REVIEW

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Small heat shock proteins from extremophiles: a review

Abstract Many microorganisms from extreme environments have been well characterized, and increasing access to genomic sequence data has recently allowed the analysis of the protein families related to stress responses. Heat shock proteins appear to be ubiquitous in extremophiles. In this review, we focus on the family of small heat shock proteins (sHSPs) from extremophiles, which are α-crystallin homologues. Like the α-crystallin eye lens proteins, sHSPs act as molecular chaperones and prevent aggregation of denatured proteins under heat and desiccation stress. Many putative sHSP homologues have been identified in the genomic sequences of all classes of extremophiles. Current studies of *shsp* gene expression have revealed mechanisms of regulation and activity distinct from other known *hsp* gene regulation systems. Biochemical studies on sHSPs are limited to thermophilic and hyperthermophilic organisms, and the only two available crystal structures of sHSPs from *Methanocaldococcus jannaschii*, a hyperthermophilic archaeon and a mesophilic eukaryote, have contributed significantly to an understanding of the mechanisms of action of sHSPs, although many aspects remain unclear.

Keywords α-Crystallins · Extremophiles · Molecular chaperones · Small heat shock proteins · Subunit assembly

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Introduction

Several decades ago, stress responses were first observed when chromosome puffs and the induction of specific proteins were observed in *Drosophila* under heat stress (Ritossa 1962). This discovery has led to numerous studies, and inducible stress responses appear to be pervasive in virtually all organisms. Although stress responses include all of the processes that organisms have developed to survive when they are exposed to environmental challenges, such as heat stress, desiccation, chemical stress, or starvation, the effector proteins are almost all referred to generically as heat shock proteins (HSPs). HSPs are diverse sets of proteins, usually classified based on their molecular weights, including, for example, HSP100, HSP90, HSP70, HSP60, and the small HSPs (Trent 1996). With some exceptions, each of these molecular weight classes includes the members of a single protein family or related protein families. Many of these proteins function as molecular chaperones, catalyzing the refolding of denatured proteins and assisting the folding of newly synthesized proteins, or else preventing protein aggregation. HSPs have been identified and characterized from various Bacteria and Archaea, and, like most organisms, they generally possess more than one set of HSPs.

In this review we have focused on the small heat shock proteins (sHSPs) from extremophiles. These sHSPs have the smallest subunits of all HSPs, with a monomeric molecular weight ranging from 15 to 40 kDa, and most of them appear to form multimeric complexes in vivo. Although they are one of the most diverse groups of HSPs, the majority of sHSPs share amino acid sequence similarity with the vertebrate eye lens α-crystallin proteins. Several lines of evidence indicate that both α-crystallins and sHSPs are molecular chaperones (Horwitz 1992; Jacob et al. 1993; Muchowski and Clark 1998; Muchowski et al. 1999; Haslbeck 2002). Currently, complete genome sequences from at least 25 extremophiles have been reported

Table 1 A survey of sHSP genes and proteins from organisms in the three phylogenetic domains. High pI sHSPs are shown in bold

Organism	Number of homologues	Accession number(s)	Isoelectric point	Molecular weight (kDa)
M. thermautotrophicus[a]	1	AAB85357	4.75	17.0
M. acetivorans STR C2A[a]	3	NP_618465	4.82	17.5
		NP_615107	4.44	17.7
		NP_619401	**9.87**	**31.2**
M. mazei Goe1[a]	4	NP_633443	4.71	17.8
		NP_632985	4.70	11.7
		NP_632984	4.30	11.0
		NP_632507	4.64	17.8
M. jannaschii[a]	1	Q57733	4.72	16.5
M. kandleri AV19[a]	1	AAM01219	4.82	19.0
S. solfataricus[a]	2	NP_343781	4.97	20.1
		NP_343935	**8.84**	**14.1**
S. tokodaii[a]	2	NP_376442	5.06	21.01
		NP_377625	**8.98**	**15.04**
A. pernix[a]	2	APE1950	5.58	20.9
		APE0103	4.86	18.4
P. aerophilum[a]	3	NP_560543	5.27	20.6
		NP_560503	7.53	13.1
		NP_559894	6.95	14.4
T. acidophilum[a]	2	CAC11613	4.58	21.0
		CAC11993	**9.27**	**14.4**
T. volcanium[a]	3	NP_111503	4.57	21.1
		NP_393915	5.10	43.9
		NP_111294	**9.22**	**14.3**
A. fulgidus[a]	2	G69411	4.64	20.4
		B69496	5.02	16.5
P. abyssi[a]	1	NP_126108	4.89	20.2
P. furiosus[a]	1	NP_579612	5.11	20.2
P. horikoshii[a]	1	D71196	4.91	20.8
Thermococcus KS-1[a]	1	BAB40930	5.60	20.0
Halobacterium NRC-1[a]	5	AAG20020	4.00	18.5
		AAG18726	4.06	14.3
		AAG20865	4.14	14.2
		AAG19995	5.12	12.4
		AAG18869	3.94	15.4
A. aeolicus[b]	1	A70411	5.09	17.2
T. elongates[b]	1	NP_681663	5.12	16.6
T. tengcongensis[b]	1	NP_624099	5.11	17.1
S. enterica[b]	1	NP_456260	5.31	17.7
O. sativa[c]	1	CAA43210	6.55	16.9
T. aestivum[c]	1	CAA45902	6.56	16.9
H. sapiens α-crystallins[c]	2	NP_000385	6.15	19.9
		NP_001876	7.37	20.2

[a]Archaea
[b]Bacteria
[c]Eukaryotes

(Robb 2003) and, through annotation of these genomes, putative *shsp* genes have been identified (Table 1). However, biochemical characterization is still limited to thermophilic and hyperthermophilic organisms. The deduced amino acid sequences from putative *shsp* genes revealed that the sHSPs from extremophiles share motifs and domain structures such as the amino terminal and α-crystallin and carboxy-terminal domains found in other sHSPs.

The expression of sHSPs has been studied in several extremophiles, and both positive and negative regulation have been found. Currently, there are a few reports on the mechanisms of *shsp* gene regulation in thermophiles from both Bacteria and Archaea (Kojima and Nakamoto 2002; Vierke et al. 2002). These studies reveal novel regulators and *cis*-acting sequence elements in *shsp* gene expression. It appears that gene expression of sHSPs in these thermophiles is negatively regulated and that the process may be analogous to the negative control of *shsp* gene expression in other organisms.

