Review

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Small heat shock proteins: recent developments

Abstract: Small heat shock proteins (sHSPs) are abundantly present in many different organisms at elevated temperatures. Members of the subgroup of alpha crystallin domain (ACD)-type sHSPs belong to the large family of protein chaperones. They bind non-native proteins in an ATP-independent manner, thereby holding the incorporated clients soluble for subsequent refolding by other molecular chaperoning systems. sHSPs do not actively refold incorporated peptides therefore they are sometimes referred to as holdases. Varying numbers of sHSPs have been documented in the different domains of life and dependent on the analyzed organism. Generally, diverse sHSPs possess more sequence similarities in the conserved ACD, whereas the N- and C-terminal extensions are less conserved. Despite their designation as sHSPs, they are not solely present during heat stress. sHSPs presumably help to protect cells under various stresses, but they were also found during development, e.g., in embryonic development of higher plants which is associated with ongoing seed desiccation. The functional and physiological relevance of several different sHSPs in one organism remains still unclear, especially in plants where several highly similar sHSPs are present in the same compartment. The wide range of biotic and abiotic stresses that induce the expression of multiple sHSP genes makes it challenging to define the physiological relevance of each of these versatile proteins.

Keywords: heat stress response; small heat shock proteins.

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Introduction

Years ago it was reported that organisms respond to an increase in temperature with the induction of a specific set of genes (1). High temperature can cause severe problems and is therefore often accompanied by structural and metabolic rearrangements within an organism, including membrane stiffening or change in protein composition on a cellular level (2). Temperature-induced occurrence of unfolded proteins causes a variety of different responses within a cell to maintain functionality and to circumvent harmful accumulation of protein aggregations (3). One response – the induction of the synthesis of small heat shock proteins (sHSP) – provides a transient and highly dynamic protein based environment that is able to bind thermally instable proteins (clients) in an ATP-independent way. Thereby irreversible protein denaturation and aggregation is prevented (4, 5). Few ACD-type sHSPs are found in unicellular organisms like archaea, bacteria or yeast (6–8), more than ten in humans (9, 10) and even more in plants (11–13). The general believe that bacteria have only a low number of sHSPs has been refuted by the identification of multiple sHSPs in α-proteobacteria like *Rhizobium* and *Bradyrhizobium* species (14). Proteins of the here reviewed sHSP family are characterized by their small monomeric sizes (12–45 kDa), the conserved ACD of about 80–100 residues (15–17), formation of large oligomers and an ATP-independent chaperone activity (4). The conserved ACD is flanked by a variable, not conserved N-terminal domain and a short C-terminal extension that can also be absent in some cases. In most cases the presence of sHSPs led to protection from heat-induced aggregation but not to activity preservation of thermally instable proteins (5, 18, 19). At heat stress (HS) many different proteins are produced, but not all comprise the mentioned ACD sHSP-defining characteristics. This review summarizes exemplarily chosen aspects of different methods of transcriptional and translational control, as well as structural and functional similarities and singularities of ACD-type sHSPs.

Regulation of sHSP gene expression

sHSP gene expression in eukaryotes

In eukaryotes the transcription of sHSP genes is regulated by a number of different HS transcription factors

(Hsfs). They bind to specific consensus sequences in front of HS-induced genes, so-called heat shock elements (HSE). In particular, in plants a complex world of different Hsfs has emerged (20–22). As an immobile organism in a varying habitat, a plant has to cope with and adapt to special challenges, such as day-to-night temperature differences or the changing availability of resources like light or water. These circumstances could have promoted the functional diversification of Hsfs and sHSPs in plants. Despite their designation as sHSPs, these proteins are not solely present at HS. Expression is induced at the levels of transcription and translation in response to various stresses like osmotic, cold and salt stress, amino acid analogues and pathogen attack (12, 23). Independent from any occurring stress, a development dependent presence of selected sHSPs is also observed. Regulated by specific Hsfs and a modified HSE in case of seed maturation (24–26), some sHSPs are produced during the development of the petals, pollen and seeds (25, 27, 28). In general the complex Hsf world shows a great plasticity in interactions with one another, which specifies the response (26, 29, 30). The well-studied example of three tomato Hsfs (Hsf A1, A2, B1) illustrates the functional diversification and cooperation of plant Hsfs. All together, these form a triad for subsequent responses at different stress phases. HsfA1 is the master regulator of the heat shock response (HSR). HsfA1/B2 heterooligomers trigger the HSR, but HsfB1 has a dual function. It can maintain the HSR alone, or during the recovery phase it restores, in cooperation with housekeeping transcription factors, the housekeeping gene transcription. A subsequent HS during the recovery phase induces heterooligomer formation of HsfA1 and HsfA2, which leads to a rapid recovery of the HSR (20, 22, 31, 32). In particular, during repeated cycles of HS, interactions between specific sHSPs and Hsfs might influence and coordinate the HSR by modulating the intracellular localization and activator function of Hsfs (Figure 1). In tomato Hsp17.4-CII functions as co-regulator and cytoplasmic retention factor of HsfA2, thereby the sHSP exerts a repressive effect on the transcriptional activator activity of the bound Hsf (33). However, only a few cases of interactions between sHSPs and Hsfs are documented. Interactions between Hsp70 or Hsp90 and Hsfs – especially at the beginning and end of the HSR – are more frequently described (31, 34, 35). In animals, sHSPs are associated with a variety of different HS-independent responses comparable to plants. In contrast to plants, animals developed more specialized tissues and cell types, so the development of more complex regulatory concepts should not be surprising, but it appears that compared to plants the multiplicity of Hsfs and sHSPs is much smaller in other

