CMLS, Cell. Mol. Life Sci. 59 (2002) 1649–1657
1420-682X/02/101649-09

CMLS Cellular and Molecular Life Sciences

sHsps and their role in the chaperone network

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Abstract. Small Hsps (sHsps) encompass a widespread but diverse class of proteins. These low molecular mass proteins (15–42 kDa) form dynamic oligomeric structures ranging from 9 to 50 subunits. sHsps display chaperone function in vitro, and in addition they have been suggested to be involved in the inhibition of apoptosis, organisation of the cytoskeleton and establishing the refractive properties of the eye lens in the case of α-crystallin. How these different functions can be explained by a common mechanism is unclear at present. However, as most of the observed phenomena involve nonnative protein, the repeatedly reported chaperone properties of sHsps seem to be of key importance for understanding their function. In contrast to other chaperone families, sHsps bind several nonnative proteins per oligomeric complex, thus representing the most efficient chaperone family in terms of the quantity of substrate binding. In some cases, the release of substrate proteins from the sHsp complex is achieved in cooperation with Hsp70 in an ATP-dependent reaction, suggesting that the role of sHsps in the network of chaperones is to create a reservoir of nonnative refoldable protein.

Key words. sHsp; α-crystallin; protein aggregation; protein folding; protein structure; chaperone.

Introduction

Small heat shock proteins (sHsps) represent a widespread family of stress proteins (table 1). They are grouped together based on their low monomeric molecular masses (≈15–40 kDa) and stretches of sequence homology mostly in the COOH-terminal part of the proteins (α-crystallin-domain; [1, 2]; fig. 1). In comparison with other Hsp families, the overall homology among sHsps is rather low. The number of sHsps in different species varies significantly, with up to 30 sHsps in plants, where sHsps appear in the cytosol and in all organelles [3]. Also, the abundance of sHsps varies considerably, depending on the cell types and organisms studied. In addition, expression depends on growth conditions, developmental state, differentiation and the oncogenic status of the cell. Under stress conditions sHsps may comprise more than 1% of the cellular protein [4]. Overexpression of eucaryotic as well as procaryotic sHsps has been shown to convey thermoresistance to the organisms, which leads to prolonged survival during and after exposure to normally lethal temperatures [5–7]. Another common feature of sHsps is their high oligomeric, globular structure [8–11]. The number of subunits and the degree of flexibility in the complex varies in different species. While Hsp16.5 from *Methanoccocus janashii* forms a stable hollow shell of 16 subunits [8], the α-crystallin complex and sHsp from *Bradyrhizobium janponicum* are more flexible and exchange subunits [9, 12].

Furthermore, like other chaperones, sHsps contribute to the balance between cell survival and cell death by preventing protein aggregation during heat shock. They display chaperone function in vitro, suppressing the aggregation of a large set of substrate proteins [8, 13–18]. Nevertheless, many sHsps at least in pro- and lower eukaryotes are dispensable for cell viability and growth under physiological or stress conditions [19, 20].

In vivo properties of sHsps

Under physiological conditions, the abundance of sHsps varies according to cell type and organism studied [21]. In addition, the expression of sHsps depends on development, growth cycle, differentiation and the oncogenic status of the cell [21–28]. A well-characterised example of developmental induction is the accumulation of sHsps during

Table 1. Representative members of the sHsp family.

