advances in our understanding of the sHSPs. Here we describe the current knowledge of the structure and dynamics of these remarkable molecular chaperones and their interaction with target proteins.

2 The Dynamic Architecture of sHSPs

Proteins are inherently highly dynamic entities [25–27], and an appreciation of how their different structural forms interconvert is necessary to understand how they carry out their cellular roles [24]. These fluctuations can span picoseconds to days,

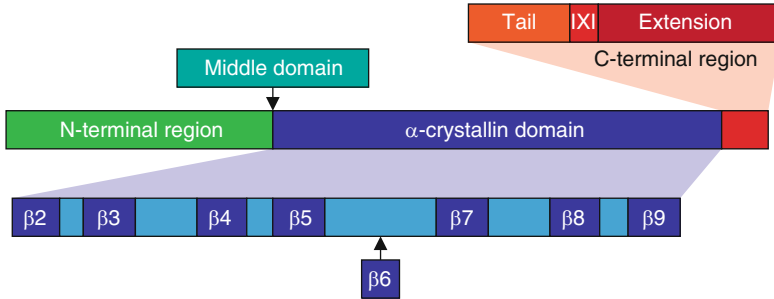


Fig. 1 Domain architecture of the sHSPs. The defining element and most highly conserved region of the sHSP sequence is the α -crystallin domain, which is flanked by the variable N- and C-terminal regions. The α -crystallin domain is composed of seven or eight β -strands, for metazoans or non-metazoans, respectively [37]. In the latter, the sequence between $\beta 5$ and $\beta 7$ contains an additional, and distinct, $\beta 6$ strand. In the metazoans this loop region is shortened, and instead the $\beta 7$ strand is elongated, into a “ $\beta 6 + 7$ ” strand. This leads to two alternative modes of dimerisation for the sHSPs (see Fig. 2). The C-terminal region is split into two parts, referred to here as the “tail” and “extension”, separated by an IXI motif. The N-terminal region by contrast has no obvious subdivisions. In HSP26, however, limited proteolysis has revealed a “middle domain” which is inserted between the N-terminal region and α -crystallin domain [35]

have diverse amplitudes and span all levels of protein organisation [28]. Furthermore, the emerging consensus is that sparsely populated “excited” states are frequently responsible for the molecular recognition events underpinning biological function [29–33]. The sHSPs represent a particularly intriguing illustration of this dynamical paradigm: although they share common features, these chaperones undergo intrinsic motions and conformational rearrangements on a wide range of both spatial and temporal scales.

2.1 sHSP Primary Structure

The sHSP family is characterised by the presence of an “ α -crystallin” domain [34], derived from the eponymous mammalian sHSP. This central domain is flanked by N- and C-terminal regions (Fig. 1). As perhaps to be expected for a family as large as the sHSPs, exceptions to this basic subdivision exist, including the presence of a “middle domain” [35] or multiple α -crystallin domains [36]. A comprehensive bioinformatics analysis of more than 8,700 sHSP sequences has revealed sHSPs to be composed of on average 161 amino acids [2], corresponding to approximately 17.9 kDa. With an average length of 94 residues, the α -crystallin domain typically composes the bulk of the sequence (approximately 58%). The N-terminus has an average length of 56 residues (35%), whereas at 10 residues (6%) the C-terminus is much shorter.

As the defining element of the sHSP family, the α -crystallin domain is the most conserved region of the sHSP sequence. Interestingly, genomic data has revealed there to be an under-representation of aromatic residues, and an over-representation

of charged amino acids in this domain [2]. Additionally there are notable positions of particularly high conservation, for example the “disease arginine” (at position 120 in α B-crystallin) [37], mutation at which results in a variety of pathologies [38].

The C-terminal region is generally considered to be of two segments, termed here as the “tail” and “extension” [39], which are separated by a highly conserved IXI motif (Fig. 1). The extension appears to be present primarily in higher eukaryotes [39]. In some members of the sHSP family, e.g. human HSP20 [40] and *Taenia saginata* TSP36 [36], the entire C-terminal region is absent. The N-terminal region is however essentially omnipresent and, in the main, considerably longer. It displays almost no sequence conservation, and is responsible for the majority of the sequence variation between sHSPs in the same organism [2]. Additionally, sites available for post-translational modification appear to be found largely in this part of the protein [41]. It is quite possible that this variability of the N-terminus may have a role to play in ensuring that a cell’s cohort of sHSPs can recognise a wide range of target proteins.

2.2 The Protomeric α -Crystallin Domain Dimer

High-resolution structures have been very hard to come by for the sHSPs, and the vast majority stem from isolated α -crystallin domains, truncated of the terminal regions. All of these structures, however, reveal a common basic fold of the α -crystallin domain, namely an immunoglobulin-like β -sandwich comprising up to nine β -strands (Fig. 2). The different structures align very well, and are replicated in the two structures solved for full-length, oligomeric sHSPs [42, 43]. However a significant difference can be seen between the structures from animals relative to other organisms (Fig. 2). In the structures determined for plant [43], archaeal [42, 44], and bacterial [45] sHSPs dimerisation occurs via reciprocal donation of the β 6 strand, located in a loop, into the β -sandwich of a neighbouring monomer. By contrast, in the mammalian sHSPs the β 6 strand has fused with β 7 [46–48] into an elongated “ β 6 + 7” strand which had previously been suggested by spectroscopic experiments, and predicted to enable dimerisation [49–51].

Interestingly, SAXS data has indicated that this dimeric interface observed in truncated forms of the mammalian sHSPs has significant flexibility [52]. Further insight comes from X-ray crystallography which has found three distinct alternative registers formed by the paired β 6 + 7 strands, causing a translation in the dimer interface, two residues at a time, spanning approximately 15 Å [46, 48, 53] (Fig. 2). These polymorphic states are termed, in order of decreasing overlap between anti-parallel β 6 + 7 strands, AP_I, AP_{II} and AP_{III} [48]. Solid-state nuclear magnetic resonance spectroscopy (NMR) revealed that dimerisation of α B-crystallin mediated by β 6 + 7 pertains also to full-length protein, but to date only one register, AP_{II}, has been observed [54]. While relating hydrogen/deuterium exchange rates determined for full-length α B-crystallin [55] to the structure of the truncated dimer certainly reveals the interfaces to be dynamic, it remains to be elucidated to what