Structural analysis has been a critical factor leading to recent insights into sHSP functions. Currently two crystal structures from unrelated organisms, *Methanocaldococcus jannaschii* and *Triticum aestivum* (wheat), have been reported (Kim et al. 1998a; van Montfort et al. 2001). It appears that although the amino acid sequence similarity of these two proteins is low and their quaternary structures are dissimilar, the monomeric structures of these proteins are almost identical. This leads to the hypothesis that many sHSPs share a common building-block structure but that their mechanisms of action may be defined by their individual quaternary structures depending on the species and target proteins. Due to limited experimentation, the mechanisms of action of sHSPs remain unclear. However, it appears that most sHSPs from extremophiles are molecular

chaperones that can protect denatured proteins from aggregation under very severe conditions or, in some cases, are able to refold denatured proteins (Kim et al. 1998c; Roy et al. 1999; Laksanalamai et al. 2001; Usui et al. 2001). The structures of these sHSPs are discussed in a subsequent section.

Complete genome sequences reveal putative sHSPs from extremophiles

By examining the available complete genome sequences, about 30–40 putative sHSP genes have been identified from extremophiles (Table 1). As mentioned previously, small heat shock proteins share significant sequence similarity with α-crystallin proteins found in vertebrate eye lenses (Caspers et al. 1995; de Jong et al. 1998). The copy number of sHSP encoding genes has been found to be variable among species, and most species contain more than one subunit (Caspers et al. 1995). Although sHSPs are ubiquitous proteins found throughout the three domains, sHSPs or α-crystallin homologues have not been found in the genomes of pathogenic bacteria such as *Mycoplasma genitalium*, *Haemophilus influenzae*, and *Helicobacter pylori* (Fleischmann et al. 1995; Fraser et al. 1995; Tomb et al. 1997; Narberhaus 2002). Interestingly, most plants appear to encode multiple sHSPs (Waters and Vierling 1999; Scharf et al. 2001; Sun et al. 2002).

Most thermophilic and hyperthermophilic archaea contain only one or two sHSP homologues, with the exception of *Pyrobaculum aerophilum*, where three sHSP homologues have been identified. Three sHSPs were also found in the genome of *Thermoplasma acidophilum*. However, one of these sHSPs appears to be related to the two ATPase domains of ArsA from *Escherichia coli* (Ruepp et al. 2001). Two hypothetical proteins from the Sulfolobus tokodaii genome (NP_377625 and NP_376442) appear to be small heat shock protein homologues (Table 1). *Sulfolobus solfataricus* and *S. tokodaii* thus appear to have complements of one 14–15 kDa and one 20–21 kDa sHSP each. Thermophilic bacteria reported so far, however, contain only a single sHSP homologue. The mesophilic methanogens *Methanosarcina acetivorans* and *Methanosarcina mazei* GoE1 contain three and four sHSP homologues, respectively. However, the 34-kDa sHSP from *M. acetivorans* (NP_619401) is a basic protein that does not appear to belong to the α-crystallin-type HSPs. The genome sequence of *Halobacterium* NRC-1 revealed the highest paralogy (Ng et al. 2000) (Table 1). Although the five sHSPs from *Halobacterium* are not highly conserved, they all belong to the α-crystallin family. Unlike some other HSPs, such as HSP60, in which heterologous complex formation between different subunits has been clearly demonstrated (Waldmann et al. 1995; Nitsch et al. 1997; Yaoi et al. 1998), there is no evidence regarding sHSP complexes with heterologous subunit composition.

Characteristics and relationship of sHSPs from extremophiles

Various studies of sHSP and α-crystallins reveal that the proteins contain three distinguishable regions. The α-crystallin domain containing 80–100 amino acid residues is flanked by a variable amino terminal and a short extension of the carboxy-terminal domain (Caspers et al. 1995; de Jong et al. 1998; Kim et al. 1998a). The sHSPs from extremophiles appear to have this characteristic as well (Fig. 1). Generally, sHSPs even from closely related groups of organisms reveal low sequence similarity, including multiple sHSPs from the same organisms (Fig. 1). In extremophiles with more than one sHSP homologue, pairwise comparisons between sHSPs from the same organisms revealed that the sHSPs are between 20% and 50% identical. On the other hand, closely related organisms from hyperthermophiles such as the Thermococcaceae family, *Pyrococcus furiosus*, *Pyrococcus abyssi*, *Pyrococcus horikoshii*, and *Thermococcus* KS-1 appear to have sHSPs with amino acid sequence identity of more than 75% (data not shown). The N-terminal regions are very diverse and there are several insertion-deletion regions among sHSPs. The N-terminal domain is also disordered in the crystal structure of *Methanocaldococcus jannaschii* sHSP (Mj-sHSP) (Kim et al. 1998a). Within the α-crystallin domain, according to the alignment shown in Fig. 1, there are three conserved regions throughout eukaryotes, bacteria, and archaea. The most conserved motif of the α-crystallin-type HSPs (AxxxxGvL) (Caspers et al. 1995; de Jong et al. 1998; Narberhaus 2002) has also been observed in all of the sHSPs from extremophiles (Fig. 1). The motifs PGxxked and the two-residue region (LP), which are located upstream from the first motif, are also conserved throughout the sHSPs within the three domains. The carboxy-terminal extensions are not highly conserved among sHSPs, but most contain several charged amino acid residues (E and K). This domain is involved in multi-subunit assembly.