	Protein	Organisms	Structure	Chaperone activity	Comments
Archaea	Hsp16.5	*M. jannaschii*	24mer	+	[8]
Prokaryotes	IbpA/IbpB	*E.coli*	oligomer	+	found in 'inclusion bodies' [41]
	Hsp16.3	*M. tuberculosis*	9mer	+	surface antigene, 'trimer of trimers' [15]
Eukaryotes					
Yeast	Hsp26	*S. cerevisiae*	24mer	+	temperature regulated [10, 19, 20]
	Hsp42	*S. cerevisiae*	oligomer	n.d.	[106]
Invertebrates	Hsp27/26/ 22/23	*D. melanogaster*	n.b.	n.d.	constitutively expressed, hormone induced, developmentally regulated [35]
	Hsp16-2	*Caenorhabditis elegans*	4mer	–	[107]
Vertebrates	αA-/αB-crystallin	all vertebrates	32mer	+	eye lens protein, phosphorylated [9]
	Hsp25/27	mammal	16-32mer/ oligomer	+	thermoprotection, phosphorylated [16]
	p20 (Hsp20)	mammal	oligomer	n.d.	Hsp27 homologue [86]
	HspB2/HspB3	mammal	oligomer	n.d.	muscle [30]
Plant	Hsp22	*Glycine max*	n.b.	n.d.	ER, endo membrane protein [108]
	Hsp18.1	*P. sativum*	oligomer	+	cytosolic, class I [14, 72]
	Hsp22	*P. sativum*	n.b.	n.d.	mitochondrial [109]
	Hsp21	*A. thaliana*	oligomer	n.d.	chloroplasts [3, 110, 111]
	Hsp16.9	wheat	12mer	n.d.	temperature-dependent structural changes [11]

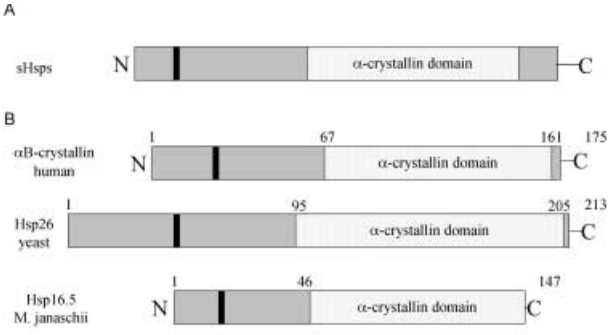

Figure 1. Comparison of schematic domain structure of different sHsps. (*A*) General domain structure. (*B*) Comparison of different sHsps. Blue, variable regions; pointed, homologue regions; black: Phe-Pro rich region; numbers, according to amino acid composition.

seed maturation [3, 29]. Under physiological conditions the levels of sHsps in different cell types range from nonexistent to about 1% in the case of human Hsp20 in striated muscle, heart and diaphragm cells [4, 30]. Studies on the expression of sHsps under heat shock and other stress conditions have shown that this group of Hsps is among the most strongly induced heat shock proteins [26–33]. In addition to the quantity, the number of sHsps also varies significantly in different species, with up to 30 sHsps in plants, where sHsp appear in the cytosol as well as in all organelles in multiple isoforms [3] (table 1).

The overexpression of sHsps has been reported to convey thermoresistance in a number of organisms and cell types [6, 7, 34–37]. In cyanobacteria, the photosynthetic apparatus is protected against heat shock by the constitutive expression of sHsps [7]. Interestingly, a *Synechocystis* sp. mutant deleted for its single sHsp gene, Hsp16.6, showed a defect in membrane stabilisation during heat stress, indicating the importance of sHsps at high temperatures in this organism [38]. In contrast, the yeast homologue Hsp26 does not seem to have a demonstrable function in thermoprotection or during development [19, 20]. The deletion of the sHsps in *Escherichia coli* is not lethal, nor does it show a phenotype [39]. Only in conjunction with a mutation in the major Hsp70 protein DnaK a defect was detected [38].