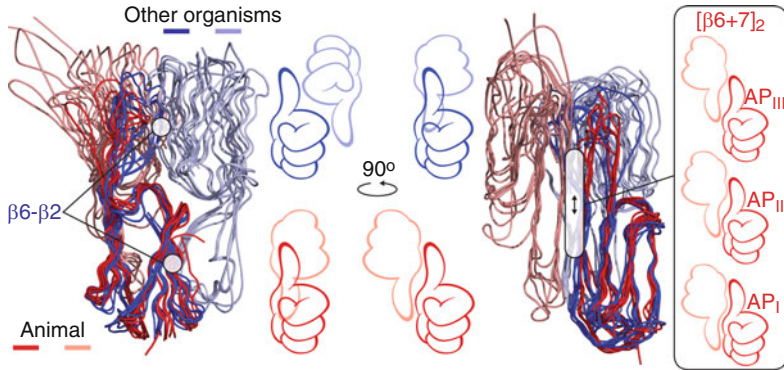


Fig. 2 Distinct dimeric α -crystallin cores. The overall fold of the α -crystallin domain is an immunoglobulin-like β -sandwich with a protruding loop, reminiscent of a “thumbs-up” hand gesture. The structures are highly conserved amongst sHSPs, and accordingly monomers from animals (red) and other organisms (blue) align very well. The corresponding dimeric partners (light red and light blue, respectively), however, are found in distinctly different locations, rotated $\approx 180^\circ$ relative to each other. This is as a result of the non-metazoan proteins dimerising through reciprocal interaction between $\beta 6$ and $\beta 2$ strands; whereas the metazoan proteins dimerise through their extended $\beta 6 + 7$ strands. The latter dimerisation form has been observed in three distinct registers, termed AP_I, AP_{II} and AP_{III}

extent multiple AP interfaces are populated in the oligomers at equilibrium in solution, and whether they interconvert. However, irrespective of these registry shifts, it is clear that, despite very similar basic monomer structures, two distinctly different modes of dimerisation have evolved across the kingdoms of life.

2.3 Heterogeneous N-Termini; Dynamic C-Termini

In contrast to the recent wealth of structural insight into the α -crystallin domain, equivalent information about the termini remains relatively limited. In the crystal structure of *Methanocaldococcus jannaschii* HSP16.5 none of the N-termini are resolved [42]; however EM data revealed additional density within the central cavity of the oligomer [56]. In HSP14.0 from *Sulfolobus tokodaii* two crystal forms were obtained, with the N-termini resolved in one but not the other [44]. Similarly, in the crystal structure of the *Triticum aestivum* HSP16.9 oligomer, half of the N-termini are unresolved; the remainder are structured and found in the centre of the oligomer [57]. Atomic models generated for the N-termini of HSP16.5 [58] and α B-crystallin [59, 60] using sparse spectroscopic restraints, and the N-termini resolved in the crystal structures of HSP16.9 [43], TSP36 [61] and HSP14.0 [44], reveal a propensity to form helical secondary structure.

In apparent contradiction with these results, in hydrogen/deuterium exchange studies of two plant sHSPs, HSP16.9 and *Pisum sativum* HSP18.1, rapid

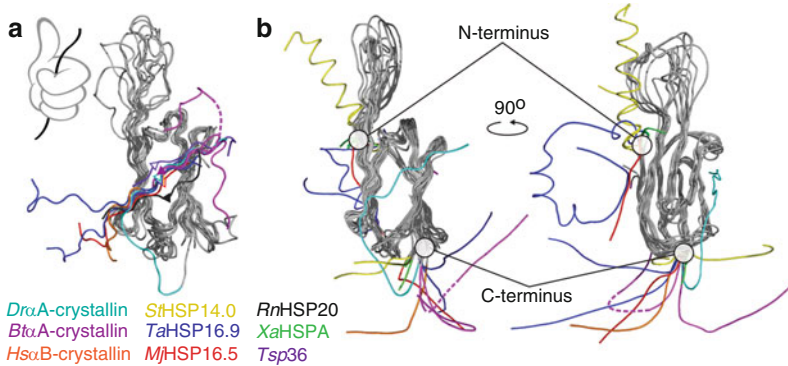


Fig. 3 Variability in the termini. C-termini make inter-dimer contacts, binding over the groove between $\beta 4$ and $\beta 8$ strands; by analogy with Fig. 2, the α -crystallin core “hand” grasps the C-terminal “string” from an adjacent monomer (a). This interaction has been observed in all crystal structures in which the C-terminal IXI motif is resolved. In other cases (*Tsp36*, *RnHSP20*) the groove is instead occupied by residues from the N-terminus. Notably both directions of binding have been observed (direction indicated by *triangles* placed in the location of the X in the IXI, *solid arrows* bind *top-right* to *bottom left*). Note that this interaction can even be intra-molecular, as observed in the structure of *DrαA*-crystallin (*cyan*). The angle which the C-terminus makes from the α -crystallin domain is very variable (b). Illustrated are all C-termini resolved in sHSP crystal structures, and it is notable that this variability in angle is found even for the same protein, either in the same oligomer (*TaHSP16.9*) or in different crystal forms (*StHSP14.0*, *BtαA*-crystallin). N-terminal residues are observed in fewer structures, but also reveal heterogeneity. This ability of the terminal regions to adopt different orientations is likely crucial for the sHSPs’ ability to populate multiple oligomeric states

exchange of the N-terminal backbone amides was observed [62, 63]. Even those positions involved in inter-dimer contacts approached complete exchange with 100 s, and only a single exchanging population was observed [63]. This reveals that all 12 of the N-termini in these dodecameric proteins are essentially equivalent, but does not rule out that at any given moment a sub-population thereof may be structured and form relatively transient interactions. Taking these results in combination with the evidence from the crystal structures suggests that the N-termini populate multiple slowly inter-converting conformations in the centre of the oligomer, perhaps helping to maintain the integrity of the assemblies [64]. Such intrinsic heterogeneity could conceivably be important in recognising and binding variable target proteins by presenting diverse geometries for interaction [65].

In the structures of HSP16.9 and HSP16.5 the C-terminal tails were revealed to span between dimers, such that the IXI motif binds into a groove between strands $\beta 4$ and $\beta 8$ (Fig. 3a). Similar “cross-linking” interactions mediated by the C-termini were observed both in X-ray structures of α -crystallin constructs lacking the C-terminal extension [48] and solid-state NMR data on the full-length protein [54], both obtained at temperatures below freezing. Interestingly, however, for the α -crystallins at physiological temperature in solution it appears that the IXI motif is actually predominantly detached from the oligomer [66–68]. This apparent

contradiction can be rationalised by the strong temperature dependence of fluctuations of the tail [66], and points to a careful thermodynamic regulation of the IXI binding [69]. Unlike the tail, NMR studies have revealed that the C-terminal extension, for those sHSPs in which it is present, is intrinsically disordered and tumbles freely in solution [70, 71]. This region of sequence is primarily hydrophilic and is thought to facilitate the detachment of the remainder of the C-terminus [72]. Considering that α -crystallins with truncated extensions are associated with cataract [73], a picture emerges in which the dynamics of the C-terminus are crucial to chaperone function, potentially through regulating access to the β 4/ β 8 groove in a form of “auto-inhibitory” regulation [43, 54].