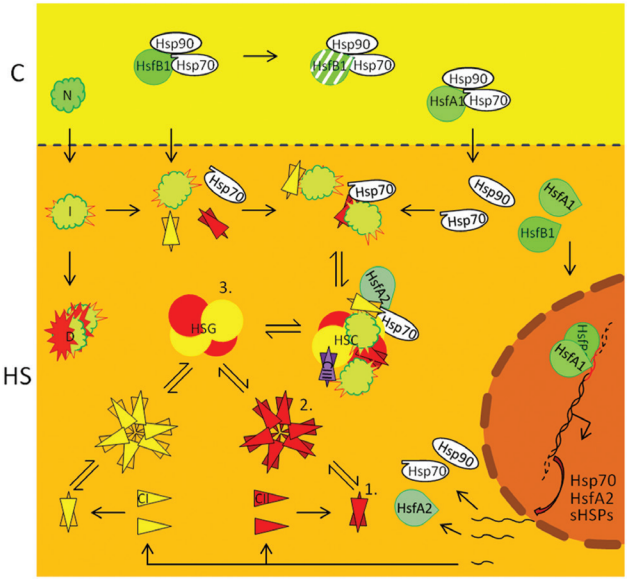

Figure 1 Schematic model of HSG and HSC formation and their influence on acclimation.
The HSR was extensively studied in tomatoes. Under control conditions (C) Hsp70 binds to HsfA1 (the major regulator of the HSR) and turns it inactive. Hsp90 binds and marks HsfB1 for rapid degradation. At HS, thermally instable proteins lose their native structure (N), whereby increasing amounts of intermediate products (I) start to attract Hsp70 and Hsp90. These high molecular weight chaperones try to prevent the aggregation of denatured proteins (D) and the released Hsfs can now activate the expression of HS regulated genes. New produced sHSPs and Hsp70 support in aggregation protection. sHSPs are highly dynamic proteins, so it is unclear whether they incorporate clients as (1) dimer, (2) oligomer or (3) in granular form. There is some evidence that at least for class I the dimeric form binds to unfolding proteins and further assembles in the other two structures, which presumably serve as storage forms. sHSPs of class II were described to be impaired in their dissociation into dimers. Thus, differences to the simplified scheme presented here are possible. The incorporation of clients in HSGs promotes the formation of HSCs. These structures exert an indirect regulative function by storage of the HsfA1/HsfB1 dependently produced HsfA2. Under recovery conditions Hsp70 and Hsp101 refold the incorporated proteins and the HSEs occupied by Hsfs are cleared. Hsp90 promotes rapid degradation of HsfB1 (to some extent HsfB1 also contributes in restoring the transcription of housekeeping genes) and Hsp70 binds to HsfA1 again. At a repeated HS during the recovery, HsfA2 assists HsfA1 (again released from Hsp70) in rapid transcriptional reactivation of HS-dependent genes.

organisms, including humans (36). Yeast comprises one Hsf and animals normally comprise three-to-four Hsfs, and their activity is often regulated by interactions of the Hsf with high molecular weight chaperones, e.g., Hsp70 (37–39). Thus, the basic mode of HSR regulation in animals seems comparable to plants even though differences are found. In animals, all Hsfs are constitutively present and they are often regulated by phosphorylation (40). In

plants, the appearance of some sHsfs is HS-induced, and it was believed for a long time that phosphorylation of Hsfs is a unique feature to animals. However, recent findings described phosphorylation-dependent localization and stability of HsfA2 in *Arabidopsis* (41).