It has been suggested that there is a shift of the average isoelectric point (pI) of the proteome toward basic values as the optimum growth temperature increases to minimize protein aggregation with variable growth temperature (Kawashima et al. 2000). In extremophiles, most sHSPs appear to be acidic, with the pI ranging from 4 to 6 (Table 1). However, a small number of sHSPs appear to be very basic proteins with pIs of more than 8 (indicated in bold in Table 1). This suggests a possible alternative role of these sHSPs as nucleotide binding proteins as found in the HSP15 from *E. coli* (Korber et al. 1999, 2000; Staker et al. 2000). Nevertheless, when they occur in extremophiles, basic sHSPs are always accompanied by at least one paralogue with an acidic pI in extremophiles with complete genome sequences (Table 1). Nucleotide binding by basic sHSPs therefore remains an important topic to be investigated.
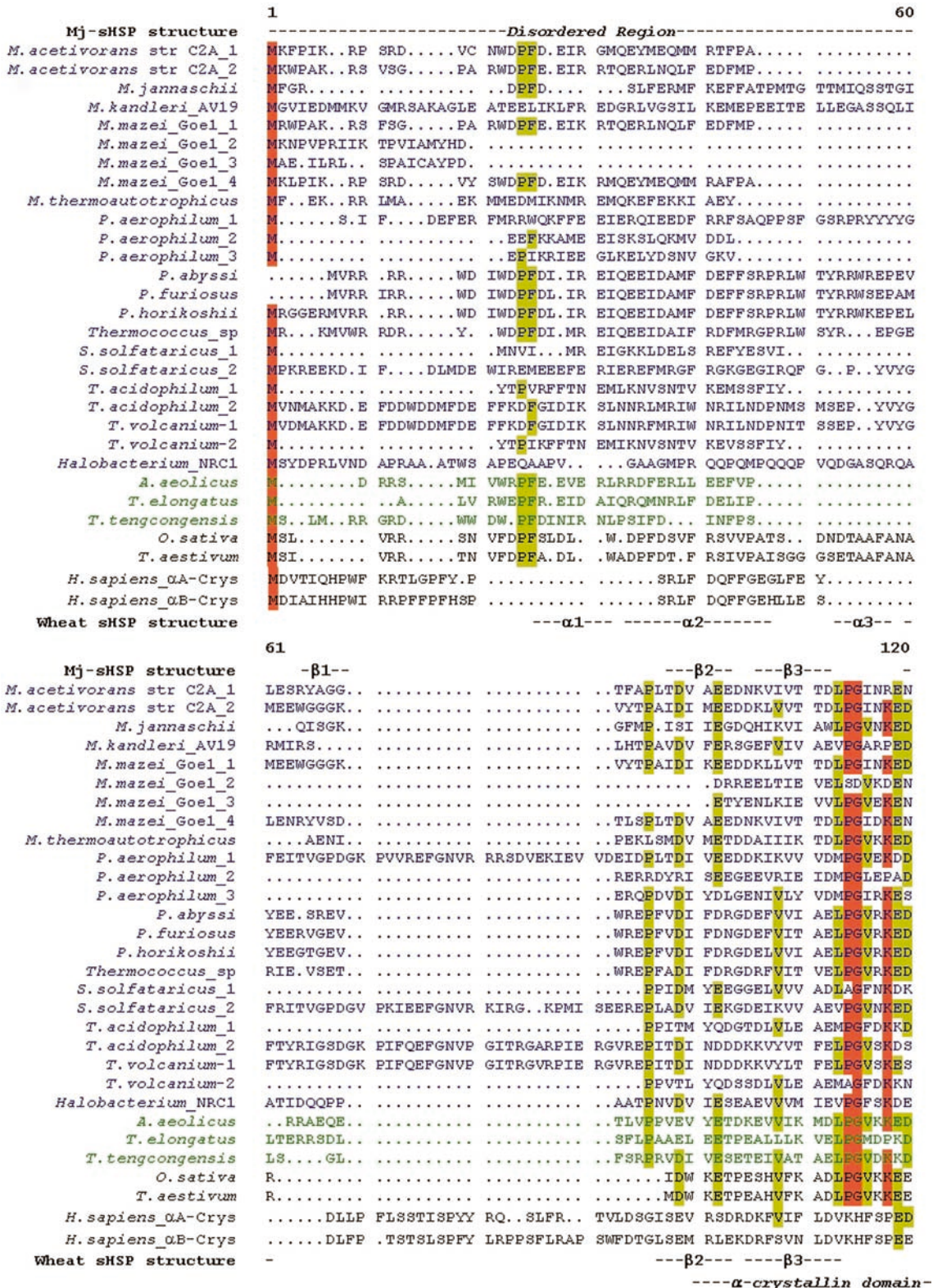
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Mj-sHSP structure        1                                                      60
                         -----------------------Disordered Region-----------------------
M. acetivorans str C2A_1 MKFPIK..RP SRD.....VC NWDPFD.EIR GMQEYMEQMM RTFPA..... ..........
M. acetivorans str C2A_2 MKWPAK..RS VSG.....PA RWDPFE.EIR RTQERLNQLF EDFMP..... ..........
M. jannaschii            MFGR...... .......... ..DPFD..... .....SLFERMF KEFFATPMTG TTMIQSSTGI
M. kandleri_AV19         MGVIEDMMKV GMRSAKAGLE ATEELIKLFR EDGRLVGSIL KEMEPEEITE LLEGASSQLI
M. mazei_Goe1_1          MRWPAK..RS FSG.....PA RWDPFE.EIK RTQERLNQLF EDFMP..... ..........
M. mazei_Goe1_2          MKNPVPRIIK TPVIAMYHD. .......... .......... .......... ..........
M. mazei_Goe1_3          MAE.ILRL.. SPAICAYPD. .......... .......... .......... ..........
M. mazei_Goe1_4          MKLPIK..RP SRD.....VY SWDPFD.EIK RMQEYMEQMM RAFPA..... ..........
M. thermoautotrophicus   MF..EK..RR LMA.....EK MMEDMIKNMR EMQKEFEKKI AEY....... ..........
P. aerophilum_1          M......S.I F....DEFER FMRRWQKFFE EIERQIEEDF RRFSAQPPSF GSRPRYYYYG
P. aerophilum_2          M......... .......... ..EEFKKAME EISKSLQKMV DDL....... ..........
P. aerophilum_3          M......... .......... ..EPIKRIEE GLKELYDSNV GKV....... ..........
P. abyssi                ......MVRR .RR.....WD IWDPFDI.IR EIQEEIDAMF DEFFSRPRLW TYRRWREPEV
P. furiosus              ......MVRR IRR.....WD IWDPFDL.IR EIQEEIDAMF DEFFSRPRLW TYRRWSEPAM
P. horikoshii            MRGGERMVRR .RR.....WD IWDPFDL.IR EIQEEIDAMF DEFFSRPRLW TYRRWKEPEL
Thermococcus_sp          MR...KMVWR RDR.....Y. .WDPFDI.MR EIQEEIDAIF RDFMRGPRLW SYR...EPGE
S. solfataricus_1        M......... .......... .MNVI...MR EIGKKLDELS REFYESVI.. ..........
S. solfataricus_2        MPKREEKD.I F....DLMDE WIREMEEEFE RIEREFMRGF RGKGEGIRQF G..P...YVYG
T. acidophilum_1         M......... .......... .YTPVRFFTN EMLKNVSNTV KEMSSFIY.. ..........
T. acidophilum_2         MVNMAKKD.E FDDWDDMFDE FFKDFGIDIK SLNNRLMRIW NRILNDPNMS MSEP..YVYG
T. volcanium-1           MVDMAKKD.E FDDWDDMFDE FFKDFGIDIK SLNNRFMRIW NRILNDPNIT SSEP..YVYG
T. volcanium-2           M......... .......... .YTPIKFFTN EMIKNVSNTV KEVSSFIY.. ..........
Halobacterium_NRC1       MSYDPRLVND APRAA.ATWS APEQAAPV.. ...GAAGMPR QQPQMPQQQP VQDGASQRQA
A. aeolicus              M........D RRS.....MI VWRPFE.EVE RLRRDFERLL EEFVP..... ..........
T. elongatus             M........ ....A.....LV RWEPFR.EID AIQRQMNRLF DELIP..... ..........
T. tengcongensis         MS..LM..RR GRD.....WW DW.PFDINIR NLPSIFD... INFPS..... ..........
O. sativa                MSL....... VRR.....SN VFDPFSLDL. .W.DPFDSVF RSVVPATS.. DNDTAAFANA
T. aestivum              MSI....... VRR.....TN VFDPFA.DL. .WADPFDT.F RSIVPAISGG GSETAAFANA
H. sapiens_αA-Crys       MDVTIQHPWF KRTLGPFY.P .......... ......SRLF DQFFGEGLFE Y.........
H. sapiens_αB-Crys       MDIAIHHPWI RRPFFPFHSP .......... ......SRLF DQFFGEHLLE S.........
Wheat sHSP structure                           ---α1--- ------α2-------      --α3-- -