2.4 Oligomeric Assembly Is Mediated by Flexible Terminal Interactions

While the sHSPs are prefixed by “small” due to their low monomeric molecular mass, relative to the other heat-shock proteins (HSPs), this is something of a misnomer. The sHSPs are typically oligomeric, with the majority studied so far comprising 12 or more subunits and having masses in excess of 200 kDa [9], making them among the largest of the HSPs. Though only two high-resolution structures (for HSP16.5 and HSP16.9) exist for such oligomers, in both cases they are stabilised by inter-dimer connections formed by the terminal regions of the protein as well as specific interactions between the α -crystallin core building blocks [42, 57].

In these structures the C-termini decorate the surface of the oligomer, holding it together by bridging between the α -crystallin domains of adjacent dimers [42, 57] (Fig. 3a). It is notable that the angle made between the domain and the tail is variable, even within the same oligomer [57] (Fig. 3b). Such flexibility in the tail is mediated by a “hinge-loop” just C-terminal to the core domain [48, 74] and, it is tempting to speculate, explains how the sHSPs can be found in a range of oligomeric forms [75]. Furthermore, it is notable that for the α -crystallins the area of sequence around the IXI is palindromic [46, 48], which may allow an additional degree of variability in assembly. The versatility of the C-terminus is reminiscent of that in the coat protein VP1 whose conformational flexibility mediates its variable assembly in simian virus 40 [76].

In the case of TSP36, which lacks a C-terminus, the β 4/8 groove in the α -crystallin domain is instead occupied by N-terminal residues [61]. The presence of IXI motifs in both the C-terminus and extreme N-terminus of a number of sHSPs, such as the α -crystallins, raises the possibility that there may be some extent of inter-changeability between the two termini. Alternatively, the structure of HSP16.9 demonstrated the ability of the N-termini to extend across the central cavity of the oligomer, intertwined in a pairwise manner [57]. Considering HSP16.9 is a monodisperse dodecamer, this may amount to a specific interaction acting to lock the protein into a single oligomeric stoichiometry, reminiscent of the role played by the protein P30 in the bacteriophage PRD1 [77].

Conceivably, as the N-termini in the main appear heterogeneous, it is possible that they interact with each other relatively non-specifically, driven together by hydrophobic interactions [64]. Constructs of the α -crystallins, truncated of the N-terminus but retaining the C-terminus, were only able to form oligomers to very low abundance relative to sub-oligomeric species [48]. However, in the case of HSP16.5, the protein was still observed as an oligomer after removal of the N-terminus [78], but was completely disassembled after further removal of the C-terminus [79]. It appears therefore that the N-termini are not necessarily required for oligomerisation, but contribute to the thermodynamic stability of the resultant assemblies. While the importance of the N-terminus therefore appears to vary between sHSPs, it clearly has a role to play in defining oligomerisation, the details of which warrant further investigation.

2.5 sHSPs Assemble into Multiple Polyhedral Topologies

Members of the sHSP family populate a continuum of oligomeric states, from monodisperse to extremely polydisperse [56]. Notably, plant sHSPs typically exist as single oligomers, generally dodecamers [80]. Conversely, many mammalian sHSPs co-populate a wide range of oligomeric states at equilibrium; for example the α -crystallins adopt all possible stoichiometries between approximately 10 and 50 subunits [81, 82]. Between these two extremes, sHSPs have been characterised that populate an intermediate number of oligomeric states, with certain amongst them preferred, such as for example *Saccharomyces cerevisiae* HSP26 [83] and Acr2 from *Mycobacterium tuberculosis* [84]. This tendency towards polydispersity has proven to be a major hindrance in the structural characterisation of sHSPs [22].

Nonetheless, the X-ray structures determined for sHSPs at conditions in which a single oligomeric state was populated provide considerable insight. HSP16.5 crystallised as a 24mer, in which the subunits are assembled into an octahedron, with a protomeric dimer comprising each edge (Fig. 4a) [42]. Remarkably, insertion of additional residues in the N-terminus resulted in an expanded symmetric oligomer, with 24 dimers assembled into a cuboctahedron [75] (Fig. 4a). The dodecamer of HSP16.9, by contrast, assembles into a “double-ring” topology, i.e. two triangular rings stacked on top of each other (Fig. 4a) [43]. Docking of a dimer into the electron microscopy (EM) reconstruction of Acr1 from *M. tuberculosis* reveals an alternative arrangement for dodecameric sHSPs, namely a tetrahedron (Fig. 4a) [84]. This striking oligomerisation into polyhedral geometries reveals the important observation that all the α -crystallin core dimers within the oligomers are essentially equivalent structural environments, connected via terminal interactions (Fig. 4b). It is plausible that this characteristic results in there being no great energetic difference for a dimer residing in a specific oligomeric stoichiometry, and therefore enables multiple oligomeric states to be populated at equilibrium [66]. As polyhedral arrangements result in dimers being arranged symmetrically while also