sHSP gene expression in prokaryotes

The transcription of HS-activated genes in prokaryotes often underlies negative regulating sequence elements, such as CIRCE or HAIR elements which control and influence the expression of heat shock genes (42). The CIRCE system (controlling inverted repeat of chaperone expression) is one of the most widely distributed negative *cis* acting DNA elements in heat shock regulation. HrcA (heat regulation at CIRCE) binds as a repressor to CIRCE and inhibits transcription of HS-dependent genes (43–45). The inhibitory effect of HrcA itself depends on GroESL and during HS the depletion of the GroE pool by denatured proteins renders HrcA inactive, leading to elevated transcription of CIRCE-controlled heat shock genes (46–48). The prokaryotic transcription of genes is regulated by sigma factors, exchangeable RNA polymerase subunits, which specifically recruit the polymerase for gene transcription. In the well-studied *Eschericha coli* model, protein quality control is regulated by sigma32 (σ^{32}), also known as heat shock sigma factor RpoH (49–51). In several α-proteobacteria, two or more paralogs of RpoH exist, suggesting functional divergence of this alternative transcription factor (52). In *Rhodobacter*, an exemplarily chosen member of this bacterial class, HS response underlies the specialized sigma factor RpoH(I), whereas the close paralog RpoH(II) controls the oxidative stress response (53, 54). In *E. coli*, DnaK (Hsp70), GrpE (Hsp24) and DnaJ (Hsp40) sequester RpoH under non-stress conditions and DnaK promotes RpoH degradation by the FtsH protease. HS-induced unfolding of thermally instable proteins leads to the recruitment of DnaK from the bound sigma factor (51, 55). This chaperone-mediated negative feedback control allows the alternative transcription factor to sense the cellular folding state. The response is further indirectly modulated by the RNA binding protein Hfq (host factor required for replication of the RNA phage Qβ), which is also involved in stress acclimation (56, 57). Hfq associates with small regulatory RNAs (sRNAs) to promote their base-pairing with target mRNAs, thereby the sRNA-mRNA pairing affects the translation rate and lifetime of the targeted transcript (58). In this case, it controls the translation efficiency of several HS-dependent gene products including *dnaK* mRNA translation thereby regulating the DnaK level within the cell (57). In addition to the already complex network, the amount of RpoH in a cell is directly regulated by the *rpoH* mRNA itself in a temperature-dependent mechanism. The *rpoH* mRNA and also some sHSP gene products belong to a group of RNA thermometers, which respond at high temperature with exposure of the ribosomal binding site. The highly structured mRNA unfolds at high temperatures and provides ribosomal subunits access to the Shine Dalgarno sequence for translation initiation (59–61). Other examples are the Hsp17 thermometer (62) or so-called ROSE (repression of heat shock gene expression) elements in rhizobial species, which control heat shock gene product translation in a similar way (48, 63, 64). As described for *Agrobacterium tumefaciens*, it is also possible that several occurring sHSP genes are regulated differently in a replicon dependent manner. While the sHSP gene on the linear chromosome turned out to be regulated by RpoH, transcripts of the sHSP genes on the *A. tumefaciens* plasmids were under the control of ROSE sequences in their 5′ untranslated region (65). In summary, this tight and optimized regulation of the cytoplasmic stress response is important for unicellular organisms to cope with suddenly occurring stress and recovery conditions in an adequate way.

Localization and physiological relevance

Most organisms except plants do not possess an extended ACD-type sHSP family though exceptions are found. To date, 10 different sHSPs were described for humans and even more for some other vertebrates like birds or fish (9, 66). These sHSPs were found in various cell types, from eye lenses to sperm cells (10) and also in diverse compartments from cytosol, medial golgi apparatus to mitochondrial membranes (67, 68). Nevertheless, the existence of highly similar sHSPs in one cellular compartment forming a superfamily with distinguishable subfamilies [Figure 2; (69–71)] is a unique feature of plants (12, 72, 73). In particular, the diversity of plant sHSPs displays that these proteins can be localized virtually to all organelles of a cell, such as the chloroplasts, mitochondria, nucleus, endoplasmatic reticulum and peroxisomes (11, 72, 74). Although the cytosolic groups of sHSPs do often not possess any familiar nuclear localization signal (NLS), studies indicated that these sHSPs are distributed between cell plasma and nucleus (12). So far, the physiological function of normally cytosolic sHSPs in the nucleus is often