Mj-sHSP structure        61                                                    120
                         -β1--                      ---β2-- ---β3---          -
M. acetivorans str C2A_1 LESRYAGG.. .......... ..TFAPLTDV AEEDNKVIVT TDLPGINREN
M. acetivorans str C2A_2 MEEWGGGK.. .......... ..VYTPAIDI MEEDDKLVVT TDLPGINKED
M. jannaschii            ...QISGK.. .......... ..GFMP.ISI IEGDQHIKVI AWLPGVNKED
M. kandleri_AV19         RMIRS..... .......... ..LHTPAVDV FERSGEFVIV AEVPGARPED
M. mazei_Goe1_1          MEEWGGGK.. .......... ..VYTPAIDI KEEDDKLLVT TDLPGINKED
M. mazei_Goe1_2          .......... .......... .......... .DRREELTIE VELSDVKDEN
M. mazei_Goe1_3          .......... .......... .......... .ETYENLKIE VVLPGVEKEN
M. mazei_Goe1_4          LENRYVSD.. .......... ..TLSPLTDV AEEDNKVIVT TDLPGIDKEN
M. thermoautotrophicus   ....AENI.. .......... ..PEKLSMDV METDDAIIIK TDLPGVKKED
P. aerophilum_1          FEITVGPDGK PVVREFGNVR RRSDVEKIEV VDEIDPLTDI VEEDDKIKVV VDMPGVEKDD
P. aerophilum_2          .......... .......... ..RERRDYRI SEEGEEVRIE IDMPGLEPAD
P. aerophilum_3          .......... .......... ..ERQPDVDI YDLGENIVLY VDMPGIRKES
P. abyssi                YEE.SREV.. .......... ..WREPFVDI FDRGDEFVIT AELPGVRKED
P. furiosus              YEERVGEV.. .......... ..WREPFVDI FDNGDEFVIT AELPGVRKED
P. horikoshii            YEEGTGEV.. .......... ..WREPFVDI FDRGDELVVI AELPGVRKED
Thermococcus_sp          RIE.VSET.. .......... ..WREPFADI FDRGDRFVIT VELPGVRKED
S. solfataricus_1        .......... .......... .....PPIDM YEEGGELVVV ADLAGFNKDK
S. solfataricus_2        FRITVGPDGV PKIEEFGNVR KIRG..KPMI SEEREPLADV IEKGDEIKVV AEVPGVNKED
T. acidophilum_1         .......... .......... .....PPITM YQDGTDLVLE AEMPGFDKKD
T. acidophilum_2         FTYRIGSDGK PIFQEFGNVP GITRGARPIE RGVREPITDI NDDDKKVYVT FELPGVSKDS
T. volcanium-1           FTYRIGSDGK PIFQEFGNVP GITRGVRPIE RGVREPITDI NDDDKKVYLT FELPGVSKES
T. volcanium-2           .......... .......... .....PPVTL YQDSSDLVLE AEMAGFDKKN
Halobacterium_NRC1       ATIDQQPP.. .......... ..AATPNVDV IESEAEVVVM IEVPGFSKDE
A. aeolicus              ..RRAEQE.. .......... ..TLVPPVEV YETDKEVVIK MDLPGVKKED
T. elongatus             LTERRSDL.. .......... ..SFLPAAEL EETPEALLLK VELPGMDPKD
T. tengcongensis         LS....GL.. .......... ..FSRPRVDI VESETEIVAT AELPGVDKKD
O. sativa                R......... .......... .....IDW KETPESHVFK ADLPGVKKEE
T. aestivum              R......... .......... .....MDW KETPEAHVFK ADLPGVKKEE
H. sapiens_αA-Crys       ......DLLP FLSSTISPYY RQ..SLFR.. TVLDSGISEV RSDRDKFVIF LDVKHFSPED
H. sapiens_αB-Crys       ......DLFP .TSTSLSPFY LRPPSFLRAP SWFDTGLSEM RLEKDRFSVN LDVKHFSPEE
Wheat sHSP structure     -                              ---β2--- ----β3----
                                                        ----α-crystallin domain-
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Fig. 1 Amino acid sequence alignment of sHSPs from the archaea (*blue*), bacteria (*green*), and eukaryotes (*black*). Secondary structures at the top and bottom were indicated based on the Mj-sHSP and wheat sHSP crystal structures, respectively. Dimeric interaction domains of Mj-sHSP and wheat sHSP as shown in Fig. 2a, b are indicated by the two horizontal *blue bars*. The alignment was performed with Pileup in GCG Wisconsin Package, version 10

Regulation of sHSP expression

The patterns of sHSPs and α-crystallin expression appear to be different in the three domains. For instance, in mice, αB-crystallin and HSP25 were reported in several