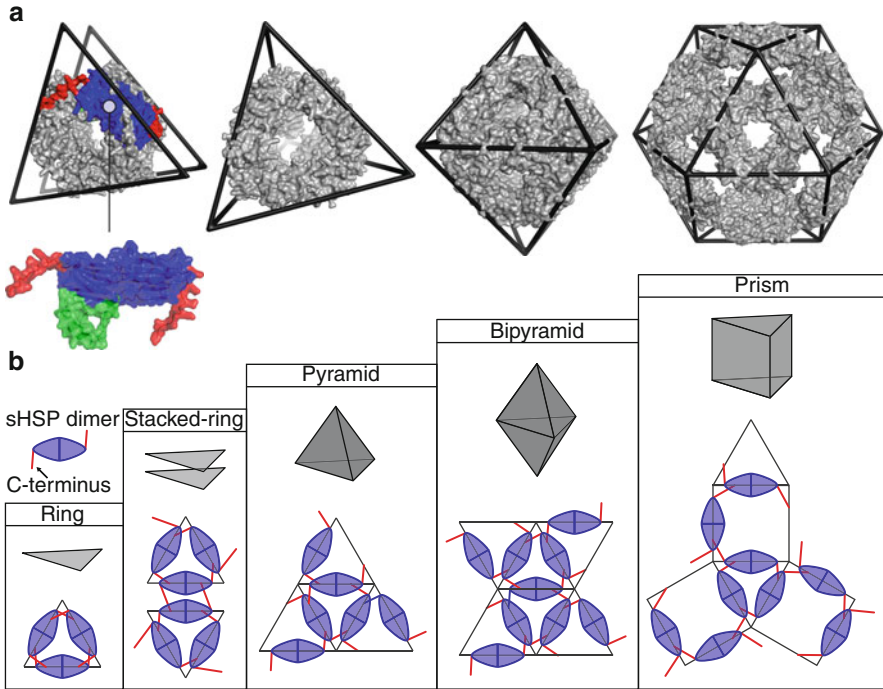


Fig. 4 Polyhedral architecture of the sHSPs. The structures, from *left to right*, of HSP16.9 (12mer), Acr1 (12mer), HSP16.5 (24mer), and a modified HSP16.5 (48mer) display striking polyhedral geometries (**a**). They assemble as a double ring, tetrahedron, octahedron and cuboctahedron, respectively (*in sequence, left to right*). In each case the α -crystallin domain dimers (*blue*) are collinear with the edges of the polyhedron, as illustrated for HSP16.9. The dimers are held together by extended C-termini (*red*, cf. Fig. 3), and the N-termini (*green*) are sequestered on the inside of the oligomers. This assembly of core dimers into polyhedra via C-termini can be illustrated schematically using nets of several classes of polyhedra (**b**). Nets are shown based on a triangular base-unit, but could easily be drawn for larger polygons in these classes (e.g. a square pyramid). Similar nets can be drawn for any given polyhedron such that all the C-termini are “satisfied”, binding dimers together at the vertices

satisfying similar terminal interactions, it has been proposed that polydisperse sHSPs share these scaffolds [85].

2.6 Hybrid Approaches to Determine the Structure of Polydisperse sHSPs

The inherent polydispersity and plasticity of sHSPs are a significant impediment to their structural characterisation. These characteristics are likely however to be important to their cellular function, for example in preventing the unwanted crystallisation of the α -crystallins despite their high concentration in the eye lens [86], but

accordingly render the determination of X-ray structures of these proteins extremely challenging [22]. This has in recent years led to the application of both novel and integrated structural biology approaches to the sHSPs. The size limit of traditional solution NMR approaches has been circumvented to provide insights into the α -crystallins, either by examining truncated forms [50] or regions of marked flexibility [70, 71]. Moreover, the large oligomeric species have even be examined directly by means of solid-state NMR [54, 59], or via selective labelling of amino acids in methyl transverse relaxation optimised spectroscopy (TROSY) solution NMR [66] and electron paramagnetic resonance (EPR) approaches [51, 87, 88].

These varied strategies all provide structural information, ensemble-averaged onto the monomer level. In order to translate these insights onto the quaternary structure, studies have combined NMR data with that obtained from EM [59, 85], small-angle X-ray scattering (SAXS) [54, 59] or ion-mobility spectrometry (IM) [85], all of which report on the oligomeric form. Particular challenges are posed in the cases of polydisperse sHSPs and techniques are required which can separate, and address individually, the constituent oligomeric states. Single-particle analysis of EM data provides the opportunity to generate three-dimensional reconstructions of particles after their sorting according to size [89, 90]; currently however, the highest resolution of separation for macromolecular assemblies is afforded by MS approaches [91]. In the case of α B-crystallin, the archetypal polydisperse sHSP, early EM analysis revealed a broad range of oligomeric sizes and masses, with apparently variable symmetries [92, 93]. MS enabled the identification and relative quantification of the underlying individual oligomeric states, revealing a broad distribution of stoichiometries centred around ~ 28 subunits [81, 82] (Fig. 5a).

Recently, an EM reconstruction for α B-crystallin was obtained by assuming that the principal oligomer states shared common symmetry elements [94]. This was combined with solid-state NMR [59] and cross-linking MS [60] data to generate model oligomers constructed from hexameric sub-complexes [59, 60]. An orthogonal approach, using IM-MS to discriminate between candidates based on a variable polyhedral architecture, and cross-validation with EM data, revealed alternative structures for this protein [85] (Fig. 5a). While these studies have reported plausible models for α B-crystallin, they differ in terms of symmetry and size distribution. While definitive structures therefore remain elusive, it is clear that the emergence of novel and hybrid approaches have provide new impetus to the structural study of this notorious target for structural biology.

2.7 sHSP Oligomers Can Transition Between Compact and Expanded Forms

Aside from the variability in quaternary structure afforded by polydispersity, it appears that sHSPs oligomers themselves can exist in multiple conformations (Fig. 5b). A cryoEM study of HSP26 revealed two distinct populations of 24mers, differing by approximately 5% in diameter [90, 95]. Additionally, subunit

