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Small Heat Shock Proteins OR: A Subgroup of Molecular Chaperones

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Abstract: Small heat shock proteins (sHsps) have chaperone-like activity and can be found in almost all organisms. The most prominent sHsp is the vertebrate eye-lens protein α -crystallin. Typically, sHsps assemble into large, dynamic oligomers, which constantly exchange subunits. *In vitro*, they prevent the formation of insoluble aggregates of thermally or chemically denatured proteins. Since sHsps have no refolding activity, substrate proteins must be passed on to other chaperones in order to regain enzymatic activity. The dynamic nature of the sHsp complexes is critical for the interaction with substrate proteins. In this review, we reported on functional and structural properties of sHsps and the role these proteins play in the cellular chaperone network.

Key words: Small heat shock protein (sHsps), molecular chaperones, protein folding

Molecular chaperones and protein folding

Protein folding in living organisms: To fulfill their biological function the large majority of proteins must fold into their unique three-dimensional structures. As initially formulated by Anfinsen, the information for correct folding is coded by the amino acid sequence^[1]. This principle was firmly established by refolding experiments *in vitro*^[2]. However, *in vitro* conditions are hardly comparable to the intracellular environment. During translation, non-native features of nascent polypeptide chains are exposed while they leave the ribosomal exit tunnel and before stable three-dimensional structures can be formed. The growing chain is thus prone to aggregation^[3]. Another important aspect is macromolecular crowding. The total concentration of proteins in the cytosol of *E. coli* cells is in the range of 200-300 g L⁻¹, which is predicted to enhance aggregation by unspecific interactions between non-native polypeptide chains^[4]. Efficient protein folding *in vivo* thus requires assistance of highly specialized machineries, so called chaperones, that fulfill different tasks in protein folding and quality control under normal and stress conditions.

Chaperone-assisted protein folding under normal and stress conditions: Chaperones have been defined as “a family of cellular proteins which mediate the correct folding of other polypeptides and in some cases their assembly into oligomeric structures, but which are not

components of the final functional structures”^[5]. Molecular chaperones are involved in both, co- and posttranslational protein folding. They restrict the inappropriate interaction of other proteins by binding to exposed hydrophobic patches and thus prevent the formation of deleterious protein aggregation. Aside from this function, some chaperones accomplish specific tasks, for example protein export and assembly of macromolecular structures^[6,7]. It was estimated that about 30% of total prokaryotic proteins in the cytoplasm transit through the major chaperone machineries DnaK (Hsp70) or GroEL (Hsp60) before adopting their final fold^[8].

DnaK and GroEL as well as their eukaryotic homologues Hsp70 and Hsp60 can be found in almost all organisms and cell types. The first line of defense against protein misfolding are chaperones that directly interact with ribosomes like the *E. coli* Trigger Factor (TF), a protein with peptidylprolyl-*cis-trans* isomerase and chaperone-like activity^[9]. TF binds to nascent chains as short as 57 amino acids. A role in mediating posttranslational folding has not yet been demonstrated^[8]. In contrast to TF, *E. coli* DnaK is not directly associated with ribosomes. Nevertheless, binding to nascent chains was demonstrated^[10]. Both, DnaK and the chaperonin GroEL have an important function in folding polypeptides after they are released from the ribosome.

Beside their function in *de novo* protein synthesis, chaperones play an important role during cellular stress. Stress conditions can have severe impacts on the delicate

structure of proteins. Formation of protein aggregates is a well-documented phenomenon during heat stress conditions. Protein aggregates most probably originate from partially unfolded proteins that un-specifically interact via exposed hydrophobic residues that are normally buried inside the three-dimensional structure. Heat stress leads to the fast transient expression of so-called heat shock proteins (Hsps), among them a high number of chaperones and proteases. In contrast to normal conditions, *de novo* protein folding now occurs mainly via the chaperone-assisted pathway^[11]. Other environmental factors known to induce Hsps are pH, changes in osmolarity, UV irradiation and the presence of toxic compounds such as ethanol, antibiotics, aromatic compounds or heavy metals^[12]. DnaK and GroEL, constitutively expressed in most cells, are highly induced under heat shock conditions. Other Hsps like the prokaryotic small heat shock proteins (sHsps) are typically expressed after heat shock and not detectable at ambient temperatures^[13].