Understanding of the precise cellular role of sHsps is still lacking. Some procaryotic sHsps were found to be tightly associated with intracellularly aggregated endogenous proteins [40] and with inclusion bodies which are formed during high-level protein expression. In *E. coli*, the two endogenous sHsps were therefore termed inclusion body proteins (IbpA and IbpB) [41]. Taken together, these results suggest a biological function of sHsps in intracellular protein aggregation. sHsps have repeatedly been reported to act as promiscuous molecular chaperones. On the other hand, there is evidence that sHsps can specifically interact with certain components of the cytoskeleton such as actin [42–44] or intermediate filaments [45]. It

could well be that the general chaperone properties leading to the stabilisation of thermolabile intracellular proteins under stress conditions and the specific interaction with the cytoskeleton, possibly preserving cell shape and integrity, are two related properties contributing to protection against stress.

sHsps in the context of diseases

An increasing number of diseases have been described where mutations and modifications of sHsps exert their pathological effect by altering protein folding or assembly. Increased expression of sHsps and especially αB-crystallin has been observed in several neurodegenerative disorders, such as Alzheimer's disease [46, 47], Alexander disease [48, 49] or Creutzfeld-Jakob syndrome [50, 51]. A common feature of all these neurodegenerative disorders is the deposition of improperly folded protein in fibers, inclusion bodies or plaques in the nervous system [52]. sHsps are typically found in association with these insoluble protein aggregates, including intermediate filaments and ubiquitin [53]. Also, increased expression of both α-crystallin and Hsp27 was observed in the context of these diseases. Interestingly, in scrapie-infected mouse neuroblastoma cells, induction of Hsp27 and Hsp72 by metabolic stress is blocked, but Hsp73 and Hsp90 expression is enhanced [54]. This observation could indicate that Hsp27, together with Hsp72, is specifically involved in the pathogenesis of the prion disease.

Elevated expression may thus be considered not only as a strategy for survival during unfavorable times but also as a potential mechanism of defense against diseases, which makes sHsps an interesting target for novel therapeutic approaches [55, 56]. Preliminary experiments have already shown the feasibility of gene therapy for high-level expression of sHsps [57].

In summary, the function of altered sHsp expression in the course of several seemingly unrelated diseases is still enigmatic. A common feature of many of the described disorders is the accumulation of misfolded protein [52, 58].

Structure of sHsps

All sHsps investigated so far share a conserved region of ~90-residue 'α-crystallin domain' in the C-terminal part of the protein. This region is flanked by a N-terminal hydrophobic region, quite divergent in sequence and length and a short C-terminal extension [33, 59] (fig. 1).

One of the most striking features of sHsps is their organisation in large oligomeric structures, comprising 9 – ~50 subunits. For three members the structure of these complexes has been solved (fig. 2), revealing hollow, globule-like structures with outside diameters of 120 and 190 Å. While the quaternary structure of Hsp16.5 from *Methanococcus jannaschii* is well defined [8], the oligomers formed by α-crystallin and Hsp27 show more structural variability and the ability to acquire and release subunits [9]. Interestingly, wheat Hsp16.9 assembles into a dodecameric double disk (fig. 2). Each disk is organised as a trimer of dimers. Comparing all structures of sHsps solved so far, they support the idea that the dimer is the smallest exchangeable unit. This minimal dimeric building block seems to be conserved and may

