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Review

The small heat shock proteins and their clients

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Abstract. Small heat shock proteins are ubiquitous proteins found throughout all kingdoms. One of the most notable features is their large oligomeric structures with conserved structural organization. It is well documented that small heat shock proteins can capture unfolding proteins to form stable complexes and prevent their irreversible aggregation. In addition, small heat shock proteins coaggregate with aggregation-prone proteins for subsequent, efficient disaggregation of the protein aggregates. The release of substrate proteins from the transient reservoirs, i.e. complexes and aggregates with small heat shock proteins, and their refolding require cooperation with ATP-dependent chaperone systems. The amphitropic small heat shock proteins were shown to associate with membranes, although they do not contain transmembrane domains or signal sequences. Recent studies indicate that small heat shock proteins play an important role in membrane quality control and thereby potentially contribute to the maintenance of membrane integrity especially under stress conditions.

Keywords. Molecular chaperone, heat shock protein, cyanobacterium, inclusion body, membrane fluidity, non-bilayer phase forming lipids, heat shock lipid, microdomain organization.

Introduction

Small heat shock protein (Hsp), which is also called or abbreviated as low-molecular-mass Hsp, small stress protein, sHsp, or α-Hsp was detected as one of the six major Hsps in *Drosophila* by SDS-polyacrylamide gel electrophoresis (PAGE) [1]. Identification/sequence analysis of paralogs and orthologs of small Hsps revealed that small Hsps exhibit considerable similarity to the α-crystallin of the vertebrate eye lens [2–4]. Since this pioneering work on small Hsps, a large number of related proteins have been identified. Although small Hsps whose monomer sizes range from 12 to 43 kDa are the most diverse in structure amongst the major molecular chaperones, they are characterized by a sequence of about 100 amino acid residues called the α-crystallin domain [5]. This domain is usually preceded by an N-terminal arm/region of variable length, and followed by a C-terminal tail/extension. *In vitro* experiments showed that small Hsps as well as other molecular chaperones such as Hsp90 (HtpG) and Hsp70 (DnaK) bind proteins in non-native conformations, thereby preventing substrate aggregation [6, 7].
Small Hsps are ubiquitous proteins found throughout three domains [8, 9]. Small Hsps and group II chaperonins exist in the genomes of hyperthermophilic archaea, while the major chaperone classes Hsp100 and Hsp90/Hsp83 are absent [10]. Small Hsps are ubiquitous in terms of cellular localization as well as the biological world. For example, they are located in cytoplasm, nucleus, chloroplast, mitochondria, and endoplasmic reticulum in higher plant cells [11]. Often, multiple members of the small Hsp family are present in one cellular compartment.

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Thus, they should have diversified functions. However, small Hsp homologs are not found in the genomes of marine cyanobacteria such as *Prochlorococcus marinus* (CyanoBase, http://www.kazusa.or.jp/cyanobase/) or in the genomes of pathogenic bacteria such as *Mycoplasma genitalium, Haemophilus influenzae* or *Helicobacter pylori* [8, 9].

Small Hsps bind a wide range of cellular substrates and are implicated in many different cellular functions and cellular defenses against many different stresses, such as high temperature and oxidative stresses (Fig. 1). Thus, they have attracted much attention. Thorough reviews on small Hsps as well as an entire book devoted to small Hsps have been published [8, 12]. Recent articles have reviewed α-crystallin [13] as well as small Hsps from plants [14], chloroplasts [15] and extremophiles [9]. The involvement of small Hsps in disease and their potential for therapeutic intervention was explored in a recent review article [16]. Structure and functional aspects of small Hsps have also been reviewed [17, 18]. Thus, we have tried not to repeat what has been already reviewed, but rather to focus on the most unusual properties of this major molecular chaperone, that is, its interaction with protein aggregates and membrane lipids. In this review, we would like to argue that the interaction with these insoluble materials is essential for *in vivo* function of small Hsps.

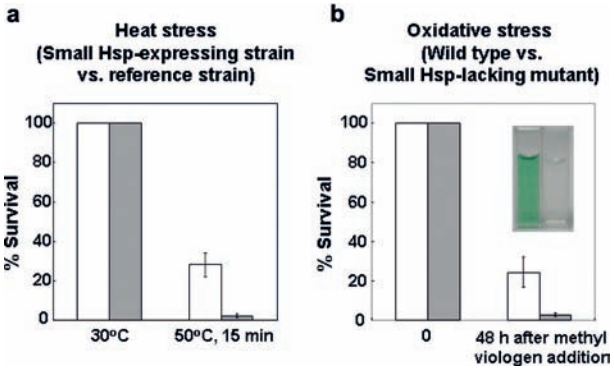

Figure 1. Small Hsp functions in stress tolerance. (*a*) A cyanobacterial strain (*Synechococcus* strain ECT16-1, shown as white columns) that overexpresses small Hsp, HspA, acquires more thermotolerance than a reference strain (*Synechococcus* strain ECT, shown as dark columns). Cells grown at 30 °C were shifted directly to 50 °C for 15 min in the light. Survival rate was assessed by colony-forming ability. These data were taken from Nakamoto et al. [94]. (*b*) HspA (or Hsp17) is required for the methyl viologen-induced oxidative stress. Wild-type (the cyanobacterium *Synechocystis* strain PCC 6803, shown as open columns) and *hspA* mutant (*Synechocystis* strain HK-1, shown as dark columns) cells were incubated at 30 °C in the presence of 5 µM methyl viologen under a light intensity of 30 µE/m^2/s for 48 or 60 h. The survival rate of cultures incubated for 48 h was assessed by colony-forming ability. The mutant's culture was completely bleached after 60 h of the incubation (inset), while the wild-type culture remained blue-green.