Despite the armada of chaperones that counteract protein misfolding and aggregation, a reasonable fraction of cellular proteins never reach their native state and aggregate. Recently, it was demonstrated that even proteins from stable aggregates can be released and refolded to their native state by the action of DnaK and ClpB^[11]. Protein aggregates that can not be rescued are degraded by proteases. Whenever quality control fails, damaged proteins accumulate as aggregates, a process associated with amyloid diseases^[14].

The molecular chaperone network and its major components: The key players of a typical prokaryotic, cytosolic chaperone system are the ATP-dependent DnaK/J and GroEL/ES machineries. Both chaperones were shown to prevent protein aggregation and to refold partially unfolded substrates.

E. coli DnaK acts as a monomer but is assisted by two additional components, DnaJ (Hsp40) and GrpE. In the ATP-bound stage, DnaK can bind to small hydrophobic stretches of substrates. The peptide is captured by ATP hydrolysis. The co-chaperone DnaJ is involved in ATP hydrolysis and recruitment of DnaK to the substrates. In *E. coli*, GrpE induces the release of ADP from DnaK. After rebinding of ATP, a new peptide can be bound^[8].

GroEL belongs to the class of chaperonins. For many organisms, *groEL* gene expression is essential under all growth conditions. The mode of action of the barrel-shaped GroEL complexes is completely different from

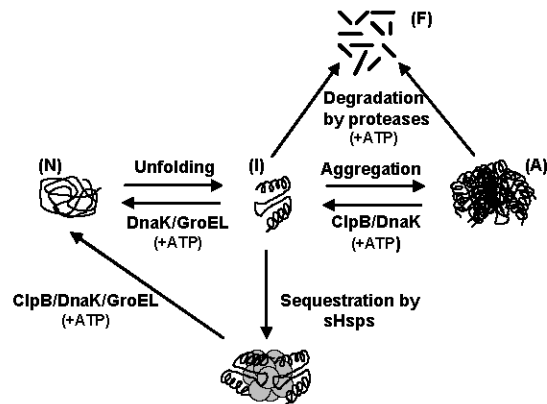


Fig. 1: Heat shock proteins protect the cell from deleterious protein aggregation. To prevent the accumulation of protein aggregates during stress situations, heat shock proteins act in a cooperative manner. Proteins that are not properly folded are either refolded or degraded. The abbreviations are: Native protein (N), unfolding intermediate (I), protein aggregates (A) and protein fragments (F)

DnaK. Two heptameric rings that are stacked to each other form a chamber. Substrates that bind to the apical site are transported into the cavity and freely fold before release. The co-chaperone GroES (Hsp10) forms a lid that upon binding assists in transporting the substrate to the cavity mainly by inducing conformational changes in GroEL. Opening and closing of the cage is triggered by hydrolysis and binding of ATP^[9].

In order to increase their efficiency, chaperones act in a concerted manner (Fig. 1). GroEL is likely to receive several of its substrates from DnaK. A genetic analysis showed that *E. coli* TF and DnaK cooperate in folding newly synthesized proteins^[9]. Recently, it was demonstrated that DnaK and the Hsp100 family member ClpB cooperatively mediate disaggregation of large insoluble protein complexes^[11]. Severe stress that leads to fast unfolding and aggregation might overwhelm the DnaK/GroEL systems. Under such conditions, sHsps function as a back-up system. No active refolding activity was reported for sHsps. Instead, they prevent protein aggregation by sequestering unfolded substrates and keeping them in a re-foldable state. *In vitro* studies demonstrated that malate dehydrogenase bound to *E. coli* sHsps (IbpB) can be transferred in a sequential manner to DnaK and GroEL leading to refolding and regain of enzymatic activity^[15]. Furthermore, incorporation of IbpB into insoluble protein aggregates facilitated the disaggregation and refolding by ClpB and DnaK^[16,17].