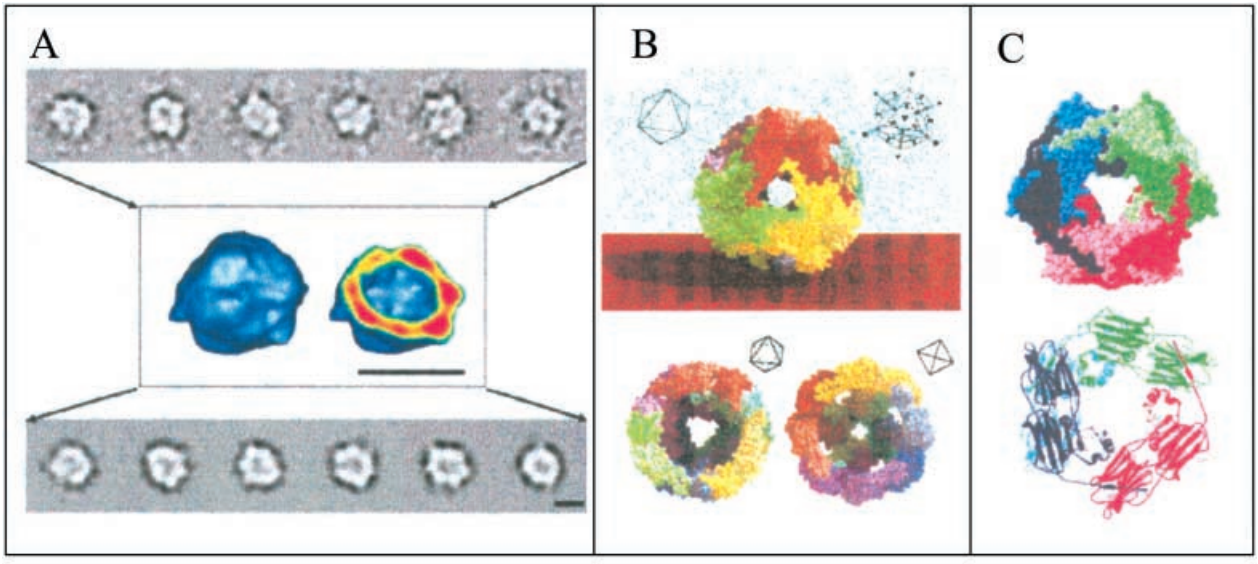

Figure 2. Comparision of the three-dimensional structure of sHsps. (*A*) Cryoelectron microscopy structure of α-crystallin [9]. The outer diameter of the 32mer is 18 nm (black = 15 nm). (*B*) Crystal structure of Hsp16.5 from *M. jannaschii* [8]. 24mer with an outer diameter of 12 nm. (*C*) Crystal structure of wheat Hsp16.9 [11]. Dodecamer, arranged as two disks, 9.5 nm wide and 5.5 nm high.

represent the functional unit concerning chaperone properties (see below) [10, 11]. Furthermore, the fold of this dimeric building block resembles the fold of human p23 a co-chaperone of Hsp90 [11]. Therefore, further investigations on a putative sHsp-Hsp90 cooperation might be very interesting.

The wheat Hsp16.9 structure shows that four hydrophobic sites become exposed on disassembly of the oligomer into dimers, a C-terminal binding groove and an N-terminal interaction site [11]. Another general property of the different structures is localisation of the N-terminal regions of the sHsps in the interior of the oligomeric structures. In the Hsp16.5 and the α-crystallin oligomer, these unordered regions sequester inside the sphere, and in the case of Hsp16.9 they are buried in the interior of the oligomeric structure. Thus, the N-terminal, disordered, hydrophobic part of the proteins might represent the substrate binding site of the sHsps (see below) [11].

sHsps as molecular chaperones

A wide diversity of factors lead to enhanced expression of sHsps and Hsps in general [60]. Many of these agents and factors are known to affect the proper conformation and therefore the function of proteins in general, leading to the accumulation of nonnative protein in cells [61–64]. Interestingly, sHsps which are sometimes termed junior chaperones [65] comprise the most effective chaperone family concerning the efficiency of substrate binding per oligomeric complex [65] (see below).

One of the first indications of sHsps being implicated in chaperoning protein folding was the finding that an ammonium sulfate fraction, enriched in plant sHsps, could prevent soluble proteins from thermal denaturation in vitro [66]. Several sHsps and α-crystallin have subsequently been shown to be able to suppress aggregation of thermally denaturing protein such as β- and γ-crystallin in the case of α-crystallin and model enzyme substrates [10, 67, 68]. Interaction with heat-inactivated citrate synthase (CS) was shown for Hsp27, Hsp25, α-crystallin, cytosolic sHsps from pea, yeast Hsp26 and Hsp16.3 from *Mycobacterium tuberculosis* [9, 10, 13–15].