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                        121                                                            180
Mj-sHSP structure       --β4--  ---β5----        --β6---      ---β7----    -α1-- -β8-
M. acetivorans str C2A_1  IELSLKD.NF LVISASKGKE EENEKEGYLR KE.RSF.MRY YREIPLPKGV .TEEGATAQL
M. acetivorans str C2A_2  VEINLKE.DM LEISAKTGEE KETEEEGYLR KE.RAY.TQF YRAVRLPVGV .KEEGSTAKM
M. jannaschii             IILNAV.GDT LEIRAKRSPL MITESERIIY SEIPEE.EEI YRTIKLPATV .KEENASAKF
M. kandleri_AV19          VQRVRAGERF. VEITA...NI PKMR.EGEAK TRER.VTGEV RRRIDLPEKI N.PSAVSAKC
M. mazei_Goe1_1           VEINLKE.DM LEISAKTGEE KESEEEGYLR RE.RAY.TQF YRAVRLPSSV .REEGACAKM
M. mazei_Goe1_2           ITLMMHENSF YIKAFSETVE .......... .....YLGSF F..LDGPVD. ..PEKAVAAN
M. mazei_Goe1_3           ISFKVVEDGF YVKATKEGVE .......... .....YTDSY A..VCCPIS. ..PEKATATY
M. mazei_Goe1_4           VELNLRE.NL LVISAQKGKE EETEKEGYLR KE.RSF.MRY YREIPLPDNV .TEDGATAQL
M. thermoautotrophicus    INIELTE.NT ISISAVFEEE VEIKEADFIK RE.RKY.GEA KREMRLPEKI .RVEDASAKF
P. aerophilum_1           IKLYISEDGK TLTIDAKGKE R......... ...RY HKEIKLPAAV D.PSKAKATY
P. aerophilum_2           IALSVTKDGT ALRA.....E GA........R GER.....KY SKFIRLPVKI D.PSTATALY
P. aerophilum_3           IKVRVYDRAI EVIASPATPE GAGKP...LR RERISN.FPV SRKIEVPFRL R.VESAKAVY
P. abyssi                 IKVRVTE.DS VVIEATVRRE KELEEEGAVR IE.RYY.SGY RRVIRLPEEV .IPEKAKAKY
P. furiosus               IKVRVTE.DT VVIEATVRRE KELEREGAVR IE.RYF.TGY RRAIRLPEEV .IPEKAKAKY
P. horikoshii             IKVRVTE.DS VVIEAIVRRE KELEEEGAVR VE.RYY.SGY RRVIRLPEEV .IPEKAKAKY
Thermococcus_sp           IKLRVTE.DT VVIEAQMRRE KELEQEGAIR IE.RYY.SGY RRVIRLPEEV .IPEKAKARY
S. solfataricus_1         ISVRLSAQNE LIINA....E REIQYIGTKY ATQRP..LKI HKVIRLPVKV KRDSQVTAKY
S. solfataricus_2         IKVKVTNGGK KLVITAKSED R......... ....QY YKEIDLPAEV D.EKAAKANF
T. acidophilum_1          IKITVE.KNV LTLRA....E RKREY.RSVY IDQRV..DKV FKVVRLPVDV DQAS.ISAKY
T. acidophilum_2          IDLKISE..Y NITLNVDEDQ K......... ...KY YKSIDFDYKL K.PETATAKF
T. volcanium-1            IDLKISE..Y NITLNVDEDQ K......... ...KY YKSIDFDYKL K.PETAVAKF
T. volcanium-2            IKVSVN.KNV LTISA....E RKREY.STVY IDQRV..DKV YKVVRLPVEI EQQD.ISAKY
Halobacterium_NRC1        ITVQADASN. LYVTAERSND PQMGIEGDDN VLLRECPDRL ERAITLPNYI D.PGEATAEH
A. aeolicus               IEITVKE.NA VHVRAERKEE KEEKTENIIR AE.RFY.GVI ERVIPLPVEV .DPEKAEAKY
T. elongatus              IDVQVTA.EA VSISGERKSE TKTETEGMKR TE.FRY.GKF QRVIPLPVRI .QNTSVKAEY
T. tengcongensis          IEINVYD.NI LEIKGQTSVD EEKEDKNYYI RE.RYY.GSF ARRIELPAEV .DPERTTAKF
O. sativa                 VKVEVEEGNV LVISGQRSKE KEDKNDKWHR VE.RSS.GQF MRRFRLPENA .KVDQVKAGL
T. aestivum               VKVEVEDGNV LVVSGERTKE KEDKNDKWHR VE.RSS.GKF VRRFRLLEDA .KVEEVKAGL
H. sapiens αA-Crys        LTVKVQDD.. .FVEIHGKHN ERQDDHGYIS RE.......F HRRYRLPSNV DQSALSCSLS
H. sapiens αB-Crys        LKVKVLGD.. .VIEVHGKHE ERQDEHGFIS RE.......F HRKYRIPADV DPLTITSSLS
Wheat sHSP structure      ---β4--- ---β5---        --β6-      ---β7---       --β8-
                          ----------------------α-crystallin domain----------------------