Small Hsps form large, dynamic oligomers

Under normal conditions, small Hsps generally form large oligomers up to ~0.8 MDa in size [19–22]. There seem to be variations in the apparent stability of the oligomers. Small Hsps such as Hsp16.3 from a eubacterium, *Mycobacterium tuberculosis* [23], Hsp16.5 from a hyperthermophilic archaebacterium, *Methanococcus jannaschii* [24], and wheat Hsp16.9 [25] form assemblies with defined oligomeric size, while mammalian small Hsps such as α-crystallin and Hsp25, and *Escherichia coli* IbpB exhibit a polydispersity in their oligomeric states [26–29]. The quaternary structure of α-crystallins is dynamic, which is reflected by rapid subunit exchange under native and stress conditions [26, 30]. Even the subunits of the *M. jannaschii* Hsp16.5 freely and reversibly exchange at temperatures above 60 °C [31]. The subunit exchange reaction is markedly enhanced at high temperatures, which may be a key factor in preventing protein aggregation during heat denaturation. It is reasonable to assume that oligomeric structure is important for the function of small Hsps *in vivo* since there is ample evidence that oligomerization is a prerequisite for *in vitro* chaperone activity [8, 22]. However, there is a report that a dimeric α-crystallin domain from the human αB-crystallin (αB57-157) retains significant chaperone activity [32]. Screening for random mutants of the cyanobacterial *Synechocystis* Hsp16.6 (HspA or Hsp17) that could not provide thermotolerance to cells resulted in identification of mutations throughout the protein [33]. The mutants were separated into two groups based on the stability of oligomerization. In one group, mutations destabilized the small Hsp oligomer and reduced *in vitro* chaperone activity, supporting the idea that oligomeric stability is required for small Hsp function. In the other group, however, mutations that were clustered in the N-terminus had little effect on the oligomeric structure and *in vitro* chaperone activity. Naturally occurring small Hsps, Hsp12.2, Hsp12.3 and Hsp12.6 from *Caenorhabditis elegans* are tetramers and a monomer. All of them are devoid of *in vitro* chaperone activity [34, 35]. The existence of these unusual small Hsps also suggests that there is an important function of small Hsps that does not require oligomerization or *in vitro* chaperone activity.

The α-crystallin domains of small Hsps are similar

Currently, two crystal structures of a small Hsp from *M. jannaschii* and the cytosolic class I Hsp16.9 from wheat have been solved at the atomic level. Hsp16.5 from *M. jannaschii* is a homo-oligomer of 24 subunits which form a hollow spherical complex of octahedral symmetry [36]. The sphere has an outer diameter of 120 Å and an inner diameter of 65 Å. Their α-crystallin domains,

which have an immunoglobulin fold, consist of a β-sandwich comprising two antiparallel β-sheets. One β-sheet consists of four β-strands and the other one consists of four β-strands of the same subunit and one from a neighboring subunit that engages in strand exchange to stabilize the partner monomer. In contrast to the archeal small Hsp oligomer assembly, the wheat Hsp16.9 is a homododecamer which consists of two disks, each comprising six α-crystallin domains organized in a trimer of dimers [25]. Although primary and quaternary structures are dissimilar between the small Hsps, their α-crystallin domains are similar, and both use a similar dimer as a building block in their oligomer assemblies. Thus, N-terminal arms and/or C-terminal extensions, which are highly variable regions in small Hsps [37], are likely determinants for different small Hsp oligomer assemblies. In support of this, a truncated version of human αB-crystallin that contains only of the α-crystallin domain forms dimers [32]. Hsp26, a small Hsp from the cytosol of *Saccharomyces cerevisiae*, also forms a dimer when it lacks the N-terminal domain [38]. Truncation of 11 amino acid residues from either the N or the C terminus led to the formation of dimeric *E. coli* IbpB [39], indicating that both terminal regions may be important for the oligomer assemblies of this small Hsp. Hsp12.6 from *C. elegans*, which possesses an α-crystallin domain with very short N- and C-terminal regions, is monomeric [34]. Thus, the central α-crystallin domain alone may not be sufficient for dimerization. Studies on the interaction of full-length or C-terminally truncated HspH from *Bradyrhizobium japonicum* with various truncated mutants of HspH also showed that the central α-crystallin domain alone is not sufficient for dimerization [40]. In contrast to previous observations [23], a recent study supports the idea that the common basic unit of all small Hsps is a dimer [41] and that Hsp16.3 from *M. tuberculosis* does not form a nonamer composed of a trimer of trimers [23], but instead a dodecamer like the wheat small Hsp [41]. Small Hsps from other eubacteria [42], including thermophilic bacteria [43], were reported to build complexes consisting of ~24 subunits like the archaeal small Hsp.