The underlying mechanism of the interaction of sHsps with nonnative proteins is still enigmatic. Most of the sHsps studied form very stable complexes with unfolded polypeptides [10, 13, 69–71]. The stoichiometry of the complexes between sHsps and nonnative protein seems to be dependent on the substrate investigated [72]. However, several reports suggest a maximum binding capacity of one denatured protein molecule per subunit or dimer of the sHsp oligomers [10, 72–74]. In the case of Hsp26, formation of the complex is a highly cooperative process [10]. Another interesting feature of sHsps is their wide substrate range. sHsps can support the folding of thermally and chemically denatured proteins, ranging from at least 4 to 100 kDa [13–15, 68, 70, 71]. For example, at physiological temperatures, α-crystallin interacts with the reduced and aggregation-prone B chains of insulin, and with melittin, a peptide from bee venom which adopts an unordered conformation [74]. Interestingly, sHsps also bind structured folding intermediates which are prone to aggregation [16, 69, 75, 76].

The mechanism of substrate binding is thought to be based on a change in the hydrophobicity profiles of several sHsps under stress conditions. α-crystallin and Hsp26 bind increasing amounts of the hydrophobic dyes 8-anilino-1-naphtalene sulfonate (ANS) [10, 76, 77] and bis-ANS [78] at temperatures above 30 °C, suggesting that α-crystallin undergoes a temperature-dependent structural change that increases surface hydrophobicity. In agreement, wheat Hsp 16.9 exposes two hydrophobic sites, during temperature-dependent disassembly of the oligomer into dimers [11].

However, data on the two possible binding sites of plant sHsps and site-directed mutagenesis seem to contradict the hypothesis that hydrophobic interactions are exclusively involved in substrate binding [72, 79]. The putative binding site identified by cross-linking [72, 80] contains several highly conserved aliphatic residues with intervening charged amino acids. This alternating polar-non-polar sequence suggests that efficient binding of denatured protein is mediated by hydrophobic residues interacting with exposed nonpolar amino acids of the substrate, while charged amino acids might serve to optimally space the residues [72]. In this context, Smulders et al. (1995) could show that the D69S mutant of αA-crystallin shows decreased chaperone activity, indicating that the charged Asp69 might be involved in protein binding [81]. Furthermore, mutations at position R116 in αA-crystallin and R120 in αB-crystallin proved to be responsible for genetic disorders [82, 83]. These mutations affected the residue, equivalent to arginine 107 in the α-crystallin domain of *M. jannaschii* Hsp16.5.

Regulation of functional properties

Chaperones are often regulated by cofactors or ATP. In the case of sHsps, it is still debated whether ATP binding and hydrolysis are involved in functional regulation or whether other mechanisms are sufficient, whereas in the case of αB-crystallin ATP binding leads to structural rearrangements and exposure of hydrophobic surfaces [84, 85]. In contrast to these findings, the chaperone activity of sHsps in vitro was so far found to be independent of ATP binding and hydrolysis, and an intrinsic mode of regulation was shown for yeast Hsp26 and wheat Hsp16.9 [10, 11]. Under physiological conditions, high oligomeric complexes exist which are supposed to be an inactive storage form (fig. 3).