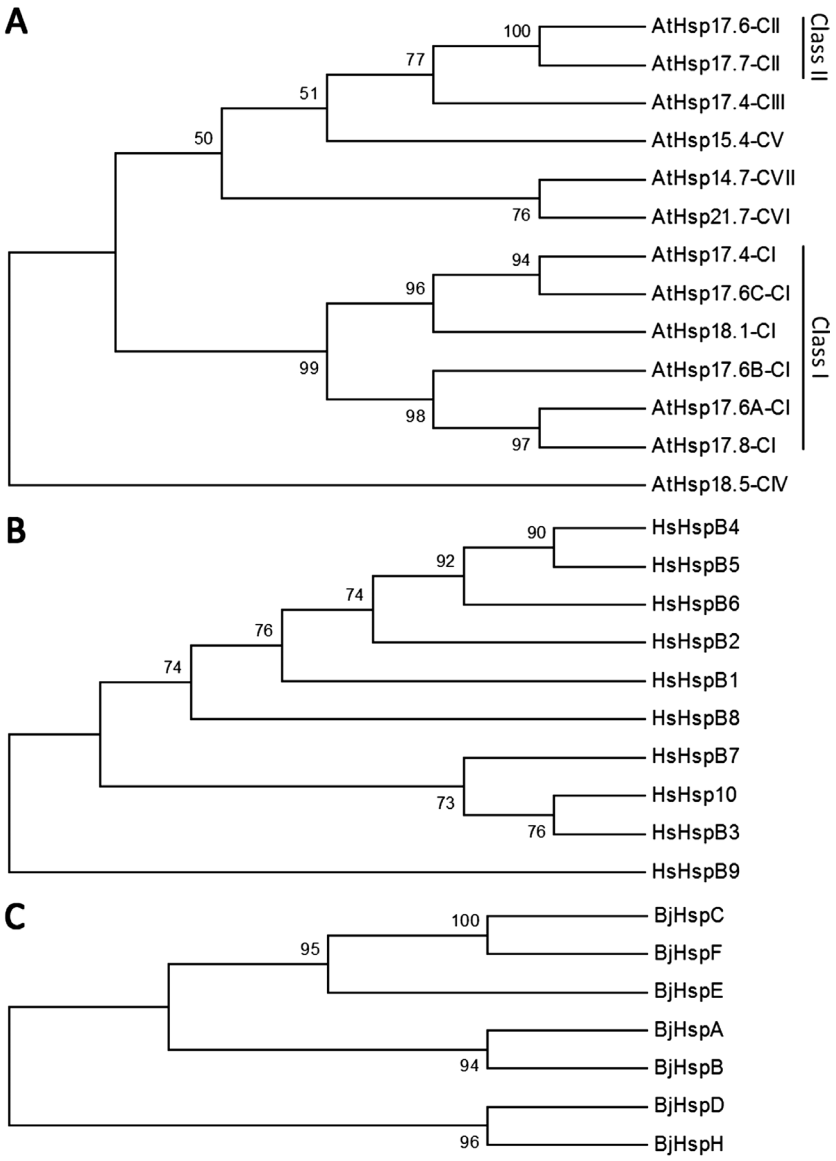

Figure 2 The cytosolic sHSP group of plants.
Comparing (A), (B) and (C) illustrates the unique feature of plants by possessing several highly related sHSPs in one compartment (cytosol) that can be sorted in different classes (CI–CVII). In other species with several sHSPs (e.g., humans or Bradyrhizobium) this phenomenon is absent. Nucleotide sequences (codons) were aligned with clustalW and the evolutionary history was inferred using the maximum parsimony (MP) method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The MP tree was obtained using default settings. Evolutionary analyses were conducted in MEGA5. The two major cytosolic sHSP classes are marked. At, *Arabidopsis thaliana*; Hs, *Homo sapiens*; Bj, *Bradyrhizobium japonicum*.

unclear. However, several sHSPs with a typical NLS are known (CIII in Figure 2), which are specifically targeted to the nucleus (11, 72, 75). This multiplicity of sHSPs in one organism, especially in plants, complicates a clear separation of the *in vivo* function of single sHSPs. Nevertheless, sHSPs appeared to be beneficial for different aspects of life, in particular under protein misfolding and other cell metabolism destabilizing conditions. As described for the golgi localized Hsp-16.1 of *Caenorhabditis elegans*, sHSPs are associated with acclimation processes and can protect from heat-stroke associated neurodegeneration (68). In addition to acclimatization, members of this group of proteins were reported to be involved in several other protection mechanisms, such as during the encystment and diapauses in *Artemia franciscana* (76–78). In humans, the well-studied αA- and αB-crystallins (CRYAA and CRYAB) prevent from cataract formation [summarized in (79, 80)]. Mutations in these and other human sHSPs are associated