Temperature change and phosphorylation control the activity of some small Hsps

It appears that temperature-regulated exposure of hydrophobic surfaces [44, 45] and temperature-dependent reversible dissociation of oligomers into smaller subunits such as dimers [25, 46, 47] are involved in the chaperone mechanism of small Hsps. The wheat Hsp16.9 structure analysis predicts that several hydrophobic sites become exposed on disassembly of the oligomer into dimers [25]. Thus, it is reasonable to assume that the hydrophobic interface sites for the small Hsp oligomer assembly are exposed due to its disassembly in response to heat stress and then become available for substrate binding, resulting in activation of the small Hsp. In fact, the yeast Hsp26 becomes activated at elevated temperatures and forms large, well-defined Hsp26-non-native substrate protein complexes [47]. This activation coincides with dissociation of the Hsp26 oligomers into dimers, leading to a proposal of the dissociation-mediated activation mechanism (Fig. 2a). An oligomer may be a stable storage form of the active dimers. To test whether the formation of dimers is essential for activation, a point mutant of Hsp26, in which the residue serine 4 of the N-terminal domain was replaced by cysteine, was constructed [48]. Crosslink of neighboring subunits via the disulfide bridge formation between the cysteine residues in the mutant resulted in inhibition of the subunit exchange and dissociation of Hsp26 oligomers into dimers at high temperatures. However, the mutant exhibited chaperone activity indistinguishable from that of the wild-type. These results indicate that the activation of Hsp26 at elevated temperatures does not require dissociation into dimers. They proposed that the structural changes necessary for shifting Hsp26 from a low to a high affinity state for binding non-native proteins occur without dissociating the oligomer (Fig. 2b). In addition to elevated temperature, phosphorylation of serine residues of animal small Hsps also results in decreasing oligomer size [22, 49, 50]. In contrast with the high-temperature-induced dissociation, phosphorylation low-

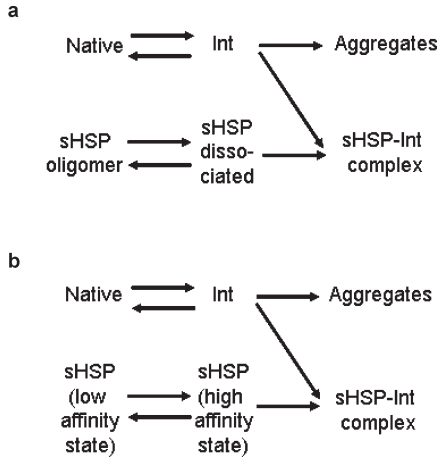

Figure 2. Models for the chaperone function of a temperature-controlled small Hsp. (*a*) Under normal conditions, small Hsp (sHSP) exists in an inactive oligomeric form. Upon heat shock, sHSP dissociates to dimers, which are able to bind non-native proteins (Int) to form sHSP-non-native protein complexes (sHSP-Int complex). (*b*) Under normal conditions, small Hsp (sHSP) exists in an inactive oligomeric form. Heat shock leads to conformational changes within the oligomer, thus shifting sHSP from the low- to the high-affinity state. The activated sHSP oligomer binds non-native proteins (Int) to form sHSP-non-native protein complexes (sHSP-Int complex).

ers both *in vitro* chaperone activity and stress tolerances *in vivo* [51, 52]. This mechanism may be important for an organism with constant temperature to regulate small Hsp activity through dissociation of oligomers.

The N-terminal region of small Hsps may be involved in substrate binding

Several regions and residues have been postulated to be involved in the chaperone function. Both the N-terminal (described below) and the C-terminal regions [53–55] have been suggested to be involved in chaperone activity. Depending on a small Hsp and/or its target, both or either one of the regions may be involved in substrate binding. Here, we present reports that show the N-terminal region of small Hsps as one of the putative substrate binding sites. As described above, the substrate binding sites are proposed to be hydrophobic interface sites for small Hsp oligomer assembly. Structural analysis on wheat Hsp16.9 showed that its N-terminal region constitutes a higher assembly subunit interface site [25]. Mutational analysis indicates that N-terminal regions of the other small Hsps are also involved in the oligomer assembly. Truncation of the N-terminal regions of Hsp16-2 from *C. elegans* [56], yeast Hsp26 [38], HspF/HspH from *B. japonicum* [40] and Hsp16.5 from *M. tuberculosis* [57] results in dissociation to monomers/tetramers, dimers, dimers/octamers and dimers/trimers, respectively. Similar results were obtained on the N-terminally deleted mammalian α-crystallins [58]. Peptide mapping of a bound hydrophobic probe showed that the N-terminal region of pea Hsp18.1 is hydrophobic and becomes exposed upon heat shock [45]. Thus, the N-terminal region of small Hsps is hydrophobic and involved in oligomerization. N-terminal truncation from *B. japonicum* HspF/HspH, yeast Hsp26 and *M. tuberculosis* Hsp16.3 resulted in loss of *in vitro* chaperone activity [38, 40, 57], while removal of the C-terminal region from *C. elegans* Hsp16-2 and *M. tuberculosis* Hsp16.3 had little or no influence on *in vitro* chaperone activity [56, 57]. Furthermore, the N-terminal region alone, either as a synthesized peptide or in a fusion protein with glutathione S-transferase (GST), was capable of interacting with denaturing proteins [57]. *In vivo* studies also support the idea that the N-terminal region is important for chaperone function under heat stress. Expression of a GST-fusion protein with only the N-terminal 78 amino acid residues of rice Hsp16.9 in *E. coli* enhanced thermotolerance, while that with the C-terminal 108 amino acid residues did not [59]. These observations suggest that the N-terminal region is important for chaperone function and contains a substrate binding site.