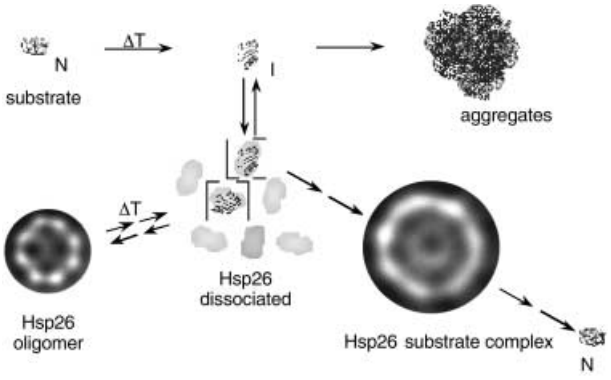

Figure 3. Model of the chaperone function of Hsp26. Under physiological conditions Hsp26 exists in an inactive oligomeric storage form. Upon heat shock (ΔT) unfolding of native protein (N) leads to the formation of nonnative, aggregation-prone intermediates (I). Hsp26 dissociates into smaller complexes which are able to bind nonnative proteins (I), thus preventing their aggregation. Well-defined Hsp26 · nonnative protein complexes assemble subsequently from the dissociated species. After release of the substrate protein, Hsp26 reassociates to the inactive storage form under physiological conditions.

Elevated temperatures lead to the dissociation of these complexes into dimers. In the case of Hsp26, the dimer specifically recognizes and binds nonnative polypeptide chains, resulting in the cooperative formation of large, well-defined Hsp26-substrate complexes [10].

It is tempting to speculate that the heat-induced dissociation and activation of Hsp26 and Hsp16.9 represents an early and simple mechanism which developed into a more sophisticated system of functional regulation during the evolution of higher eukaryotes. Instead of a direct temperature-sensing system, additional signals could now be integrated into the activation process of the respective sHsp. Phosphorylation is a widespread modification of mammalian sHsps, which seems to have functional consequences. Like heat in the case of Hsp26, the phosphorylation of sHsps seems to be associated with changes in the oligomeric structure of sHsp complexes. Depending on the cell-type, the extracellular stimulus and the time after stimulation, phosphorylation of Hsp27 correlates with either dissociation or further increase in size of oligomers [86–89]. The phosphorylated, dissociated form of Hsp27 shows no chaperone activity [90], and thermoprotection is decreased [91]. Interestingly, larger oligomeric complexes of Hsp 25 were observed upon heat treatment and, for a nonphosphorylatable mutant of Hsp25, after tumor necrosis factor α treatment [92]. In addition, Hsp25 showed chaperone activity as a hexadecamer and as a large mega-Dalton complex [93]. The mammalian sHsp-related protein Hsp20 dissociates when phosphorylated. This may mediate cellular signaling processes that lead to vasorelaxation [94]. Furthermore, phosphorylation of sHsps seems to alter the interaction with actin [95–97].

From the data above, one may speculate that phosphorylation is necessary but not sufficient for dissociation of mammalian sHsp oligomers. At present, only a few members of the diverse family of sHsps have been investigated in detail. It could well be that in addition to dissociation and phosphorylation, other factors could contribute to regulation of the oligomeric size of sHsps and thus modulate function via structural changes.

sHsps in the context of the cellular chaperone network

Taken together, sHsps seem to be potent and efficient chaperones in that they are able to selectively bind nonnative proteins in large quantities per oligomeric sHsp complex. However, in general no release of bound substrate had been observed. For α-crystallin, it was suggested that irreversible binding of other lens crystallins, which might occur in the aging lens, prevents aggregation and light scattering [81, 98–100]. Along these lines, Lee et al. suggested that in the case of plant sHsps, bound substrate was held in a soluble form for subsequent degradation [14]. In the living cell it seems detrimental to entertain a chaperone system which traps nonnative proteins without allowing reactivation once a stress period is over. A first hint that sHsp·substrate complexes were not dead-end, but productive intermediates was the discovery that mitochondrial citrate synthase (CS) could be dissociated and reactivated from Hsp25·CS complexes by oxaloacetate (OAA), a stabilising ligand of CS [16]. Thus, Hsp25 is able to bind to thermally inactivated proteins and trap them in a soluble and folding-competent state until permissive folding conditions are restored. While the above-mentioned experiments used the ligand OAA, in the cell a physiological release factor would be required.