sHsps: A distinct family of molecular chaperones

sHsps are ubiquitous proteins: sHsps form a distinct family of molecular chaperones. They occur in all organisms with the exception of a few bacteria. Under heat or other stress conditions, they are among the most strongly induced heat shock proteins^[18,19]. The numbers of sHsps varies significantly from organism to organism. In contrast to prokaryotes where in general only one or two sHsps are present, some eukaryotic genomes encode a high number of these proteins.

Mammalian sHsps can be found in the cytoplasm and nucleus of many different cell types^[20]. Nine sHsps are transcribed in humans, among them the major eye lens components α A and α B-crystallin^[21]. α -Crystallins are in part responsible for maintaining lens transparency by sequestering denatured proteins. An important finding was the association of increased levels of α B-crystallin with neurological diseases like Alzheimer's, Creutzfeld-Jacob and Parkinson's disease. A common feature of these disorders is the deposition of improperly folded proteins in fibers, inclusion bodies or plaques in the nervous system^[22]. The plant *Arabidopsis thaliana* encodes 19 sHsps^[23]. Plant sHsps are divided into six classes according to their primary structure and localization in the cytosol, nucleus, plastids, endoplasmic reticulum and mitochondria. Plant sHsps are highly induced under heat and other stress conditions but are also involved in a broad range of different cellular processes such as embryo development or fruit maturation^[24].

The absence of sHsps from some prokaryotic genomes correlates in part with genome size and more importantly the life style and the temperature a bacterium usually faces. Several bacteria lacking sHsps are intracellular pathogens of vertebrates and are normally not exposed to drastic temperature changes. In such organisms, sHsps might be dispensable. In contrast, high numbers of sHsps are found in some rhizobial species which is in accordance with their existence outside of warm-blooded hosts^[25,26].

Functions of sHsps: Diverse stressors lead to partial un-folding or misfolding and subsequent harmful aggregation of proteins. Generally it is believed that the main function of sHsps is the sequestration of aggregation-prone proteins during such phases of stress. Almost all sHsps described so far were shown to prevent a broad range of model substrates from thermally or chemically induced aggregation, among them the prominent human eye-lens protein α -crystallin, the *E. coli* inclusion body protein IbpB and several sHsps of *B. japonicum*^[27-29]. In contrast to most other molecular

chaperones, refolding is not a competence of sHsps. Sequestered substrate proteins form a re-foldable protein reservoir and can be recycled after stress release. *In vitro* studies demonstrated that bound substrates can be refolded and regain enzymatic activity in collaboration with the ATP-dependent DnaK and GroEL chaperone machineries^[15,30,31]. Recently, it was shown that *in vitro* dissociation and refolding of substrates incorporated in such aggregates relies on a disaggregation mechanism most efficiently mediated by ClpB/DnaK. Studies with Δ *ibpAB* and Δ *clpB* mutant cells demonstrated that this functional triad might as well play an important role *in vivo*^[16,17].

Several publications suggest an involvement of sHsps in thermo tolerance. Heterologous expression of sHsps conferred thermotolerance to eukaryotic and prokaryotic cells^[32,33]. Overexpression of IbpA and IbpB increased the resistance of *E. coli* cells towards heat and superoxide^[34]. Heat sensitivity in absence of sHsps was reported for *Neurospora crassa*, *Synechocystis* and *E. coli*^[35-37]. Direct evidence for involvement of the chaperone function of sHsps in thermotolerance was reported in the case of *Synechocystis* Hsp16.6. Replacement of wild-type Hsp16.6 with mutants that were demonstrated to be inactive chaperones *in vitro* did not restore the observed defect of heat-treated cells^[38]. Interestingly, *Synechocystis* sHsps seem to play a dual role. In addition to their traditional role as member of the protein folding network they may function in stabilization of heat-stressed membranes^[39]. Association with membranes was also reported for Hsp16.3 of *Mycobacterium tuberculosis*. It is the major membrane protein of these bacteria and contributes to cell envelope thickening during oxidative conditions^[40,41].