Small Hsps interact with a denatured protein to prevent its aggregation

Bovine α-crystallin, murine Hsp25 and human Hsp27 were shown to suppress thermally induced aggregation of various enzymes/proteins such as alcohol dehydrogenase, citrate synthase, α-glucosidase, and β- and γ-crystallins [6, 7]. They also promoted the functional refolding of these proteins after guanidine hydrochloride or urea denaturation. This refolding may not be due to folding catalysis by small Hsps since reactivation of the enzymes in the presence of small Hsps was slower than that in its absence [7]. ATP has no effect, positive or negative, on chaperone activity [7, 60, 61]. To date, it has been demonstrated *in vitro* for a number of different small Hsps that they are able to capture thermally or chemically denatured substrates and prevent formation of aggregates. The substrate-binding efficiency varies depending on the temperature and model protein substrate studied [8].

Small Hsps form a soluble complex with substrate proteins, creating a transient reservoir of substrates for subsequent refolding by ATP-dependent chaperone systems

Under conditions in which small Hsps prevent aggregation of substrate proteins, the substrates form complexes with small Hsps [23, 45, 47, 56, 62]. Furthermore, after a shift to non-stress conditions, there is no spontaneous refolding of substrates [23, 43, 45]. The large, soluble small Hsp/substrate complexes appear to be stable for hours [45, 62, 63], just as complexes of yeast Hsp26 with its substrates are more stable than the Hsp26 oligomers [38] alone.

Indications that these thermally induced, small Hsp/substrate complexes are not dead-end products derives from the observations that heat-denatured mitochondrial citrate synthase bound to mammalian Hsp25 can be reactivated by Hsp70 [62] and that heat-denatured firefly luciferase bound to Hsp18.1 from pea can be reactivated in the presence of rabbit reticulocyte or wheat germ extract in an ATP-dependent process [45]. These results also suggest that small Hsps collaborate with other molecular chaperones in the refolding of bound denatured proteins. The *E. coli* small heat-shock protein IbpB also assists the refolding of denatured proteins in the presence of other chaperones, and the mechanism of substrate transfer from IbpB to other molecular chaperones was studied in detail [64]. IbpB-stabilized malate dehydrogenase and lactate dehydrogenase are specifically delivered to the DnaK/DnaJ/GrpE chaperone system, while GroEL/GroES chaperonins do not interact directly with IbpB-released proteins. Kinetic and gel-filtration analysis showed that

denatured malate dehydrogenase transfers from IbpB to DnaK/DnaJ/GrpE, then from DnaK/DnaJ/GrpE to GroEL/GroES, and finally forms an active enzyme. The slow dissociation of malate dehydrogenase from the IbpB/malate dehydrogenase complex is the rate-limiting step. From these studies with eukaryotic and prokaryotic small Hsps, small Hsps are proposed to hold unfolding proteins in a soluble, folding-competent state, creating a transient reservoir of substrates for subsequent refolding by ATP-dependent chaperone systems, DnaK/DnaJ/GrpE or Hsp70/Hsp40. The substrates may refold with the help of the molecular chaperones when physiological conditions are restored.

Small Hsps coaggregate with non-native proteins

As described above, many *in vitro* studies have shown that small Hsps prevent protein aggregation by forming soluble complexes with aggregation-prone substrate proteins. However, small Hsps are frequently found in insoluble, salt- and detergent-resistant fractions incorporated with many other proteins, under *in vivo* conditions. Early studies showed that small Hsps are major components of heat shock-induced aggregates in animal cells [65, 66] as well as in plant cells [67]. In *E. coli*, they (i.e. IbpA and IbpB) were found to be tightly associated with inclusion bodies formed during heterologous protein production [68]. Although these aggregates with small Hsps incorporated as major ingredients were detected more than 2 decades ago, the function of small Hsps in the aggregates, if any, were only recently evaluated. Surprisingly, small Hsps appear to coaggregate with other heat-denatured proteins in order to mediate/chaperone refolding of these proteins in cooperation with other molecular chaperones, as described below. How is it possible that a molecular chaperone suppresses aggregation of denatured proteins and at the same time becomes involved in the formation of a large, insoluble aggregate? What factor determines which function small Hsp will take, suppression of aggregation or formation of aggregates? One of the factors may be the ratio of small Hsps to substrate since *in vitro*, the apparent size of small Hsp/substrate complexes increases as the ratio of small Hsps to substrate decreases [69]. Thus, an insoluble small Hsp/substrate complex is formed when small Hsp is overloaded with non-native substrates [70]. This may be the situation under severe thermal stress. The insoluble small Hsp/substrate complexes are very stable protein aggregates without significant spontaneous substrate release [69]. However, substrates can be released in the presence of ClpB/DnaK/DnaJ/GrpE/ATP or Hsp104/Hsp70/Hsp40/ATP.

Small Hsps cooperate with ClpB (Hsp104) and DnaK/DnaJ/GrpE (Hsp70/Hsp40) in order to refold aggregated proteins