with a wide range of diseases, the majority of which are neuropathies and myopathies (81–84). Besides possible interactions with elements of the cytoskeleton (85–87), different members of the chaperone family are found to be present at sites of inflammation (88, 89) and cellular defects like ischemic acute renal failure (90) or excitotoxic lesions (91). As a general protective effect, it was reported that high levels of sHSPs extend the life span of some organisms (92–94). In *Synechocystis* a sHSP (Hsp17) was found to be involved in membrane stabilization (95, 96), in *Chlamydomonas* Hsp-22 in photosystem protection (97) and AtHsp17.8-CI in *Arabidopsis* facilitated the protein reallocation from the cytosol to the outer chloroplast membrane (98). sHSPs with methionines as redox sensor motif (e.g., AtHsp25.3-P in *Arabidopsis*) were described in chloroplasts (99). Chloroplast-localized sHSPs often show a temperature-dependent membrane association (100, 101), maybe acting in a comparable way as described for Hsp17 from *Synechocystis* (102). In general, sHSPs can interact with a wide variety of proteins *in vivo*, thereby preserving a wide range of cellular functions (103). In plants they assist during several developmental processes, such as seed development (25), and it is assumed that they confer enhanced desiccation tolerance to the embryo (104). In prokaryotes, only a lower number of different sHSPs within an organism are usually detected (with exceptions), however wide effects on different physiological functions were found. In *E. coli* sHSPs were first described associated to inclusion bodies (105), therefore named 'inclusion body associated proteins' (IbpA/IbpB). But besides their responsiveness to unfolded proteins during heterologous protein expressions, both proteins are involved in heat and oxidant protection mechanisms (106, 107). In addition, sHSPs with unusual functions are known. In *Bacillus subtilis*, an ACD-containing sHSP (CotM) was identified as a spore coat protein (108, 109). The plant pathogen *A. tumefaciens* uses HspL as virulence promoting factor (110–112). Thus sHSPs exert pleiotropic effects throughout the life and it cannot be excluded that new functions will be discovered. Schematic structures of some of the described ACD-containing sHSPs are summarized in Figure 3. Table 1 summarizes the accession numbers of all discussed sHSPs.

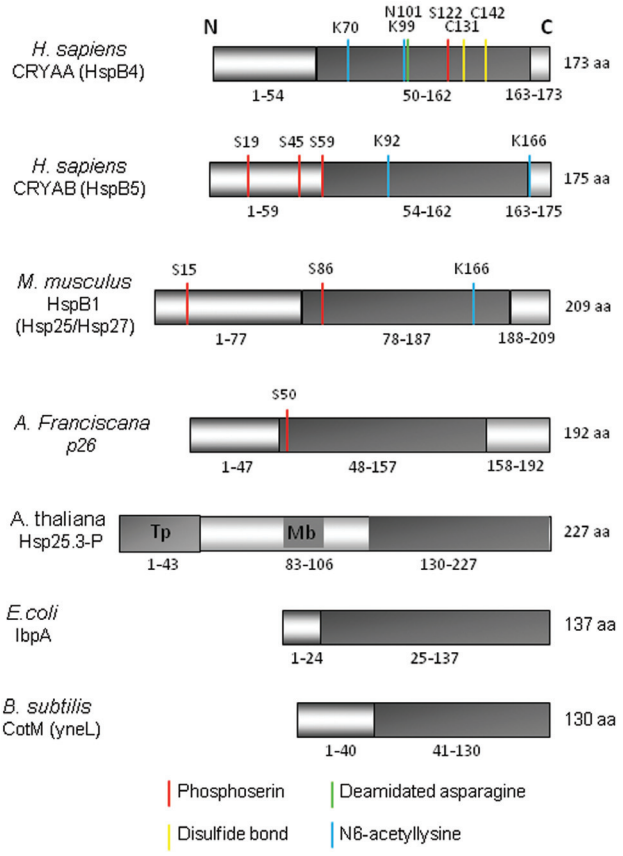

Figure 3 Comparative schematic composition of ACD-type sHSPs from different organisms.
Indicated amino acid positions (aa) for shown domains and modifications were taken from following protein accession numbers: *Homo sapiens* αA crystallin (CRYAA/P02489), *Homo sapiens* αB crystallin (CRYAB/P02511), *Mus musculus* HspB1 (P14602), *Artemia franciscana* p26 (O44112), *Arabidopsis thaliana* HSP25.3-P (P31170), *Escherichia coli* IbpA (P0C054), *Bacillus subtilis* CotM (Q45058). Zn^{2+} binding histidine residues of αA/B crystallin are not shown. Tp, transit peptide; Mb, methionine bristle.