A clue for the contribution of different chaperones to this reaction came from in vivo experiments. It was demonstrated that the overexpression of Hsp70 led to a deceleration of the inactivation of marker proteins in transfected cells [101, 102], whereas in the presence of Hsp27, the inactivation of the marker protein was not influenced, and both the substrate and the chaperone were found to be insolubilized. However, when these cells were transferred to permissive conditions, both proteins became soluble again, and the cells recovered much faster than in the absence of Hsp27. This is reminiscent of the finding that procaryotic sHsps are found specifically associated with inclusion bodies and intracellular aggregates of overexpressed proteins [42].

In agreement with these findings, it was shown in vitro that the chaperone Hsp70 allowed reactivation of the sHsp-bound substrate in the presence of ATP [16, 72]. In addition, for Hsp18.1 from pea, the ATP-dependent re-

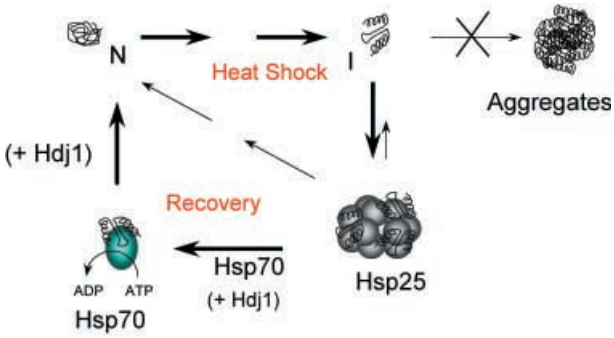

Figure 4. Model for the chaperone function of Hsp25 under heat shock conditions. Unfolding of native protein (N) leads to the formation of nonnative, aggregation-prone intermediates (I). In the presence of Hsp25, these unfolding intermediates can be bound in high quantity, in a soluble and refoldable state. Even at permissive temperatures the nonnative protein stays bound. However, in the presence of Hsp70, ATP, and possibly cofactors like Hdj-1, sHsp-bound protein can be released and refold into its native state.

lease and refolding of bound luciferase by different Hsp70 and Hsp40 was demonstrated [72, 103]. Evidence for the release of substrate proteins bound to sHsp were also shown for *E. coli* IbpB. Here, bound malate dehydrogenase and lactate dehydrogenase were specifically transferred to the DnaK-DnaJ-GrpE system and subsequently reactivated [104]. Sequencing of the genome of the thermophilic eubacterium *Thermotoga maritima* revealed another piece of evidence for the cooperation of sHsps with Hsp70. Here the genes for Hsp70 and a 17.0-kDa sHsp were found in one operon [105].

Based on these data, a model that allows sHsps to integrate in the cellular chaperone machinery was proposed (fig. 4). Under stress conditions Hsp25 binds nonnative proteins, prevents their irreversible aggregation and complexes them in a refoldable state. Upon restoration of physiological temperatures, the nonnative protein will either dissociate from the complex spontaneously, or ATP-dependent chaperones such as Hsp70 will complete the refolding. Whether other factors such as Hsp70 co-chaperones or additional Hsp families are involved in this process remains to be seen. Another open question is whether the proposed model (fig. 4) can be generalised for all organisms.

Conclusions

With good reason, sHsps have been called ‘the forgotten chaperones’, since, although they had been implicated in a number of fundamental cellular processes in addition to the heat shock response, their function remained enigmatic. Recent experiments using in vitro protein-folding assays made it possible to define functional properties of sHsps under stress conditions. These results showed that sHsps belong to the class of molecular chaperones. The current evidence suggests that a specific function of sHsps is to effectively trap aggregation-prone folding intermediates and maintain them in a refoldable conformation. At the moment, structural information of sHsps is rather poor. Thus, the underlying conformational features responsible for the stable binding of nonnative proteins remain unknown. In the eye lens, binding of α-crystallin to other crystallins seems to be sufficient to protect the lens efficiently from irreversible aggregation processes which could cause turbidity. However, in the cytosol and potentially in organelles, cooperation with other ATP-dependent chaperone systems seems to be required to achieve productive folding, even under permissive conditions.

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