Cell survival under severe thermal stress requires the activity of the ClpB (Hsp104) chaperone. ClpB (Hsp104) can solubilize and refold aggregated proteins in concert with the DnaK/DnaJ/GrpE (Hsp70/Hsp40) chaperone system [71–74]. The DnaK/DnaJ/GrpE (Hsp70/Hsp40) chaperone system can also refold aggregated proteins, but it can act on smaller protein aggregates than can ClpB [71, 75]. ClpB and the DnaK system mediate refolding of insoluble protein aggregates with incorporated small Hsps *in vitro* and *in vivo* [69, 76]. ClpB and the DnaK system are able to disaggregate the insoluble small Hsp/substrate complexes more rapidly than the substrate aggregates without small Hsp. Small Hsp intercalation into aggregates of substrates may increase the accessibility of DnaK binding sites in substrates, and/or decrease the number of hydrophobic contacts between substrate molecules and hence the tightness of the complexes [69]. Similar interplay or cooperation of small Hsp with Hsp104 and Hsp70 (Ssa1)/Hsp40 (Ydj1) is also present in *S. cerevisiae* [77, 78]. Aggregates generated in yeast after heat stress contained Hsp26, several members of the Hsp70 family, Hsp104 and many other proteins. A majority of cellular Hsp26, but not Hsp42 (the other cytosolic small Hsp in yeast), became insoluble in response to heat shock. Resolubilization of the aggregates during recovery of cells was dependent on the presence of Hsp104 [78]. Hsp104/Hsp70/Hsp40-mediated reactivation of firefly luciferase *in vitro* was greatly increased when the firefly luciferase aggregates were produced in the presence of Hsp26. Cashikar et al. [78] showed that all the chaperones, Hsp104, Hsp70 and Hsp40, are required for reactivation of thermally denatured firefly luciferase, regardless of the Hsp26:firefly luciferase ratios. On the other hand, Haslbeck et al. [77] showed that the addition of Hsp104 alone or Hsp70/Hsp40 results in substantial reactivation of *in vitro* synthesized green fluorescent protein, thermally inactivated citrate synthase and firefly luciferase. The addition of both chaperone systems resulted in a further increase in activity of all the proteins. In all the above reactivations, formation of aggregates with small Hsps is essential for Hsp104 and/or Hsp70/Hsp40 to work efficiently. It appears that the complete set of chaperones is required when Hsp26/substrate complexes (aggregates) are formed in the presence of substoichiometric amounts of Hsp26, at late stages of the denaturing process or under severe stress. These bigger aggregates with incorporated Hsp26 may have to be disassembled by Hsp104 before the Hsp70 system refolds the misfolded proteins liberated from the aggregates. Expression of small Hsps also facilitates Hsp104-mediated solubilization of polyglutamine in yeast and ameliorates

polyglutamine toxicity in yeast [78]. The authors suggest that small Hsps bind misfolded proteins and coaggregate with them. This keeps the entire aggregate in a state that allows efficient disaggregation by Hsp104. The chaperone pathway in protein disaggregation in yeast also appears to be operating in higher eukaryotes. Consistent with the mechanism described above, a heat-resistant Chinese hamster cell line overexpressing only human Hsp27 showed an enhanced rate of recovery from nuclear protein aggregation [79]. On the other hand, heat-induced protein aggregation occurred at the same rate as control cells. These results led the authors to suggest that resolubilization of protein aggregates could be at least partly responsible for Hsp27-mediated acquisition of thermotolerance and that Hsp27 might have protein disagggregation activity. Although the heat shock granules in plants may not be the same as those in animal cells, plant small Hsps may also cooperate with the Hsp70 system. Heat shock granules purified from heat-stressed BY-2 tobacco cells contained small Hsp, Hsp70, Hsp40, tubulins and other proteins [80]. The importance of small Hsp-incorporated aggregates for thermal tolerance was studied by ectopic expression of single-chain fragment variable (scFv) antibodies against cytosolic small Hsps to generate small Hsp loss-of-function mutants by antibody-mediated prevention of small Hsp assembly *in vivo* [81]. Anti-small Hsp scFv antibodies transiently expressed in heat-stressed tobacco cells prevented the assembly of small Hsps into heat stress granula. Mesophyll cells of the transgenic plants suffered destruction of all cellular membranes and finally underwent cell death after prolonged stress at sublethal temperatures, while the control cells showed normal cell function.

Are the aggregates shelters for aggregation-prone proteins to escape from intolerable stress?

When proteins denature under mild stress, in the presence of sufficient amounts of molecular chaperones, or at early stages of the denaturing process, unfolding polypeptides may be refolded spontaneously, or with the help of the DnaK/DnaJ/GrpE (Hsp70/Hsp40) chaperone system and/or GroES/GroEL. On the other hand, when proteins denature under severe stress, in the presence of an insufficient number of molecular chaperones, or at late stages of denaturing process, unfolding polypeptides aggregate with or without small Hsps. When physiological conditions are established, the denatured proteins are extracted from the aggregates, and refolded to their native structure with the help of molecular chaperones such as ClpB (or Hsp104) and/or the DnaK/DnaJ/GrpE (or Hsp70/Hsp40) chaperone system. Small Hsps play an important role in facilitating protein disaggregation and thus the cell's acquisition of stress tolerance.

Small Hsps are more effective than other major chaperones with respect to the efficiency of substrate binding per oligomeric complex [8]. The capacity for protein binding increases further when they become aggregated. Although protein aggregates are toxic to cells since they would impair normal cellular function [82], production of protein aggregates might be 'the last choice' for a cell to tolerate very harsh conditions. Stable granules may shield target proteins from denaturing environments and degradation by proteases.

Small Hsps interact with membranes and may protect them

Recently, a potential link between small Hsps and membrane functions has been receiving growing attention. From several studies it was inferred that the cellular pool of Hsps, and small Hsps in particular, is divided into a cytoplasmic and a membraneous subfraction [see references in the following reviews – 83–87 – and in the text below]. Underlying this fact, which ascribes novel biological significance to a membrane-associated subpopulation of small Hsps, the presence of small Hsps in the membranes is widely documented.