High amounts (about 35% of total lens proteins) of the heterologomeric sHsp α AB-crystallin are present in mammalian lenses. This organ has an extremely high protein concentration ($\approx 450 \text{ mg mL}^{-1}$) but very low protein turnover. Apart from structural functions α -crystallin acts as a chaperone, preventing uncontrolled aggregation of other lens protein^[22]. Several diseases are linked with defects in mammalian sHsps. A mutation in α A-crystallin (R116C) leads to the formation of congenital cataracts in humans^[42]. In contrast to α A-crystallin, α B-crystallin is also found in other tissues than the lens. A corresponding mutation in α B-crystallin caused cataracts as well as desmin-related myopathy, a neuromuscular disorder^[43]. *In vitro* studies demonstrated that mutations of the conserved arginine led to defective chaperones^[44-48].

The function of sHsps is not restricted to heat stress or even protein folding but can be expanded to many

cellular activities such as apoptosis, developmental processes in animals, plants and bacteria or cytoskeleton modulation. The presence of sHsps in many different tissues, cell types and organelles and the association with a broad range of cell components like proteins, lipids and nucleic acids demonstrated that during evolution sHsps were adapted to fulfill specific tasks depending on their cellular environment^[25].

Structural and functional features of sHsps

The primary structure of sHsps: sHsps generally consist of three regions, the moderately conserved, characteristic α -crystallin domain (named after the human lens protein α -crystallin) and the poorly conserved N- and C-terminal extensions. The α -crystallin domain consists of approximately 80-100 amino acids. The N- and the C-terminal extensions are of variable length^[49,50]. Based on their primary structure, bacterial sHsps can be separated into two classes, A and B. Class A members are restricted to prokaryotes whereas class B proteins show homology to eukaryotic sHsps^[13].

Sequence identity of sHsps is relatively low. Extensive sequence comparisons revealed only a few universally conserved residues mainly located in the C-terminal end of the α -crystallin domain. In an amino acid alignment containing 67, mainly prokaryotic sHsps only five residues were present in more than 80% of all sequences (corresponding to G62, L111, A122, G127 and L129 of *M. jannaschii* Hsp16.5). L111 resides in a characteristic motif of the sHsp superfamily basically described as F-x-R-xxx-L, A122, G127 and L129 in a motif towards the end of the α -crystallin domain (A-xxxx-G-x-L). A third characteristic motif (I-x-I) resides in the C-terminal extension^[25,49]. Elimination of this characteristic motif either by C-terminal truncation or point mutations demonstrated its important structural and functional role in *B. japonicum* sHsps. Partial or entire removal of the two isoleucines significantly reduced complex size and led to a loss of chaperone activity. Modifications touching the isoleucine motif led to the formation of dimers^[51].

A structural overview of sHsps: The monomeric mass of sHsps is in the range of 12 to 43 kDa. Biological units of sHsps are large complexes sometimes exceeding 500 kDa^[20]. Several different types of assemblies were reported. Yeast Hsp26 and *M. jannaschii* Hsp16.5 were shown to form 24 subunit complexes. Wheat Hsp16.9 is organized as a dodecameric complex and mouse Hsp25 was reported to assemble into hexadecamers^[52-55]. A special case is Hsp16.3 of *Mycobacterium tuberculosis* since the stable nonamer is built from trimeric subunits and not from dimers or tetramers as in most other

cases^[56]. In addition to these rather monodisperse entities, several sHsps like *E. coli* IbpAB and mammalian α -crystallins are associated in polydisperse complexes^[28,57,58]. A unifying feature of sHsps is the mainly β -pleated secondary structure, as demonstrated by several circular dichroism studies^[28,59]. An exception is the monomeric rat Hsp22 exhibiting a randomly coiled structure^[60].