Structure and molecular function

The ACD-type sHSPs were originally summarized as a group of small HS-induced proteins (15–45 kDa) with an ACD and an ability to form high molecular weight complexes up to 150–800 kDa in size. sHSP oligomers are dynamic structures that often underlie temperature- or post-translational modification-dependent structural rearrangements. With few exceptions, dimers act as basic structural subunits, which further assemble into an oligomeric structure composed of an even number of subunits like 12, 24 and 36mers (113–115). Nonamers with a trimer of trimers or oligomeric structures with tetramers as subunits were also discovered, but are more unusual (116, 117). For quite a number of sHSPs from different organisms (plants, animals and prokaryotes) structural data is available, even though the crystallization of full length sHSPs is difficult. The highly dynamic N-terminal domain complicates stable positioning of sHSPs, a prerequisite for the crystallization process and subsequent data

Table 1 Summary of discussed sHSPs.

Name	Synonym	Organism	Accesion number (protein)	Length (aa)	MW (kDa)	Localization
AtHsp17.4-CI	–	*A. thaliana*	P19036	156	17.4	c/n
AtHsp17.6A-CI	–	*A. thaliana*	Q9XIE3	155	17.6	c/n
AtHsp17.6B-CI	–	*A. thaliana*	Q9ZW31	153	17.6	c/n
AtHsp17.6C-CI	Hsp17.6	*A. thaliana*	P13853	157	17.6	c/n
AtHsp17.8-CI	–	*A. thaliana*	Q9LNW0	157	17.8	c/n
AtHsp18.1-CI	Hsp18.2	*A. thaliana*	P19037	161	18.1	c/n
AtHsp17.6-CII	Hsp17.6-II	*A. thaliana*	P29830	155	17.6	c/n
AtHsp17.7-CII	Hsp17.6A	*A. thaliana*	O81822	156	17.7	c/n
AtHsp17.4-CIII	–	*A. thaliana*	Q9SYG1	155	17.4	c/n
AtHsp18.5-CIV	AtHsp18.5-CI(r)	*A. thaliana*	O64564	162	18.5	c/n
AtHsp15.4-CV	AtHsp15.4-CI(r)	*A. thaliana*	O49710	134	15.4	c/n
AtHsp21.7-CVI	AtHsp21.7-CI(r)	*A. thaliana*	Q9FIT9	192	21.7	c/n
AtHsp14.7-CVII	AtHsp14.2-P(r)	*A. thaliana*	Q6NLV0	131	14.7	c/n
AtHsp25.3-P	Hsp21	*A. thaliana*	P31170	227	25.3	p
TaHsp17.6B-CI		*T. aestivum*	Q41560	151	16.9	c
LpHsp16.1-CIII		*L. peruvianum*	Q94EN7	144	16.1	n/c
Afp26		*A. franciscana*	O44112	192	20.8	–
CeHsp-16.1		*C. elegans*	P34696	145	16.3	gmc
Tsp36	R-Tso2	*T. saginata*	Q7YZT0	314	35.6	c
MmHspB1	Hsp25	*M. musculus*	P14602	209	23.0	c/n
HsHspB1	Hsp25/27	*H. sapiens sapiens*	P04792	205	22.8	c/n
HsHspB4	CRYAA	*H. sapiens sapiens*	P02489	173	19.9	c/n
HsHspB5	CRYAB	*H. sapiens sapiens*	P02511	175	20.2	c/n
ScHsp26		*S. cerevisiae*	P15992	214	23.9	c/n
BsCotM	yneL	*B. subtilis*	Q45058	130	15.2	sc
MjHsp16.5		*M. jannaschii*	Q57733	147	16.5	c
SsHsp17		S. PCC 6803	L8AFE3	146	16.6	c/m
XaHspA		*Xanthomonas*	ADI78883	158	17.7	–
MtHspX	Acr, Nox16	*M. tuberculosis*	P0A5B7	144	16.2	c, cw
RsHsp20		*R. sphaeroides*	Q3IWA3	177	19.0	–
BjHspA		*B. japonicum*	P70917	152	17.2	–
EcIbpA		*E. coli*	P0C054	137	15.8	c, om
EcIbpB		*E. coli*	P0C058	142	16.1	c, om

c, cytoplasm; n, nucleus; m, membrane; p, plastids; gmc, golgi medial cisterna; sc, spore coat; cw, cell wall; om, outer membrane; –, not defined.