A significant earlier finding, relevant to the role of small Hsps in membrane localization, was that a subset of the 15-kDa Hsps of *E. coli* recovering from sublethal heat stress were found to interact with membranes [88]. This subset of proteins were sigma-32-dependent, and two of them, designated C14.7 and G13.5 (at that time Hsps with unknown functions), were later identified as IbpA and IbpB [89]. At the same time, the capability of IbpA and IbpB to protect cells from heat and oxidative stress was also documented [89, 90]. Based on systemic cell fractionation studies, a predominant outer membrane localization of IbpA/B was further confirmed by Laskowska et al. [91]. It was demonstrated that whereas the transcription of the *hsp*17 gene of a blue-green alga *Synechocystis* PCC 6803 is strongly regulated by subtle changes in the physical order of the membranes, most of the newly synthesized Hsp17 is associated with the thylakoid membranes [92]. Direct evidence for the physiological relevance of Hsp17 thylakoid association was presented by Lee et al. [93], who reported that inactivation of *hsp*17 results in greatly reduced activity of photosynthetic oxygen evolution in heat-stressed *Synechocystis* (see below further details about the lipid-mediated mechanism of membrane protections by IbpA, IbpB and Hsp17). Independent studies with the cyanobacterium *Synechococcus* PCC 7942 strain showed that constitutive expression of HspA, a small Hsp from the cyanobacterium *Synechococcus vulcanus,* confers cellular thermotolerance and greatly increases the thermostability of the photosystem II (PSII) electron transport system and light-harvesting phycocyanins

in the cyanobacterium [94]. The PSII electron transport system is thought to be the most thermolabile element of the thylakoid membrane [84]. Indeed, HspA stabilized subcellular structures such as thylakoid membranes under stress conditions [95]. Immunocytochemical studies showed that twice as many HspA molecules are present in thylakoids as in the cytosol [95]. Interestingly, the main localization of HspA shifted transiently to the cytoplasm after heat shock. The liberated HspA may interact with phycocyanins, the most abundant soluble proteins in the cyanobacterium. Phycobilisomes, in which phycocyanins are the major components, are deassembled during heat shock. HspA recognizes phycocyanins in deassembled phycobilisomes, probably as major cellular targets in order to protect them from irreversible denaturation, thus also providing elevated cellular survival at severe heat stress [96].

Just as in the case of *Synechocystis* PCC 6803, the expression of the small Hsp Lo18 in the lactic bacterium *Oenococcus oeni* was shown to be induced by administration of membrane fluidizer benzyl alcohol and heat stress. Moreover, it was demonstrated that a subset of Lo18 is localized in the membrane fraction, and that the actual level of its membrane association depends on the temperature upshift [97]. Hsp16.3 from *M. tuberculosis* was originally identified as an immunodominant antigen and later found to be a major membrane protein [98]. Gene knockout studies indicated that Hsp16.3 is fully required for *M. tuberculosis* to grow in macrophages. The reversible sulfoxidation/desulfoxidation of methionine residues of Hsp16.3 serves as a tool to scavenger reactive oxygen and nitrogen species abundantly present in macrophage cells, thus protecting the plasma membrane and other components of *M. tuberculosis* in the bactericidal host [99].

Early observations also supported membrane localization of the small Hsps from eukaryotic systems. *Toxoplasma gondii* subcellular localization studies revealed that small Hsps are located in different compartments. Relevant to the present review, Hsp29, a member of the five parasite small Hsps, appeared to be membrane associated by immunostaining [100]. These particular differences in immunostaining patterns suggest that the targets and function of *T. gondii* small Hsps might be fundamentally different [100]. Thylakoid association of small Hsps in the chloroplasts of heat-stressed plants was first shown by Adamska and Kloppstech [101]. Experiments with the green alga *Chlamydomonas* have revealed that elevated levels of small Hsps increase the resistance of thylakoids to light and heat damage [102]. In particular, small Hsps appeared to protect the PSII electron transport system [103]. Administration of certain small Hsps alone to submitochondrial membrane vesicles also confirmed that small Hsp function is totally responsible for the heat 'acclimation' of complex I electron transport in pre-heat-stressed plants [104].

Several members of the small Hsp family, present in mammalian cells, are also associated to membranes. HspB2, expressed in heart and skeletal muscle, was shown to associate with the outer membrane of mitochondria [105]. Elevated levels of αB-crystallin are known to be associated with a number of neurodegenerative pathologies such as Alzheimer disease and multiple sclerosis. Mutations in the αB-crystallin gene have been linked to desmin-related cardiomyopathy and cataractogenesis. The physiological function of this protein, however, is unknown. It has been suggested that increased membrane binding of α-crystallin is an integral step in the pathogenesis of many forms of cataracts [106, 107]. Both αA and αB homopolymer complexes, as well as a reconstituted 3 : 1 heteromeric complex, bind to lens plasma membranes in a specific, saturable and partially irreversible manner that is sensitive to temperature. The amount of α-crystallin that binds to the plasma membrane increases under acidic conditions but is not affected at high ionic strength, suggesting that α-crystallin binds to the fiber cell plasma membranes mainly through hydrophobic interactions. It was concluded that membrane association of α-crystallin is closely related to the loss of transparency in the lens [106, 107]. A missense mutation (R120G) of αB-crystallin has been linked to a familial form of desmin-related myopathy (DRM). Worth noting is, that an enhanced plasma membrane localization of both αB-crystallin and Hsp27 in myotubes of dexamethasone-treated DRM patients has also been shown [108]. Discrete Golgi membrane-bound fractions of αB-crystallin have been identified in rat tissues and human glioblastoma cell lines. Confocal microscopy revealed colocalization of α-crystallin with ceramide and the Golgi matrix protein GM130 in the perinuclear Golgi. It is inferred that this small Hsp, with its chaperone-like activity, may have an important role in Golgi reorganization during cell division [109].