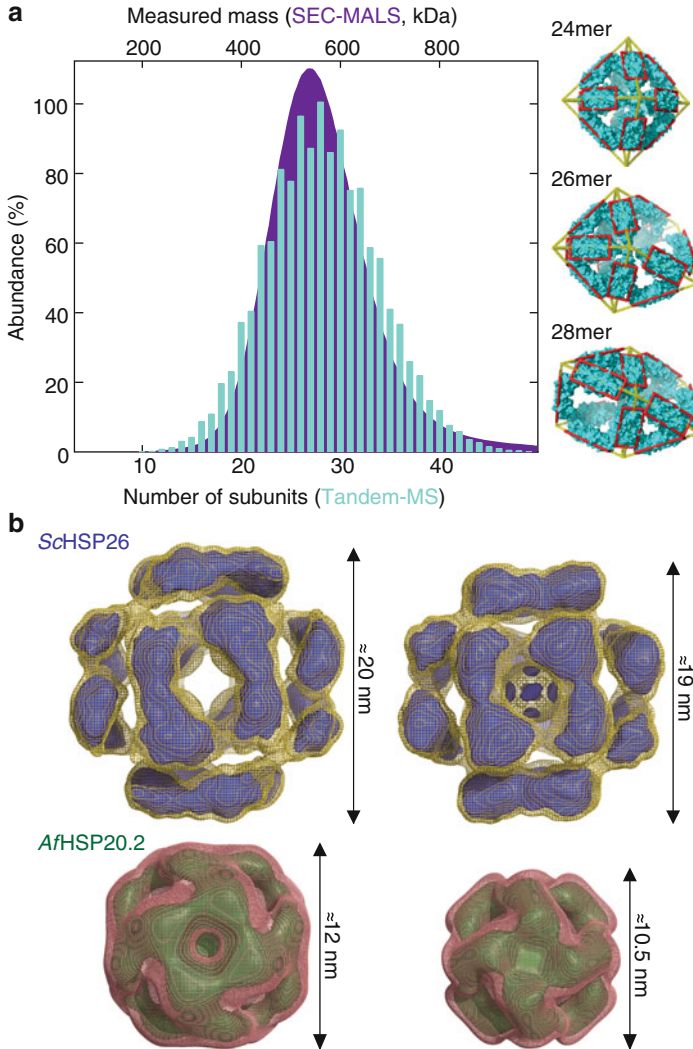


Fig. 5 Macro- and micro-heterogeneity of the sHSPs. Many sHSPs are polydisperse, populating a range of oligomeric states at equilibrium (a). The most famous example of this macro-heterogeneity is α B-crystallin, which forms oligomers spanning 200–1,000 kDa, as determined using size-exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) (*purple*) [91]. Mass spectrometry measurements have revealed the underlying distribution of oligomers (*cyan*) to be centred on 28 subunits, with those stoichiometries composed of an even number of subunits to be preferred [82]. Ion-mobility mass spectrometry measurements allowed the filtering of structural models, based on polyhedral scaffolds (Fig. 4), to reveal the likely architecture of the 24mer, 26mer and 28mer forms [85]. These structures are based on an octahedron, augmented triangular prism and gyrobifastigium, respectively (*right*). Aside from populating multiple stoichiometries, different conformations of individual sHSP oligomeric states have been observed (b). Electron microscopy investigations of both ScHSP26 and AfHSP20.2 suggested the possibility of sHSPs populating “expanded” and “compact” forms, differing in overall size but not topology. This micro-heterogeneity adds an additional level to the quaternary complexity of the sHSPs

exchange of HSP26 could only be quantitatively understood by invoking at least two separate dissociation rate constants, consistent with two types of oligomers [83]. Differences were noted between negative-stain EM reconstructions of *Arabidopsis thaliana* HSP21, in the presence or absence of cross-linker, hinting at the possibility of multiple conformations differing in terms of compactness [96]. A similar observation was also made in a negative-stain EM study of the octahedral HSP16.5 and *Archaeoglobus fulgidus* HSP20.2 24mers. For both proteins the relative proportions of the two forms varied as a function of temperature, demonstrating that the two states can interconvert [97]. Previous studies of HSP16.5 had reported some heterogeneity of the protein [56, 98, 99]. As such it appears that sHSPs not only display “macro-heterogeneity”, that is populating multiple oligomeric stoichiometries, but that these individual stoichiometries can adopt multiple quaternary structures in a form of “micro-heterogeneity”. Moreover, for HSP20.2 the larger form was predominant at temperatures both above and below the physiological temperature of the organism, suggesting a functional role for these quaternary conformational fluctuations.

The underlying structural origin of these expansions and contractions of the oligomers is unclear. There is evidence that they might stem from packing differences enforced by rearrangement of the inter-dimer contacts formed by the terminal regions [95]. For HSP26, they may be caused by its unique middle domain, which undergoes a conformational switch upon heat shock [100]. Alternatively, the α -crystallin domain dimeric building-block itself might fluctuate in length, for example due to changes in register at the interface [48, 53], which would propagate to have consequences on the overall size of the oligomer. Alternative registers have so far only been observed for mammalian sHSPs, which have an extremely labile dimer interface formed by the $\beta 6 + 7$ strand [50, 82]. However, considering that the interface of dimers formed via the $\beta 6$ loop are also easily broken [101], it is not inconceivable that a similar mechanism might also occur in sHSPs from lower organisms.

2.8 *sHSPs Co-Assemble into a Recycling Oligomeric Ensemble*

In addition to the complexity afforded by both macro- and micro-heterogeneity, it has long been known that members of the sHSP family co-assemble into hetero-oligomers *in vivo* [102]. Isolated sub-populations of oligomers re-equilibrate to the parent distribution [103] in a process mediated by the movement of individual subunits [104]. The combinations of sHSPs which are compatible for co-assembly are dependent on their evolutionary relationships [80]; however the subunit exchange of the individual oligomers appears to be a general property of these proteins [105–108]. As such, the sHSPs should not be considered as static homomeric proteins, but rather as a continually “recycling” ensemble of hetero-oligomers.

Subunit exchange occurs via the dissociation of the oligomer, with a rate strongly dependent on solution conditions [82, 105]. This reveals that the sHSPs are in a rapid equilibrium with a small population of sub-oligomeric forms. The

identity of the exchanging unit depends on the sHSP in question, with monomers [82], dimers [108] and mixtures thereof [101] all having been observed directly in high-resolution mass spectrometry experiments. This equilibrium between oligomers and smaller species is shifted towards dissociation at elevated temperatures, with appreciable concentrations of the latter observed under heat-shock conditions for some sHSPs [80, 83, 109], but not for others [80, 82]. Concomitant to this dissociation, high-order oligomers have also been observed, such that sHSPs which are monodisperse under ambient temperatures effectively become polydisperse at elevated temperature [110, 111]. These processes of assembly, dissociation and exchange are also affected by modifications to the sHSPs thought to regulate or compromise sHSP activity such as post-translational modification [72, 112, 113] or mutation [114–116]. Given these characteristics, it is tempting to speculate that such quaternary dynamics are important for chaperone function, presumably by exposing target-protein binding regions either on the sub-oligomeric species, or *en route* to dissociation [9, 57].

3 The Molecular Chaperone Function of sHSPs

As might be expected from their evolutionary diversity, sHSPs have been reported to be involved in a range of cellular processes. The role which is common to most members of the family is the ability to act as molecular chaperones [117, 118]. This function of the sHSPs was first demonstrated when it was found that α -crystallin could prevent the accumulation of aggregation-prone eye-lens proteins [119]. This *in vitro* observation was later confirmed *in vivo* when α A-crystallin knockout mice developed inclusion bodies rendering the eye lens opaque [120]. Furthermore, disruption of the α B-crystallin and the adjacent HSPB2 sHSP genes resulted in degeneration of some skeletal muscles [121]. Since the pioneering work on α -crystallin, many other sHSPs have been demonstrated to have molecular chaperone activity, and it is quite likely that the ability to interact with non-native states even underpins the mechanism of their other activities in the cell [6].