                        181                                      221
Mj-sHSP structure       --  ---β9--- ---------α2------- --β10--
M. acetivorans str C2A_1  KNGVLTVTMP RTE....... ........AL TEK...RIPI E
M. acetivorans str C2A_2  ENGVLTISLP KAQI...... ....EE PKK...KIEI E
M. jannaschii             ENGVLSVILP KAES...... ...SI KKG....INI E
M. kandleri_AV19          GRGLLIVRAP KAEA...... ...SE IEVK..PMEE E
M. mazei_Goe1_1           DNGVLTIMLP KAKI...... ....EE PKK...KIEI E
M. mazei_Goe1_2           NNGMLTIKVP YKEGFTCARF .......IHV D
M. mazei_Goe1_3           SNGVLKVNVP YQQPFEKAVD .......VKI E
M. mazei_Goe1_4           KNGVLTVTLP RTK....... ....DV TEK...RIQI E
M. thermoautotrophicus    ENGVLTVELP KVEV...... ...K. .KKQ..TLKV D
P. aerophilum_1           KNGVLSIEL. EK........ ....TE KKKKGFEIKI D
P. aerophilum_2           RHGVLIITAK KVK....... .EEEIRIPVR G
P. aerophilum_3           KDGVLQIVVG KAG....... .EYGVAELKI D
P. abyssi                 NNGVLEIRIP KKQP...... ...TK KEGEGFEVKI E
P. furiosus               NNGVLEIRVP KKHP...... ...TK KESEGFEVKV E
P. horikoshii             NNGVLEIRIP KKNP...... ...TK KEGEGFEVKI E
Thermococcus_sp           NNGVLEIEIP KKKP...... ...TK PEKEGVEVKI E
S. solfataricus_1         ENGVLTIRIP VEGS...... ...V. ...SIRI E
S. solfataricus_2         KNGVLEITLK KK........ ....AS SSDSGVDIKV E
T. acidophilum_1          QDGLLTVRMK AKDI...... ...K. ....TVEI E
T. acidophilum_2          NNGLLDVTVE KA........ ...TS KEGSGRKVSI Q
T. volcanium-1            NNGLLDVTVE KA........ ...TS KESGGRKVSI Q
T. volcanium-2            SEGILTVRMK TKNI...... ...K. ...NVEI E
Halobacterium_NRC1        VDGVCHVTVP KDEA...... ...E. .RQH..EIAF Q
A. aeolicus               ENGVLEIRIP KVHV...... ...EK EK....KIKL A
T. elongatus              KDGILHLTLP KAEE...... ...EK NRVV..KVSL A
T. tengcongensis          ENGILKITMP KLHP...... ...SK PKKR..RIDI E
O. sativa                 ENGVLTVTVP KAEV...... ...KK PEVKAIEIS. G
T. aestivum               ENGVLTVTVP KAEV...... ...KK PEVKAIQIS. G
H. sapiens αA-Crys        ADGMLTFCGP KIQTGLDATH AERAIPVSRE EKP..TSAPS S
H. sapiens αB-Crys        SDGVLTVNGP RKQ....VSG PERTIPITRE EKPAVTAAPK K
Wheat sHSP structure      - ----β9----          -β10-
                          -α-crystallin-
```

Fig. 1 (Contd.)

organs such as eye lenses, heart, stomach, and lung under non-stress conditions (Klemenz et al. 1991, 1993). Bacterial sHSPs such as those from the thermophilic cyanobacterium *Synechococcus vulcanus* or the anaerobic bacterium *Clostridium acetobutylicum* were not expressed under normal growth conditions (Sauer and Durre 1993; Roy and Nakamoto 1998; Roy et al. 1999).

Heat shock gene expression has been well characterized in mesophilic bacterial systems. Both positive and negative modes of regulation of *shsp* gene expression have been reported. In some organisms, coordinate regulation of *shsp* gene expression with other heat shock protein expression has been reported (Allen et al. 1992). However, many organisms have non-coordinate regulation of *shsp* gene expression (Servant et al. 1999, 2000; Nocker et al. 2001a, 2001b). In *E. coli,* the sHSP genes *ibp*A and *ibp*B form an operon and are positively controlled by σ^{32}, the sigma factor that regulates many heat shock genes (Allen et al. 1992). However, even in an σ^{32} defective mutant of *E. coli*, there is some accumulation of the sHSP genes *ibp*A and *ibp*B, suggesting alternative mechanisms for *ibp*A/*ibp*B regulation (Laskowska et al. 1996). Furthermore, the discovery of a σ^{54} promoter region between these two *ibp* genes upstream of *ibp*B suggested that in addition to σ^{32}, *ibp*B may also be regulated by σ^{54} (Kuczynska-Wisnik et al. 2001). Interestingly, mutants of *E. coli* lacking *ibp*A and *ibp*B show no altered phenotype. Negative control has been shown in the case of the RheA (Repressor of *Hsp* Eighteen) protein from *Streptomyces albus*, which appears to repress transcription of *hsp*18 by repressing transcription at temperatures below 30°C. The RheA protein undergoes a conformational change at elevated temperature and transcription is no longer repressed (Servant et al. 1999, 2000). In several Rhizobia, translational control of *shsp* gene expression has been reported. ROSE (Repression Of heat Shock gene Expression) is a novel type of regulatory system that functions by a *cis*-acting element positioned in the 5′-untranslated region. The ribosome-binding site is protected by formation of the stem-loop from the 3′ region of ROSE, and elevated temperature has been suggested to disrupt the secondary structure (Nocker et al. 2001a, 2001b).

Although sHSPs have been identified in extremophiles from bacteria and archaea, studies of their regulation are mainly limited to thermophilic and hyperthermophilic organisms. Thermophilic cyanobacteria such as *Synechococcus vulcanus* appear to accumulate sHSP, GroEL, and GroES when cells are exposed to heat shock from 50°C to 63°C (Roy et al. 1999). Unlike expression of other *hsps*, the regulation of *shsp* expression of cyanobacteria is not under the control of CIRCE (Controlling Inverted Repeat of Chaperone Expression) (Roy and Nakamoto 1998; Narberhaus 1999; Kojima and Nakamoto 2002). A recent study in the thermophilic cyanobacterium *Thermosynechococcus elongatus* demonstrated that there is an AT-rich inverted repeat region upstream of the *shsp* (*hsp*A) gene. The putative DNA-binding protein appears to bind the DNA in this region more efficiently in non-heat shock than in heat shock conditions (Kojima and Nakamoto 2002). This result indicates that the *shsp* expression in thermophilic cyanobacteria may be similar to that found in *S. albus*.

Hyperthermophilic archaea such as *Pyrococcus furiosus* and *Thermococcus* KS-1 have a single copy of a sHSP that is also synthesized only at heat shock temperatures (Laksanalamai et al. 2001; Usui et al. 2001). These results indicate that sHSPs may not be required at the optimal growth temperature even when this is near the boiling point, as in the case of *P. furiosus* (Laksanalamai et al. 2001). In *P. furiosus*, a putative heat shock regulator (Phr) and a *cis*-acting regulatory sequence have recently been discovered (Vierke et al. 2002). The Phr is a small protein with a molecular weight of 24 kDa and is related to Phr homologues from the other two sequenced genomes of *Pyrococcus* sp. (PH1744 and PAB0208). Double-stranded DNA is required as a binding substrate for Phr. The promoters of *hsp*20 (Pfu-*shsp*) and aaa^+ atpase, which are *phr*-regulated genes, show highly conserved regions. The transcripts of aaa^+ atpase and *hsp*20 (Laksanalamai et al. 2001) are induced by heat shock. The Phr regulator appears to regulate the expression of these genes negatively, as it can block the formation of RNA polymerase complex. Phr expression was not induced by heat shock, suggesting that the Phr protein may be required at both normal and heat shock conditions.