Hsp27 expression is highly upregulated during late pregnancy and labour, and the Ser-15-phosphorylated Hsp27 in circular muscle became primarily detectable in perinuclear and membrane regions of myocytes [110]. It was documented that while it modulates agonist-induced signal-transduction cascades in smooth muscle contraction, the phosphorylated form of Hsp27 is crucial for acetylcholine-induced translocation and association of PKCα and the *ras*-related small GTP binding protein RhoA to the plasma membrane [111]. The functionality of Hsps at the cell surface has also begun to be elucidated, with recent work focusing on Hsp-receptor interactions. Based on a novel strategy implemented for the comprehensive profiling and identification of plasma membrane proteins from various cancer cells, together with several 'classical' chaperone proteins (like Grp78, Grp75, Hsp70 and Hsp60), Hsp27 was also found to be highly abundant on the cell surface [112]. Surprisingly, none of the membrane-associated Hsps contain transmembrane domains

or signal sequences, which normally target the nascent polypeptide into the membrane or to the secretory pathway. Consequently, it is unlikely that the membrane-associated subclass of Hsps utilized the classic secretory pathway for their membrane targeting.

Membrane quality control via lipid-small Hsp interactions

Members of the small Hsp family have been implicated in an ever-growing number of cellular activities. Based on the above collection of literature data, it is tempting to speculate that a subset of small Hsps may play a role in cellular 'stress management' by acting as a membrane-stabilizing factor. Thus, the next central question that remained to be elucidated was to explore the possible mechanism(s) of small Hsp-membrane interaction.

A critical role of small Hsps in controlling the physical state, bilayer stability and thereby integrity of membranes via specific lipid interactions has basically been established with Hsp17 from *Synechocystis* PCC 6803. The interpretation that Hsp17 is an important stabilizer of photosynthetic membranes is supported by the early observation that the majority of heat-induced Hsp17 associates with thylakoid membranes [92]. Whereas altering the membrane physical state by administration of the membrane fluidizer benzyl alcohol enhanced the thermosensitivity of *Synechocystis* cells, the threshold temperature required for induction of major heat shock genes, including *hsp*17, was reduced [92]. The close correlation between membrane physical order and heat shock response documented in this and in other models ultimately led to the hypothesis that membranes act as cellular thermometers from which stress is sensed and transduced into signaling pathways, leading to the activation of heat shock genes [83–87]. As outlined elsewhere [85], at least two features make membranes a candidate for monitoring thermal changes. Membranes are among the first cellular structures that encounter external stress. Moreover, microdomain organization, phase state and fluidity of membranes are very sensitive to temperature variations, at both low [113] and high [83–87, 114] temperatures. As was shown by a combination of genetic and *in vitro* studies, Hsp17 is indeed able to play an important role in membrane quality control, and thereby potentially contribute to the maintenance of membrane integrity under heat stress conditions. Consistent with this, transmission electron microscopic studies showed that expression of *Synechococcus* HspA stabilized thylakoid membranes under elevated temperature and intensive light stress [95]. Thus, *Synechocystis* Hsp17 possesses not only protein-protective activity located either in the cytosol [33] or in membranes [95, 96], but also a previously unrecognized ability to stabilize the lipid phase of membranes. Similar to that shown for IbpB from *E. coli* [64], recombinant Hsp17 forms stable complexes with denatured malate dehydrogenase and serves as a reservoir for the unfolded substrate, transferring it to the DnaK/DnaJ/GrpE and GroES/GroEL chaperone network for subsequent refolding [115]. Unlike in case of solely GroES/GroEL-driven refolding [116] large unilamellar vesicles made of synthetic and cyanobacterial lipids were found to modulate this refolding process [115]. In addition, the interaction between Hsp17 and large unilamellar vesicles made of synthetic or cyanobacterial lipids strongly increased the membrane microviscosity. This ability of Hsp17 was documented both by measuring anisotropy changes using a fluorescent membrane probe (1,6-diphenyl-1,3,5-hexatriene, DPH) or monitoring wave number alterations of the CH_2 stretches of lipid acyl chains by Fourier transform infrared spectroscopy (FTIR). It appeared that the lipid interaction of Hsp17 is specific with some preference for the fluid lipid matrix with acidic domains. Similar to our previous findings with GroEL [116], lipid-Hsp17 interaction generally causes an elevated membrane order, but surprisingly seems to fluidize negatively charged *saturated* lipid molecular species [115]. FTIR studies with lipid/Hsp17 systems indicated considerable changes in the regions of the lipid phosphate bands (polar head-group region), the C=O band (interfacial region) and the CH_2 band (hydrophobic core) reflecting the high, and hitherto unexplored complexity of the lipid-small Hsp association [117]. To confirm that heat-induced membrane dynamical changes are causally related to the membrane lipid-Hsp17 interaction, *Synechocystis hsp*17$^-$ mutant and *hsp*17$^+$ revertants were analyzed. The more heat-sensitive mutant displayed a significantly reduced stability of thylakoid electron transfer, in parallel with a strong increase in membrane fluidity as measured by DPH anisotropy [115]. In addition, differential scanning calorimetry studies revealed that even at an extremely low protein:lipid molar ratio, Hsp17 strongly stabilizes the lamellar liquid-crystalline phase at the expense of the non-lamellar lipid phase (H_{II}), which is known to disrupt membranes under severe heat stress [114, 117]. It was suggested that together with GroEL [116, 118], Hsp17 behaves as an *amphitropic* protein and plays a dual role. Depending on its membrane or cytosolic location it may function as a 'membrane stabilizing factor' as well as a member of a multichaperone protein-folding network. As illustrated on Figure 3, membrane association of small Hsps could antagonize the heat-induced hyperfluidization of specific membrane domains and was capable to prevent formation of the membrane-disrupting non-bilayer lipid phase, simultaneously. Specific membrane domains may act as lipid-controlled stress sensors [87]. One can speculate that the association of small Hsps with membrane lipids may lead to downregulation of the ac-