3.1 *High-Capacity Holdase Function of sHSPs Sequesters Destabilised Targets from Aggregation*

It has been established for some time that the molecular chaperone role of sHSPs is to bind target proteins whose native structure is destabilised by a range of stresses [119, 122–125]. Under such conditions, these proteins can have a tendency to form amorphous or amyloid aggregate morphologies [18]. Rather than refolding the targets, as is the case for the ATP-dependent “foldase” chaperones such as HSP60, HSP70 and HSP90 [126], the sHSPs instead act in an ATP-independent manner to trap them as they unfold [119, 122–125]. The resultant complexes formed between

the sHSP and target proteins can range in mass up to several MDa [111, 127, 128]. The binding capacities of the sHSPs vary between different members of the family, but can be very high, with the chaperones capable of protecting stoichiometric quantities of target [128, 129]. The capacity appears to be somewhat dependent on the identity of the target protein [127, 129], perhaps purely reflecting the mass of the target [7, 130]. As such, the sHSPs can be viewed as high-capacity “sponges” for non-native proteins, preventing the deleterious consequences of their aggregation [131].

The majority of assays for studying sHSP activity rely on assessing the ability of the chaperone to suppress the aggregation of model proteins, due to the difficulties in purifying inherently unstable targets [132]. It is not uncommon that the apparent efficiency of protection is dependent on the choice of model protein [133]. As such these *in vitro* assays might not be expected to capture all aspects of *in vivo* function [134]. Nevertheless, the capacity of sHSPs to interact with a range of destabilised model proteins renders it likely that they have multiple targets in the cell [9, 57]. Determining the characteristics of actual cellular substrates has however been hampered by the absence of easily assayed phenotypes associated with sHSP deletions in yeast or *Escherichia coli* [135, 136]. However, a study using *Synechocystis* sp. PCC 6803, in which only a single sHSP is encoded and the deletion of which results in lack of thermo-tolerance [137], identified interactions with numerous proteins [132]. These interactors displayed no commonality in sequence or structure, and spanned functions ranging from transcription, translation, to cell signalling, and secondary metabolism [132]. A similarly heterogeneous set of targets was also observed in yeast, corroborating the apparent broad specificity of sHSPs [138]. The general chaperone function of the sHSPs therefore appears to be to act as “holdases”, sequestering target proteins and thereby impeding the deleterious consequences of their aggregation [131].

3.2 sHSP Activity Is Influenced by Environmental Conditions

Multiple different stresses have been reported to stimulate the activity of sHSPs. Primary to these is heat shock, with sHSPs generally thought to be more effective chaperones at elevated temperature. For example, HSP26 has been demonstrated to undergo significant structural and dynamical changes around 40°C, consistent with a thermal activation of the protein [83, 100, 109]. While HSP26 is unusual in containing a middle domain, HSP18.1 undergoes a similar dynamic transition in oligomerisation from an inactive “storage form” into a functional chaperone with temperature [111]. In fact chaperone functions at temperatures far in excess of those normally termed “heat shock” have been reported [139]. On the other hand, some sHSPs have been shown to retain chaperone activity below heat shock temperatures [140], demonstrating that thermal activation is not a universal requirement for sHSP function.

Solution pH is also known to affect the molecular properties of the sHSPs. Studies of α B-crystallin have revealed dramatic changes in the thermodynamics and kinetics of the inter-subunit interfaces [50, 54, 66, 82]. This is reflected in pH-dependent changes in chaperone function *in vitro* [141–143], and a role for α B-crystallin in responding to cellular acidification [144–146]. sHSPs have also been demonstrated to provide protection against toxicity from metal ions *in vivo* [147]. The α -crystallins have been shown to bind metal ions directly [48], potentially silencing any tendency to oxidise [148, 149], and resulting in modulation of their chaperone function [150, 151]. This behaviour is interesting in light of the role of the redoxins, which switch from their enzymatic function to become molecular chaperones upon oxidative stress [152, 153].

While it has been generally accepted that the function of sHSPs is as ATP-independent molecular chaperones, there have been reports suggesting that the sHSPs can bind nucleotides [154, 155]. This finding is supported by the observation of sulphate ions accumulated at the dimer interface in recent crystal structures [48, 156]. Mapping changes in residue-specific protease susceptibility of α B-crystallin upon the addition of nucleotide [157] on the structure of the core domain suggest that the location of the sulphate may represent ATP-binding sites [53]. This is in line with the notion that nucleotide binding (rather than hydrolysis) might regulate activity [154], a mode of action that contrasts with the canonical chaperones, in which the turnover of ATP drives their action [158].

Members of the sHSPs can become post-translationally modified upon stress, with phosphorylation in particular implicated in affecting their function [159–161]. Accordingly, profound effects of phosphorylation on the chaperone activity have been observed *in vitro* [112, 162–164]. However, the identity of the target protein and solution conditions both strongly influence whether the post-translational modification leads to an increase or decrease in chaperone efficacy [133]. As such, while the evidence clearly points to phosphorylation regulating the function of sHSPs, a simple description of the mechanism appears unlikely [165]. Overall, from the differences in activity observed for the sHSPs as a result of multiple and varied stimuli, a picture emerges of a family of molecular chaperones which are exquisitely and directly controlled by the insult responsible for a particular stress condition.

3.3 sHSPs Co-Operate with the Cellular Machinery to Allow Reactivation or Degradation of Targets

While the sHSPs are active under stress conditions and act to bind non-native target proteins, they do not themselves appear to enable their refolding. Instead the target protein is subsequently retrieved from the sHSP:target complex and refolded upon interaction with ATP-dependent chaperones [166–169]. This renaturation pathway involves the HSP70/HSP40 system (DnaK/J in prokaryotes), a nucleotide exchange factor, and HSP100 working in concert with the sHSPs to liberate and refold unfolded substrate proteins [170].

While it has been demonstrated that the sHSPs facilitate the disaggregation of insoluble protein deposits [135, 170–174], the mechanism by which this is achieved is presently unclear. Recent studies suggest that different sHSPs may play varying roles in the resolubilisation process [175]. It is likely that they act to compete kinetically for the inter-molecular interactions that would otherwise ultimately lead to the formation of stable aggregates, instead holding proteins in a conformation more amenable to subsequent refolding [170].