Electron microscopical studies of sHsps revealed spherically shaped complexes for several members of the sHsp family, e.g., for mouse Hsp25, yeast Hsp26, human Hsp27, pea Hsp18.1 and *B. japonicum* class A and B sHsps^[30,55,61-63]. In the case of *M. tuberculosis* Hsp16.3, a triangular structure was observed^[56]. Insights into structural details are limited. Attempts to crystallize sHsps were not very successful most probably as a result of the polydisperse and dynamic character of most sHsps oligomers. So far, two crystal structures of sHsps were solved, i.e. Hsp 16.5 of the hyperthermophile *M. jannaschii* and Hsp16.9 of wheat at resolutions of 2.9 Å and 2.65 Å, respectively^[53,64]. *M. jannaschii* Hsp16.5 is a hollow, spherical complex composed of 24 monomers with eight trigonal and six square “windows”. The amino-terminal 32 residues are highly disordered and could not be solved. Each monomeric unit is composed of nine beta-strands organized in two sheets forming a β -sandwich. One β -sheet consists of β 1, 4, 5 and 7 and the other of β 2, 3, 8 and 9 of the same subunit and β 6 of the neighboring unit. There is evidence that the internal cavity might be filled by the unordered N-terminal region. The short β 10 strand in the C-terminal end (containing the conserved I-x-I motif) is involved in oligomerisation and interacts with β 4 and 8 of a neighboring subunit. The quaternary structure of wheat Hsp16.9 is different. The dodecamer consists of two hexameric rings forming a double disk. Surprisingly, the monomeric fold with the α -crystallin domain forming a β -sandwich and the dimeric building blocks are very similar to what was observed for *M. jannaschii* Hsp16.5 despite the relatively low sequence identity. A dimeric building block might hold true for other sHsps as yeast Hsp26 and pea Hsp18.1^[65,66]. The dimer interface is primarily made by residues belonging to a long loop extending from the β -sandwich between β 5 and 7 containing the β 6-strand. The N-terminal ends of wheat sHsps are disordered in one monomer but fully resolved in the dimerisation partner showing mainly α -helical structure. The ordered N-termini of the top disk are involved in interaction with a monomer of the lower disk. One half of the C-termini are involved in interaction with a neighboring unit in the same disk whereas the others interact with units from the other disk to hold the rings together. The fact that in the plant sHsp assembly the

C-terminal extension can be oriented in two directions whereas only one conformation is observed in the archaeal assembly might be the main reason for the different quaternary structures. This example demonstrates nicely that predictions on the structure of other sHsps should be interpreted carefully. Despite a very similar fold of single units, different sHsps might adapt completely different quaternary structures.

Chaperone function of sHsps, the mode of action: Despite the growing information on structural properties of sHsps not much is known about their interaction with substrates. Many *in vitro* studies demonstrate that sHsps are able to prevent thermally and chemically induced aggregation of a broad range of unrelated model substrates^[15,27,29,52,56,62]. It is generally believed that sHsps act in an ATP-independent manner. However, a few reports claim that ATP influences sHsps either by enhancing their binding activity or by inducing substrate release^[67,68]. Gelfiltration and electron microscopical analysis of complexes formed between yeast Hsp26 or murine Hsp25 with different substrates revealed the formation of large aggregates with defined shape. The aggregate size and shape was dependent on the substrate and not on the sHsp^[69]. In contrast, Friedrich *et al.*^[70] observed that the morphology of the aggregates can be sHsp- and substrate-dependent.