Structural analysis of sHSPs from extremophiles

Most α-crystallin and small heat shock proteins assemble into high-molecular-weight complexes ranging in size from 200 kDa to more than 1 MDa (Kim et al. 1998a, 1998b; Ruepp et al. 2001). The number of subunits in sHSP complexes is extremely variable among species, and the complexes are polydisperse in size. Consequently, the analysis of sHSP and of α-crystallin structures is limited due to the continuum of these oligomeric structures (Haley et al. 1998, 2000). We have previously reported that the sHSP from the hyperthermophilic archaeon *P. furiosus* appears to be polydisperse (Laksanalamai et al. 2001) On the other hand, purified Mj-sHSP is monodisperse and forms a 24-mer with a hollow center (Haley et al. 2000).

Currently, the two crystal structures of the sHSPs from the methanogenic archaeon *M. jannaschii* and the mesophilic eukaryote (wheat) *T. aestivum* form the basis for modeling the activities of sHSPs (Kim et al. 1998a; van Montfort et al. 2001). The quaternary structures of these two sHSPs are quite dissimilar; the wheat sHSP is a dodecamer composed of a double-ring complex, whereas that of Mj-sHSP is a 24-subunit spherical complex. However, the comparison of both sHSP crystal structures reveals strikingly common features of the sHSPs. Although the amino acid sequences of these sHSPs from the organisms are divergent with about 33% identity, the dimeric building-block structure is conserved (Fig. 2a, b). The amino-terminal domain appears to be disordered in Mj-sHSP, but it is resolved in the wheat sHSP as two helices (α_1/α_2 and α_3) connected by a random coil (van Montfort et al. 2001) (Fig. 2b). From cryo-electron microscopy, it was suggested that the disordered amino-terminal domain might be located
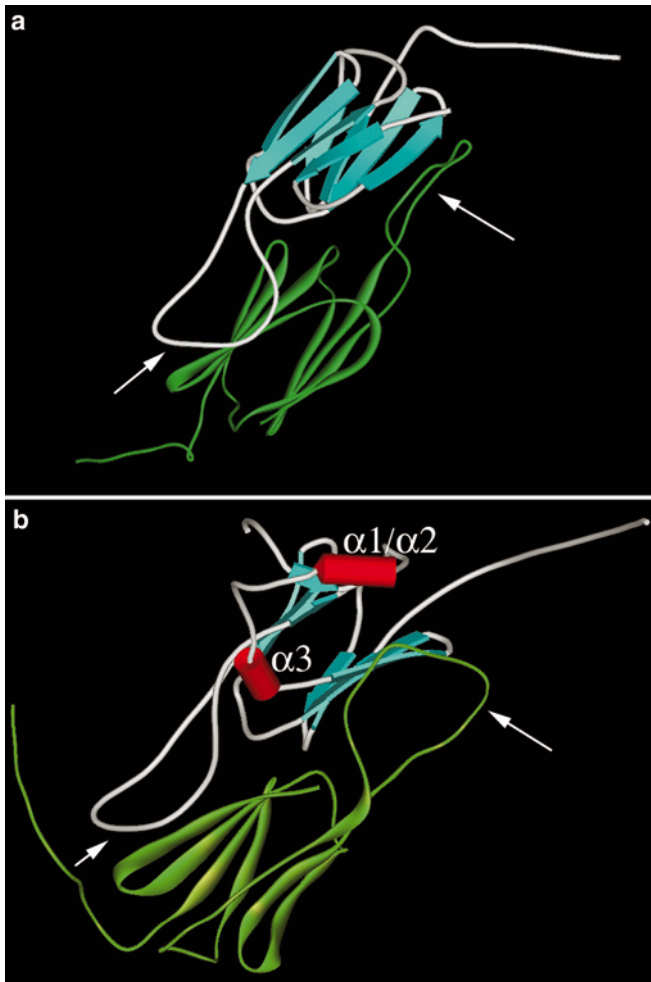
Fig. 2a, b Stabilization of dimeric complexes of sHSP by the loop domain as indicated by *arrows*. The pictures were drawn based on the crystal structures of **a** Mj-sHSP (1SHS) and **b** wheat sHSP (1GME)
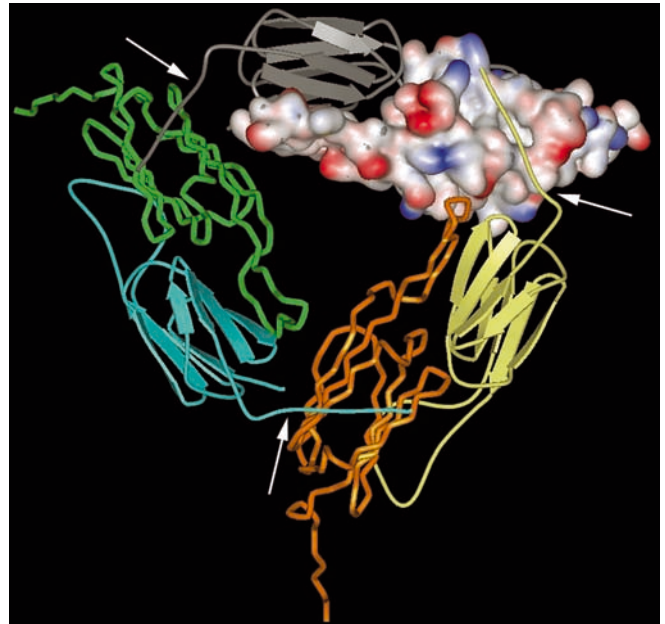
Fig. 3 Multimeric complex formation of Mj-sHSP. The *arrows* indicate the inter-dimeric interactions via the carboxy-terminal domain. Figures 2 and 3 were drawn based on the crystal structure from PDB with ViewerLite 4.2 program, Accelrys

inside the cavity (Haley et al. 2000). Another assembly format has been reported, where oligomeric complexes of murine sHSP appear to form from tetramers assembled from dimeric complexes (Ehrnsperger et al. 1999). These findings suggested that the dimeric building block may be common to sHSPs and may be essential for many sHSPs functions.

According to the available crystal structures of both sHSPs, generally, there are at least two critical interactions required to form and stabilize sHSP complexes: (1) the interactions between each monomer to form a dimeric building-block structure and (2) the interactions between each dimeric building block to form higher multimeric complexes. A long loop domain of approximately 20 residues has been found in both sHSPs between two monomers to stabilize the dimeric building block (Fig. 2a, b). Several types of interactions are involved, including hydrophobic and ionic interactions. However, the amino acid sequence comparison at this region does not show significant conservation. The

carboxy-terminal domain appears to be involved in the interaction between each dimeric building block (Fig. 3). Two orientations of the carboxy-terminal domain are found in wheat sHSP, whereas only one is found in Mj-sHSP. The difference in quaternary structure of both sHSPs may result from the hinge angle between the α-crystallin and the carboxy-terminal domains (van Montfort et al. 2001). The variable orientations of the carboxy-terminal domains probably contribute to the different assembly mechanisms and polydisperse property of other sHSPs.

Several lines of evidence suggest that subunit interactions, either association or dissociation, play an essential role in the chaperone functions of α-crystallin and sHSPs. Mammalian HSP20 is found in two complex sizes, which are dimeric and multimeric forms depending on the concentration of the proteins. The multimeric form appears to be more efficient in chaperone activity than the dimeric form (van de Klundert et al. 1998). The HSP16.3 from *Mycobacterium tuberculosis* forms a trimer of trimers and functions as a molecular chaperone (Chang et al. 1996). Several mutations of *Bradyrhizobium japonicum* sHSPs that disrupt large multimeric protein formations result in the reduction of chaperone activity (Studer et al. 2002). Interestingly, the HSP12.2, HSP12.3, and HSP12.6 from *Caenorhabditis elegans*, containing very short N-terminal and no C-terminal tails, are incapable of forming large multimeric complexes and do not posses chaperone activity (Leroux et al. 1997; Kokke et al. 1998). In *P. furiosus* sHSP (Pfu-sHSP), the carboxy-terminal domain appears to be responsible for the multimeric formation. Recent results

from the Kim laboratory indicate that non-native proteins are bound on the outside of the 24-mer sphere, provided that the temperature is above 70°C (Kim et al. 2003). Mutation studies on *M. jannaschii* sHSP suggested that the N-terminal region had no role in preventing protein aggregation, whereas deletion mutations to the C-terminal, or extensive (>12 amino acids) deletions to the N-terminus prevented chaperone activity in vitro. However, the chaperone activity was undiminished in short amino- or carboxy-terminal deletions (Kim et al. 2003). Laksanalamai et al. (2003) showed that deletion mutants that were unable to form large multimeric complexes were also unable to protect *E. coli* cells from thermal death. We have previously shown that only dimeric structures of Pfu-sHSP were formed when the carboxy and amino-terminal domains were deleted, whereas an amino-terminal deletion Pfu-sHSP lacking these residues is still able to form multimeric complexes (Laksanalamai et al. 2003). The inability to form multimeric complexes of Pfu-sHSP mutants also results in the loss of in vivo chaperone activity in *E. coli* (Laksanalamai et al. 2003). Yeast HSP26, on the other hand, forms a large complex at physiological temperature. The complex is subsequently dissociated during heat shock conditions and the HSP26 enters the functional stage (Haslbeck et al. 1999). These findings indicated clearly that subunit interactions are significant for chaperone functions of both α-crystallins and sHSPs. However, the mechanisms and characteristics of subunit assembly may vary considerably among organisms.

Extremophile sHSPs function as molecular chaperones