While the link between the sHSPs and ATP-dependent foldases is clear, recent evidence also points to their interaction with the protein degradation machinery [176]. A number of studies have linked sHSPs with the proteasome/ubiquitin pathway [177–180], and the *E. coli* sHSPs IbpA and IbpB have been shown to be substrates of the Lon protease [181]. Though this field warrants considerably more attention, the involvement of sHSPs in both the refolding and degradation pathways reveals them as crucial switching points in determining the fate of unfolded proteins [182].

3.4 sHSPs Possess Multiple Sites that Become Exposed to Bind Targets

Considerable effort has been expended in an attempt to elucidate the region of the sHSPs which interact directly with the target proteins. Different studies have implicated the N-terminal region [65, 134, 183, 184], the C-terminus [185, 186] and the α -crystallin core [149, 187, 188]. Indeed, isolated α -crystallin domains from some sHSPs have been shown to have a certain amount of molecular chaperone activity [48, 52]. Taking these results together implies that there is no single binding site within the oligomer, but rather that these are dependent on the sHSP or target protein in question [6] (Fig. 6).

A commonality observed in the majority of putative interacting regions which have been elucidated is that they are hydrophobic in nature [6]. This is unsurprising, considering that it is the exposure of complementary hydrophobic surfaces on target proteins which renders them aggregation prone. It has been shown that the number of accessible hydrophobic sites on the sHSP increase upon heat shock [80, 167, 189], prompting the question whether this is a consequence of structural rearrangements of the sHSP oligomer.

A popular hypothesis, based on cumulative evidence from studies of several different members of the family which demonstrated oligomeric dissociation at heat-shock temperatures, is that a sub-oligomeric species form of the sHSP initially binds the target [109, 137, 190]. This mechanism does not appear to be universal, however, as a number of examples have emerged showing that dissociation, or its corollary, the rate of subunit exchange, is not necessarily correlated with chaperone activity [72, 80, 82, 191, 192]. In this regard it is informative to consider the case of HSP26. An early study of this protein demonstrated it to undergo dissociation into sub-oligomeric species at heat shock temperatures, suggesting this event to predicate chaperone activity [109]. However, subsequent studies revealed that chaperone

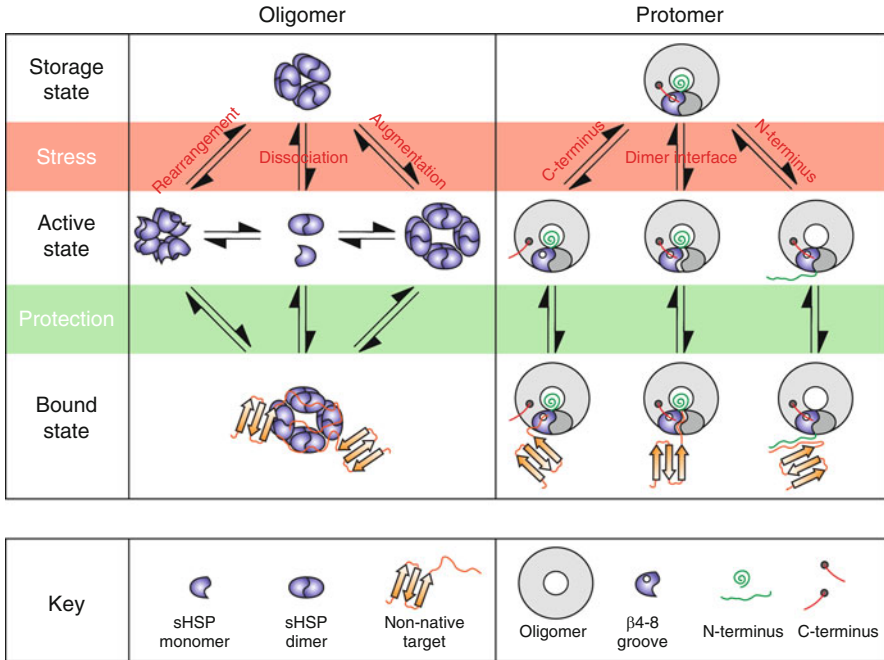


Fig. 6 Molecular basis for activation of binding of target by the sHSPs. A range of structural and dynamical changes in the sHSPs have been observed which have been ascribed to switching the chaperone into an “active” state. These are separated here according to effects on the oligomer (*left*) or protomer (*right*) level. The former include dissociation of the oligomer; a conformational rearrangement of a domain within the oligomers; or a change in oligomeric distribution. At present no universal pathway has emerged, and activation is likely to be sHSP dependent. Ultimately, and common to all sHSPs studied, large and heterogeneous sHSP:target complexes are formed. On the protomer level evidence for the exposure of the β 4– β 8 groove by detachment of the C-terminus, exposure of the dimer interface and unfurling the N-terminus has been proposed. The diversity in mechanism is likely to reflect the evolutionary diversity of both the sHSPs and their targets

activity was unaffected if the oligomer was cross-linked such that it could not dissociate [191]. Instead, a thermally regulated conformational change in the middle domain [100], which in the unrestrained protein is concomitant to changes in oligomerisation [83], has been implicated as underlying activation of this sHSP. This example illustrates that even if a protein undergoes dissociation into sub-oligomeric species at heat-shock temperatures, this does not necessarily imply that the sub-oligomeric species is the active target-binding form.

An alternative model is motivated by a recent cross-linking study between HSP18.1 and malate dehydrogenase which suggested that the N-terminal region was primarily responsible for binding [65]. However, examination of the structure of its homologue HSP16.9 reveals at least some of the N-termini to be sequestered within the centre of the oligomer [43], a location structurally incompatible with the high binding capacity of the chaperone. It is possible therefore that the N-terminal

arm unfurls from the central cavity of the protein to become exposed at heat-shock temperatures in an intrinsically disordered state which can present diverse geometries of interaction sites for binding [65]. One can even envisage a situation whereby the N-terminus acts to modulate the surface of the oligomer, similar to the way in which the specificity of protein phosphatase 1 is governed by the binding of its unstructured regulator proteins such as spinophilin [193]. Such a release of the N-termini would necessitate the loss of the directional inter-dimer constraints they form in the crystal structure of HSP16.9. Circumstantial evidence for this mechanism therefore comes from that fact that at heat-shock temperatures the oligomeric form of this protein is no longer confined to a dodecamer [110].

This change in protein partitioning, with most of the protein being re-allocated from a monodisperse oligomer at ambient temperatures into a polydisperse ensemble, arises purely from thermal motions that transfer subunits between oligomers of varying relative stability. This is particularly pronounced for HSP18.1 [111] where, interestingly, the resultant distribution of higher oligomers that was observed in this study was very similar to that populated by the α -crystallins at ambient temperatures [82, 162], conditions under which they remain chaperone-active. This raises the possibility that a polydisperse ensemble of oligomers may not only be a direct consequence of sHSP activation, but may even itself be of direct functional benefit.