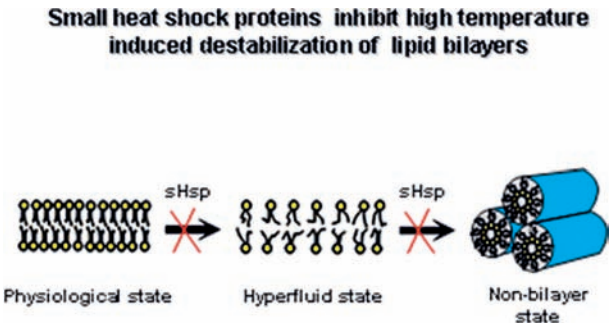

Figure 3. A subpopulation of small Hsps is actively involved in the protection of membranes in heat-stressed cells via lipid-specific association to membranes. This association aimed at normalizing the lateral packing order ('fluidity') and preventing formation of membrane-damaging non-bilayer lipid structures.

tivity of such lipid-controlled cellular thermometers, according to a hypothetic feedback mechanism [85–87].

Since lipid specificity seen with Hsp17 was also documented for αB-crystallin [117], it implies that membrane binding of small Hsps through a specific Hsp/lipid interaction may confine the location of the Hsps to one or more *membrane lipid domains*. In line with this assumption, a 'heat shock lipid', the highly saturated monoglucosyldiacylglycerol (MGlcDG) has been identified in *Synechocystis*. Out of five thylakoid polar lipid classes tested, MGlcDG, rapidly formed during heat/light stress conditions, expressed the strongest interaction with Hsp17 [119]. *Vice versa*, selective lipid interactions might also modify oligomeric organization and other, yet unrevealed features of small Hsps. Recently, missense mutations at 17 positions throughout the Hsp17 protein and a C-terminal truncation of five amino acids were identified [33] as described earlier, and subsequent biochemical assays separated these mutants into two groups. The C-terminal truncation and six mutations in the α-crystallin domain destabilized the small Hsp oligomer and reduced *in vitro* chaperone activity. In contrast, the other three mutations had little effect on oligomer stability or chaperone activity *in vitro*, although they significantly decreased small Hsp function *in vivo*. These mutations were clustered in the N-terminus of Hsp17, pointing to a previously unrecognized, important function for this evolutionarily variable domain. Furthermore, the fact that the N-terminal mutations were impaired in function *in vivo*, but active as chaperones *in vitro*, indicates that current biochemical assays do not adequately measure essential features of the small Hsp mechanism of action [33]. When point mutations of *Synechocystis* Hsp17 resulting in either a stable dissociation of small Hsp oligomers or causing reduced ability to dissociate [33, 120] were tested for their lipid interactions, it turned out that dissociation of the Hsp17 oligomer is necessary for their membrane lipid interaction [Balogi et al., unpublished results]. In favor of this finding, Zhang et al. have also shown that dissociation of the oligomers of benzyl alcohol-inducible Hsp16.3 in *M. tuberculosis* is a prerequisite for its plasma membrane binding [121]. Furthermore, the oligomeric structure of Hsp16.3 seemed to be more dynamic and flexible when it was coincubated with the mycobacterium lipids [121].

It was previously documented that deletion of *ibpA* and *ibpB* in *E. coli* did not produce a stress-sensitive mutant strain. However, overexpression of both genes increased resistance to heat and superoxide stress [89, 90]. Our recent efforts aimed at discovering the 'membrane phenotype' (i.e. identify cells possessing altered membrane permeability and fluidity characteristics) by comparison of wild-type with that of Δ*ibpAB E. coli* cells. We concluded that in accordance with the membrane (particularly outer membrane) localization of a significant portion of both IbpA and IbpB proteins, deletion of these small Hsps caused significantly elevated membrane permeability and fluidity if tested under severe (48–55 °C) heat stress conditions [Balogi et al., unpublished results]. Supporting the *in vivo* observations, lipid-small Hsp association dramatically reduced membrane fluidity, especially at high temperatures [Balogi et al., unpublished results]. We note that recently a very similar observation was made by testing lipid interaction and subsequent fluidity changes caused by the small Hsp of lactic acid bacterium, Lo18 [122].

Taken together, the above data reinforce the hypothesis that a lipid-associated pool of small Hsps may play an important role in protection of membranes under stress conditions. In recent years, our understanding of the structure-function relationship of biological membranes has changed considerably as our knowledge of lipid microdomains has expanded [86, 87, 123]. Since lipid microdomains have been widely shown to play important roles in the compartmentalization, modulation and integration of cell signaling, we have suggested that these microdomains may additionally have an influential role in stress sensing and signaling [86, 87]. Thermally controlled, lipid-mediated and transient association of small Hsps to specific membrane domains widely documented from prokaryotes to mammalian cells may also serve as part of a feedback mechanism in the regulation of heat shock genes.

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