acquisition. In many cases (e.g., *Methanocaldococcus jannaschii* Hsp16.5; *Xanthomonas citri* pv. *citri* HspA, Human Hsp27 and αB-crystallin) structures were solved without the N-terminus. The dodecameric Hsp16.9 structure from *Triticum aestivum* (118) consists of only six complete subunits (residues 2–151), while the other six subunits are missing the first 42 N-terminal residues [summarized in (119)]. However the existing data increased our knowledge regarding structural and functional aspects of different sHSPs. sHSPs belong to the large family of protein chaperones. Incorrect folded proteins are recognized and bound by sHSPs in an ATP-independent manner. The incorporated protein stays protected in high molecular weight complexes for subsequent refolding by other members of the molecular chaperone network. For client reconstitution, ATP-dependent high molecular weight chaperoning machineries are needed (120, 121), for example, members of the Hsp70/40 class (DnaK/DnaJ in prokaryotes). In the case of improperly refolded clients, a linkage to a degradation or recycling pathway would be reasonable, but was not clearly identified up to now (119). Normally ACD-type sHSPs are holdases and cannot refold incorporated clients on their own. Few exceptions were described where certain sHSPs seem to have refolding function, but these examples are rare (122). A detailed *in silico* examination found characteristic differences in segment lengths between plants, animals and bacteria (123). Generally the ACD of sHSPs seems to be a platform for flexible arms that capture clients to maintain their solubility. Taking a look at the different structural sections, the highly variable and often hydrophobic N-terminus appeared to be necessary for client binding, the

β-sheeted ACD for the dimer assembly and the C-terminal extension with the basic IXI/V motif (118) responsible for oligomer formation. But it is not possible to generalize that the N-terminus is the major client recognition and protection site and the C-terminus is responsible for the control of the oligomer size. In many cases all sections have overlapping functions (124, 125). The exact mechanism of how clients are bound and protected is not completely solved until now. At HS, different proteins bound on different sites at the N-terminal sHSP arm (126) and the proteins did not show any commonality in sequence or structure that could be a signature for a sHSP binding target (103). Based on the available data of sHSP structures, different concepts of mechanistic modus operandi are discussed. It is unclear whether heat-induced oligomer dissociation into dimers is indispensable for client recognition and protection (127) or not (113). Comparing different analyses might suggest that the functional relevance of heat-induced dissociation may vary between sHSPs and the examined organism – but at least for most of the analyzed plant sHSPs, oligomer dissociation seems to be a prerequisite for client protection (127, 128). Exceptions might be dimeric sHSPs without a dominant oligomeric structure such as AtHsp17.8-CI or AtHsp18.5-CV in *Arabidopsis thaliana*. As already described for AtHsp17.8-CI, it has (besides its chaperone activity) an additional function in protein reallocation processes within the cell and perhaps the dimeric structure facilitates this function (18, 98). The functional and physiological relevance of the presence of multiple cytosolic and nuclear localized similar proteins in plants remains a matter of debate. Aspects of cooperative function was discussed, but mainly additive not cooperative client protection was measured (129). At finding a clear connection between the oligomeric state of sHSPs and their client protection capacity, interesting results were obtained, because *in vitro* and *in vivo* observations were inconsistent with one another. A screening for mutations of *Synechocystis* Hsp17 that reduced the ability of the protein to provide thermo tolerance *in vivo* identified two groups of alterations. One group of mutations destabilized the oligomer and reduced the *in vitro* chaperone activity. The other group of mutations, especially in the N-terminus, had little effect on the oligomer stability or chaperone activity *in vitro*. As these mutations still failed to provide thermo tolerance *in vivo*, the results indicate a previously unrecognized function of the N-terminus that is not adequately analyzed by current biochemical *in vitro* measurements. Therefore, it was concluded that some *in vitro* client protection measurements are not directly comparable to the *in vivo* situation (130). Changes in structure or protection efficiency do not only depend on heat-induced dissociation events, but cold-induced changes in protection capabilities were also observed in different organisms (131, 132). In addition, the oligomeric structure of sHSPs is in many organisms often regulated by post-translational modifications. In animals and humans the phosphorylation of sHSPs resulted in different visible or measurable responses, which presumably are all connected to a modification-dependent change of the dissociation and re-association behavior of the oligomer. Very early it was found that αA- and αB-crystallin undergo a large variety of post-translational modifications, such as deamidation, racemization, phosphorylation, acetylation, glycation and age-dependent truncation (summarized in (133) and in Figure 2). Some histidine residues involved in Zn^{2+} binding were newly identified (134), as earlier studies showed an increase of activity after Zn^{2+} addition (135). For the αA-crystallin, homo- and cAMP pathway-dependent phosphorylations were described (136–138). In the murine model system, non-phosphorylated Hsp25 monomers were active in inhibiting actin polymerization, while phosphorylated Hsp25 monomers and non-phosphorylated multimeric Hsp25 particles were inactive (139). Phosphorylation and other modifications were not found in plants or prokaryotes up to now. The redox-sensing chloroplast-localized sHSPs are one exception (140). These sHSPs share an oxidant-accessible region formed by methionines, referred to as 'methionine bristle' (141). Only sulfoxidation – not phosphorylation or heat treatment – leads to changes in structure and abolished the chaperone-like activity of the tested sHSP (99, 140, 142, 143). The exchange of the methionines to sulfoxidation-resistant leucines residues leads to a loss of redox-sensing capabilities but no decrease in chaperone function. The methionines are exclusively required as sensors but not for client protection (144).