In combination these studies suggest that there are not only multiple binding sites on the oligomer but also different mechanisms for their exposure to target proteins (Fig. 6). A common thread appears to be that the native oligomer represents a chaperone-inactive “storage form”, which undergoes a transition to a chaperone-active form. Such a change may exist purely to prevent unwanted, retarding associations with non-native states during non-stress conditions. Alternatively it may signal a switch between different cellular functions, analogous to redoxins which have been observed to become chaperone active upon a stress-induced change in the oligomeric state [152, 153]. Overall, however, it is clear that there are considerable mechanistic variations in the chaperone function of different sHSPs, emphasising the broad specificity of these chaperones in protecting the proteome.

3.5 sHSP:Target Complexes are Plastic and Polydisperse

The complexes formed when sHSPs are incubated with target proteins are very large and extremely heterogeneous [127, 128]. This is in stark contrast to the defined stoichiometries of interaction observed for foldase chaperones and their targets [158]. Such heterogeneity severely hampers structural interrogations, but can be overcome using the high resolution of separation afforded by MS approaches [91]. Employing a tandem-MS strategy [81], the different complexes formed between HSP18.1 and luciferase were identified and quantified [111]. Despite these experiments being performed with an excess of chaperone, remarkably over 300 stoichiometries of interaction were observed, variable in both the number of

sHSP and target subunits [111]. In light of the observation that sHSP:target complexes scale with the amount of target protein [127], a glimpse of the bewildering polydispersity of these complexes is obtained.

The complexes are not static entities; indeed they can continue to incorporate ever-increasing amounts of substrate [111, 127, 128]. Moreover, the sHSP subunits continue to exchange with free sHSP oligomers and other complexes [127]. By contrast, target proteins appear unable to transfer from one complex to another [127]. Hydrogen/deuterium exchange experiments revealed no difference in backbone amide protection between the sHSP free in solution or in complex [62], and α -lactalbumin associated with α -crystallin is still visible in proton NMR spectra, implying that it must spend a significant proportion of its time tumbling independently in solution [194]. These data therefore reveal that the interaction between sHSP and target protein is, at least in some cases, only transient, presumably to facilitate subsequent transfer to the refolding machinery.

In addition to these inherent dynamics and variability, it also appears that sHSP:target complexes can adopt different morphologies [128, 130]. Interestingly it appears that this is target-protein dependent: the same sHSPs formed different morphologies with different targets [128, 130] but complexes of different sHSPs and the same target appear similar [128]. This can be rationalised by the observation that the sHSPs bind target proteins early during the unfolding process, when their structure is largely preserved [62, 130]. In summary, the complexes formed between sHSPs and targets are extremely polydisperse and dynamic, which renders their structural characterisation very challenging, but is likely integral to their cellular function.

4 Paramedics Within the Proteostasis Network

Molecular chaperones are crucial for the maintenance of cellular protein homeostasis [10]. sHSPs are an important part of this network, being dramatically upregulated and activated during cellular stress, and sequestering destabilised targets from aggregation. In this way, sHSPs can be regarded as the paramedics of the cell [83], rapidly stabilising the targets prior to their attention by the refolding or degradation machinery. Aside from this important role in overcoming the kinetics of aggregation, the sHSPs also act to increase the proteostasis capacity of the cell. By temporarily storing the aggregation-prone proteins, awaiting refolding or degradation, they act as a vital buffer between protein unfolding and its potentially deleterious consequences.

While the sHSPs form a crucial part of the proteostasis network [11], they themselves can be thought of as a highly dynamic sub-network. As discussed above, many studies have shown that sHSPs can adopt a range of plastic oligomeric states, containing chains from multiple different sHSPs. As many organisms express multiple sHSPs in the same cellular compartment, this will lead to their

coassembly upon synthesis. If all particular subunit arrangements are allowed the number possible of oligomers N_{Oli} is given by

$$N_{\text{Oli}} = \sum_i^n \frac{[N_{\text{sHSP}} + P_i - 1]!}{P_i! [N_{\text{sHSP}} - 1]!}$$

where N_{sHSP} is the number of sHSPs capable of coassembly and i the number of sHSP subunits in a particular oligomeric stoichiometry P .

As an example, for monodisperse hetero-dodecamers ($P_i = 12$), if there are six compatible sHSPs ($N_{\text{sHSP}} = 6$), as is the case for class I sHSPs in the Arabidopsis cytosol [3], the number of potential oligomers is 6,188. In the analogous calculation for class I cytosolic sHSPs in Californian poplar ($N_{\text{sHSP}} = 18$) [3], almost 52,000,000 combinations are possible. While what proportion of these potential hetero-oligomers are formed *in vivo* will be influenced by factors such as tissue specificity and relative expression rates and levels, the number of possibilities is nonetheless remarkable. Combined with the observation that the oligomers rapidly exchange subunits, this suggests that sHSPs should not be regarded as individual oligomers but rather an extensive and interconverting ensemble.

The very large numbers of compatible sHSPs in plants are not replicated in mammals but instead it appears that a different mechanism to achieve the same effect might be at play. In the case of α -crystallin in the eye lens, where both isoforms A and B are expressed and populate oligomeric states between 10 and 50mers [66], 1,271 combinations are possible. Additionally, α B-crystallin is found outside the lens, and is one of seven sHSPs abundant in muscle, leading to potentially over 264,000,000 different complexes. Although, specificities of interaction between sHSP pairs [195] will act to reduce this number in the cell, the polydispersity of many members of the family can act as a means to magnify the diversity of hetero-oligomers.

Indeed, in the case of plant sHSPs it has been shown that a mono- to polydisperse transition occurs under heat-shock conditions, and therefore both the large number of sHSPs and polydispersity can combine to create astonishingly large possible numbers [111]. It remains to be proven to what extent this diversity exists *in vivo*, and how it acts to regulate molecular chaperone function. It is however enlightening to consider the parallels with the immune system in higher eukaryotes, whereby a relatively small number of genes (~300) can give rise to over 10^8 different antibodies [196], allowing the recognition of the diverse structures of antigens. It is tempting to speculate that evolution of such an extensive sHSP ensemble, within the context of the wider chaperone network [197], allows organisms to protect themselves against the diversity of unfolding client proteins and thereby maintain proteostasis.

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