Typically, sHsps are highly dynamic in terms of subunit exchange and dissociation/re-association processes (Fig. 2). Several sHsps were demonstrated to reversibly dissociate in a temperature-dependent manner^[28,38,53,55,71]. The current opinion is that sub-oligomeric particles exhibit substrate binding sites. Such sites most probably have a hydrophobic character and normally are hidden within the quaternary structure. Exposition of hydrophobic sites upon higher temperatures was demonstrated by photo-incorporation of the hydrophobic dye bis-ANS for example for pea Hsp18.1 and *E. coli* IbpB^[28,31]. The recently solved structure of wheat Hsp16.9 revealed that sites responsible for stabilization of the oligomer coincide with putative substrate interaction sites. It was hypothesized that the 24mer might act as storage form at physiological temperature whereas the dimeric sub-particle represents the active chaperone unit. Sites serving in stabilization of the large complex might represent substrate interaction sites after temperature-induced dissociation^[53].

Nevertheless, thermal disassembly is not always a prerequisite for chaperone function. Several sHsps that dissociate in a temperature-dependent manner nevertheless prevent the aggregation of chemically denatured substrates even at temperatures around 25°C. In addition, several sHsps like mammalian α -crystallin,

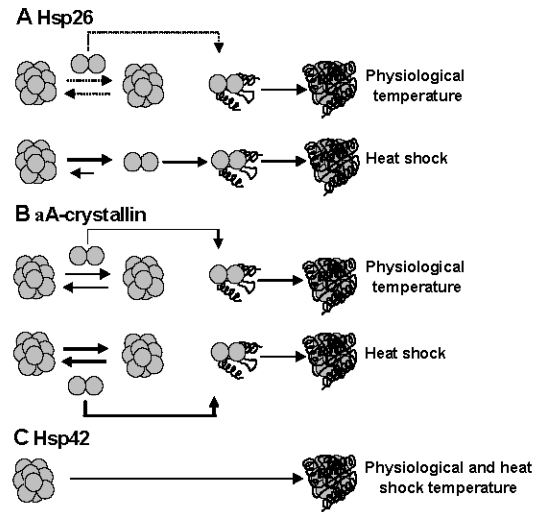


Fig. 2: Different principles of sHsp chaperone function. (A) Yeast Hsp26 exchanges subunits at physiological temperatures. Particles dissociating from the large complex are able to bind substrate proteins (dotted arrows). At heat shock temperatures the large complex dissociates into its active dimers and substrate is more efficiently bound (black arrows). (B) α A-crystallin shows fast subunit exchange at ambient temperatures. The active subunits bind to unfolding substrates (black arrows). Upon heat shock, the rate of subunit exchange increases and more active particles provide increased chaperone activity (bold arrows). © In the case of yeast Hsp42, no temperature influence on the chaperone activity was observed. The hexadecamer is most probably the active unit independent of the surrounding temperature

yeast Hsp42 or *M. jannaschii* Hsp16.5 that do not disassemble upon heat prevent the formation of insoluble substrate aggregates^[27,52,72]. These contrary findings can be explained by the dynamic behavior that is common to most sHsps. Fluorescence-labeled α A-crystallin was found to readily exchange subunits with Hsp27 and α B-crystallin but not with unrelated proteins. Both, subunit exchange and chaperone activity were increased at higher temperatures^[73-75]. In the case of the rather rigid Hsp16.5 of the hyperthermophilic organism *M. jannaschii* efficient subunit exchange was only seen at physiologically relevant temperatures above 60°C. The correlation between dynamics and chaperone activity is underlined by the fact that Hsp16.5 is a markedly inefficient chaperone at 37°C, a temperature at which no subunit exchange was observed^[76].

In contrast to prokaryotic and most plant sHsps, kinase-dependent phosphorylation was shown to have a regulatory effect on mammalian sHsps. Specific phosphorylation induced significant structural and functional changes. Dissociation of the large complex and concomitant reduction of chaperone activity has repeatedly been observed^[63,77].

In conclusion, subunits dissociating and re-associating from the large sHsp aggregates might represent the active, substrate binding units of the sHsps. Most probably, the described different yet similar dynamic strategies were evolved to trigger chaperone function at both, ambient and heat shock temperature depending on the requirement.

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