Heat shock granules

Heat shock granules (HSG) are large cytosolic complexes of up to 1–2 MDa molecular weight that are present in all tissues during heat shock (145). So far this phenomenon has only been described in plants and is therefore presumably a unique feature of plants. They are formed in the presence of certain cytosolic sHSP members, whereat proteins of the cytosolic class II family seem to be a prerequisite (75, 146). In the cytosol of plants, several sHSPs are present under HS conditions. The classification of sHSP subfamilies in plants is based on differences and

similarities in their N-terminal sequences. sHSPs with high similarities in their N-terminal sequences are summarized in same class (see also Figure 2). There are many different cytosolic sHSP classes distinguishable, whereby class I comprises the most members (e.g., 6 in *A. thaliana*) and class II is often associated to HSG formation. These irregular predominantly globular-shaped cytosolic particles with up to 40 nm in size are composed of class I and class II sHSPs, and can further assemble in larger cytosolic heat shock complexes (HSCs), involving sHSP classes I-III as well as high molecular weight HSPs, e.g., members of the Hsp70 and Hsp40 family (12). HSG may build up a basic core complex, whereby sHSPs provide binding and incorporation of misfolded cell proteins, which are stored in HSCs for subsequent refolding by high molecular weight HSPs during recovery (schematic summary of HSG and HSCs is presented in Figure 1). Other proteins such as α- and β-tubulins were also found to be associated with HSCs, but partial digestion analysis revealed that these proteins, in contrast to sHSPs and to some extent Hsp70, are probably not integral part of the complexes and are therefore hypersensitive (susceptible) to proteolysis (147). Furthermore, HSG and HSC may be indirectly involved in transcriptional control by storage of Hsfs (32). Surprisingly, neither ubiquitin nor ubiquitin-protein conjugates were detected in these structures. This does not exclude that refolded proteins undergo cellular quality control mechanisms as usual, when they are released from the granules, or a missing linkage to the degradation systems will be found in future. Earlier findings suggested that HSG are able to incorporate and protect untranslated mRNAs (145), but comparative studies on messenger ribonucleo protein (mRNPs) homeostasis, which depends on rapid transitions between different functional states (translated mRNPs, untranslated mRNPs, mRNPs under degradation), clearly highlighted that mRNP-dependent cytosolic aggregates are different to HS-induced HSG. In plant cells, different types of cytoplasmic aggregates, such as stress granules (SGs) or processing bodies (PBs), are present in addition to HSG. On one hand, neither SGs (stalled mRNPs accumulated in cytosolic aggregates) nor PBs (sites of mRNP processing) contained sHSPs. On the other hand, HSG had no mRNA incorporated (148, 149). As class I proteins are not able to form granules on their own, HSG formation represents a specific assembly process that depends on formation of class II sHSP oligomers as a prerequisite for the auto-aggregation (146, 147).

Summary and outlook

The field of sHSP research is still developing. Besides the observation that the presence of sHSPs may be beneficial for an organism to cope with unfavorable conditions, the exact cellular function of several sHSPs still has to be elucidated. How clients are recognized and bound, as well as the role of multiple sHSPs in the same compartment of one organism, is not understood in detail. The fact that most of the tested sHSPs act as holdases *in vitro* does not necessarily explain the advantage of multiple in parallel up-regulated sHSPs in one location of a cell. Functional redundancy or cooperation may be a possible explanation, as well as more specified not yet identified functions of single sHSPs. In plants, they might have a buffer function in acclimated cells comparable to compatible compounds during osmotic stress conditions, but the efforts for an organism to produce so many sHSPs under HS seems to be not worth the cost. We could expect that more efficient systems should have evolved, if sHSPs should just perform buffering. Perhaps new technical approaches (e.g., mass spectroscopy based analysis of sHSP/client interactions, NMR structures of full length sHSP monomers) help to add new details in the emerging picture of sHSP function. sHSP are already used as stabilizers in molecular biological processes and their use is discussed for clinical therapies (150, 151). Thereby the oligomeric sHSP structure shall serve as a nano cage for drug delivery. Nonetheless, more insights in sHSP structure and function are needed to fulfill all the ambitious expectations.

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