Several conclusive studies have established that α-crystallins and sHSPs from plants, mammals, or mesophilic prokaryotes are molecular chaperones (Horwitz 1992; Haley et al. 1998; Haslbeck et al. 1999; Haslbeck 2002). Currently, several studies are revealing that sHSPs from extremophiles, including those from bacteria and archaea, also function as molecular chaperones. The sHSPs from the hyperthermophiles *M. jannaschii*, *P. furiosus*, and *Thermococcus* sp. KS-1 appear to prevent other mesophilic enzymes from aggregation at extremely high temperatures (Kim et al. 1998c; Laksanalamai et al. 2001; Usui et al. 2001). The Pfu-sHSP and Mj-sHSP can prevent *E. coli* cellular proteins from aggregation above 100°C. In addition, the Pfu-sHSP significantly enhances the viability of mesophilic organisms such as *E. coli* at normally lethal temperatures (Laksanalamai et al. 2001). Interestingly, protection was not found in the sHSP from *Thermococcus* sp. KS-1 (Usui et al. 2001). A lesser, but appreciable, protection of viability in *E. coli* was reported by sHSPs from mesophilic organisms such as the chestnut, *Castanea sativa* (Soto et al. 1999). The sHSP from the thermophilic bacterium *Synechococcus vulcanus* (HspA) has been shown to be heat inducible and is also able to protect some mesophilic enzymes

from aggregating at elevated temperature (Roy and Nakamoto 1998; Roy et al. 1999). In addition, when the *S. vulcanus* HspA was expressed in the mesophilic strain of *Synechococcus* sp. PCC7642, the viability of the mesophilic strain was greatly enhanced at non-permissive temperatures. The *S. vulcanus* HspA also appears to promote the heat shock resistance of photosystem II and protect phycocyanin from heat-induced photobleaching in vivo (Nakamoto et al. 2000).

The *trans*-acting, protein-folding mechanism of sHSPs still remains enigmatic. It has been reported that *Mycobacterium tuberculosis* and *S. vulcanus* sHSPs can suppress thermal aggregation of citrate synthase and malic dehydrogenase, respectively. However, enzymatic activity was not recovered following sHSP treatment (Chang et al. 1996; Roy et al. 1999). A similar effect was also found in *P. furiosus* in protection of bovine glutamate dehydrogenase (Laksanalamai et al. 2001). These results suggest that, in these experiments, the sHSP may not be promoting protein folding, but merely holding the denatured proteins in solution. It seems likely that sHSPs might also function cooperatively with other chaperone systems. In fact, plant sHSPs restrain proteins from thermal aggregation, and these non-native proteins are subsequently refolded rapidly by other ATP-dependent chaperone system such as HSP70 (Lee and Vierling 2000).

Although it appears that most sHSPs are unable to refold non-native proteins, there is evidence showing that denatured proteins can be reactivated by some sHSPs and α-crystallin. Excessive addition of *Thermococcus* sp. KS-1 sHSP allows the refolding of chemically denatured green fluorescent protein (GFP) in an ATP-independent fashion (Usui et al. 2001). Chemically denatured citrate synthase and lactate dehydrogenase can also be reactivated by HSP17.7 and HSP18.1 from pea in an ATP-independent fashion (Lee et al. 1995). Human αB-crystallin can reactivate chemically denatured citrate synthase, and the reactivation can be enhanced by approximately twofold by the addition of 3.5 mM ATP (Muchowski and Clark 1998).

Functions of sHSPs have been investigated throughout the three domains. However, the mechanisms of action of several sHSP functions still remain unclear. Most of the studies reveal that sHSPs tend to bind non-native proteins and restrain these denatured proteins from aggregation under stress conditions. Bacterial sHSPs from *E. coli* and *Vibrio harveyi* appear to bind to cellular proteins under heat shock conditions (Laskowska et al. 1996; Klein et al. 2001). The studies using the hydrophobic probe 1,1′-bi(4-anilino) naphthalene-5,5′-disulfonic acid (Bis-ANS) suggested that hydrophobic interactions may be involved in the functions of α-crystallin sHSP (Das and Surewicz 1995; Lee et al. 1997). The Mj-sHSP crystal structure also revealed an equivalent Bis-ANS binding site as found in eukaryotic sHSP (Kim et al. 1998a). Unlike other larger HSPs in which ATP hydrolysis is required for

substrate binding and release, most sHSPs appear to function without addition of ATP (Jacob et al. 1993). However, there are some reports suggesting that ATP may enhance the chaperone function of sHSP and αB-crystallin (Muchowski and Clark 1998; Valdez et al. 2002).

Discussion and conclusions

Most sHSPs are related to vertebrate α-crystallin proteins found in eye lenses, although the overall amino acid sequence similarity is not high. The α-crystallin proteins are very important proteins especially in eye lenses, as they restrain proteins from aggregation, thereby preventing cataracts (Clark and Muchowski 2000; Derham and Harding 2002). Similar chaperone functions to those found in α-crystallins appear to be widespread in sHSPs from all three domains. Most organisms including extremophiles possess at least one heat shock-regulated homologue of sHSP, suggesting that the proteins may be important for organisms to survive under stress conditions. Their lack of expression at normal growth temperatures suggests that, in most cases, sHSPs may not be required unless stress conditions occur (Roy et al. 1999; Laksanalamai et al. 2001).

Currently, although the mechanisms of action of sHSPs from extremophiles remain enigmatic, multimeric structures and subunit interactions appear to be important for chaperone functionality. Several lines of evidence show that conformational changes or altered subunit assembly can also modulate the chaperone functions of sHSPs. The crystal structures of Mj-sHSP and wheat sHSP have provided new perspectives leading to insights into the functions of these small molecular chaperones. The dimeric structure is widely believed to be the essential basic building block of most sHSP complexes. Some sHSPs may have monodisperse assembly, but most of them produce a continuum of multidimeric complexes covering a wide range of molecular weights. In addition, novel approaches are being applied to the analysis of the sHSP functions from hyperthermophiles. For instance, mutations that suppress large-complex formation may allow structural analysis to be carried out (Laksanalamai et al. 2003). Genetic analysis and knock-out systems in hyperthermophiles, which are essential to future studies of the functions of sHSP in vivo, are now foreseeable with the recent development of genetic systems in archaea (Zillig et al. 1998; Sowers and Schreier 1999; Stedman et al. 1999; Martusewitsch et al. 2000; Stedman et al. 2000; Cannio et al. 2001; Lucas et al. 2002; Worthington et al. 2003).

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