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ORIGINAL PAPER

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Multi-subunit assembly of the *Pyrococcus furiosus* small heat shock protein is essential for cellular protection at high temperature

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Abstract The hyperthermophilic archaeon, *Pyrococcus furiosus*, expresses a small, α-crystallin-like protein in response to exposure to extreme temperatures, above 103°C. The *P. furiosus* small heat shock protein (Pfu-sHSP) forms large oligomeric complexes. Based on the available crystal structures of the *Methanocaldococcus jannaschii* and wheat sHSPs, the protruding carboxy terminal domain is probably involved in subunit interactions. We constructed Pfu-sHSP mutants to analyze chaperone function and to study multi-subunit assembly. The results confirmed that the carboxy terminus of Pfu-sHSP is involved in inter-dimer interactions, whereas the amino terminal deletion mutant still exhibited the wild-type assembly characteristics. The ability to form oligomeric complexes via the carboxy terminal domain was shown to be necessary for thermotolerance of *Escherichia coli* overexpressing Pfu-sHSP. The amino terminal domain was not required for inter-species thermotolerance.

Keywords Hyperthermophile · *Pyrococcus furiosus* · Small heat shock protein · Subunit assembly · Thermotolerance

Introduction

Many hyperthermophilic microorganisms growing at or above 100°C are known, and several have been studied in some detail. Several adaptive mechanisms of hyperthermophiles, which ensure thermotolerance of whole cells and high temperature protein stability, have emerged (Robb and Maeder 1998; Robb and Clark 1999; Vieille and Zeikus 2001). Heat shock proteins (HSPs) are inducible molecular chaperones that perform critical roles in thermal protection of cells, by preventing denatured proteins from aggregating or else refolding them to their native structures (Chang et al. 1996; Yan et al. 1997; Izumi et al. 1999). HSPs have been classified into five classes based on their molecular weight: HSP100, HSP90, HSP70, HSP60, and small HSPs (sHSPs) (Trent et al. 1994). During heat stress, several HSPs are normally induced and this can lead to acquired thermotolerance (Hightower 1991; Lindquist 1992; Trent et al. 1994).

Small heat shock proteins are HSPs of relatively small subunit size that may form multi-subunit complexes of 200–800 kDa (Kim et al. 1998). Most prokaryotic and eukaryotic sHSPs contain an α-crystallin domain within a short, very highly conserved region (de Jong et al. 1998). The number of sHSP paralogs varies among species and many species contain more than one paralog (Caspers et al. 1995). For example, *Escherichia coli* expresses two sHSPs, IbpA and IbpB (Allen et al. 1992). Archaeal sHSPs described so far are, however, limited to the euryarchaea. Based on current complete genome sequences, most archaeal species contain a single sHSP-encoding gene. However, the sulfate-reducing archaeon, *Archaeoglobus fulgidus* contains two *shsp* genes (Klenk et al. 1997). Small heat-shock protein genes are not found in the complete genome sequences of pathogenic microorganisms such as *Mycoplasma genitalium*, *Haemophilus influenzae* and *Helicobacter pylori* (Fleischmann et al. 1995; Fraser et al. 1995; Tomb et al. 1997).

Functions of sHSPs have been investigated both in vitro and in vivo. In some cases, no cellular function can be assigned. For instance, *E. coli* sHSPs were shown to be dispensable (Thomas and Baneyx 1998), whereas that of *Neurospora crassa* contributed to thermotolerance (Plesofsky-Vig and Brambl 1995). Several lines of evidence reveal that most sHSPs function as molecular

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chaperones, having the ability to prevent aggregation of denatured proteins under stress conditions. We have shown previously that the Pfu-sHSP can confer significant thermotolerance on *E. coli* expressing the protein (Laksanalamai et al. 2001). A similar effect was reported for a plant sHSP (Soto et al. 1999); however, the molecular basis for this effect is unclear.

Currently, crystal structures of two sHSPs from the archaeon, *Methanocaldococcus jannaschii* and the plant, *Triticum aestivum* have been reported (Kim et al. 1998; van Montfort et al. 2001). Both proteins are monodisperse complexes at room temperature, whereas other sHSPs or α-crystallin homologs are polydisperse. These structures revealed that although the amino acid sequences of both sHSPs are quite divergent, the folds are very similar (van Montfort et al. 2001). These studies suggested that oligomeric structures may be important for the functions of the sHSPs, which are quite divergent in phylogenetic terms. We have previously reported that, unlike the sHSPs from *M. jannaschii* and *T. aestivum*, the sHSP from *Pyrococcus furiosus* (Pfu-sHSP) is polydisperse (Laksanalamai et al. 2001). In this study, we used the Mj-sHSP crystal structure as a model to construct Pfu-sHSP domain deletion mutants. We report that the carboxy terminal region of Pfu-sHSP is critical for subunit assembly, and we show that multi-dimeric assembly is necessary for effective cellular thermoprotection by Pfu-sHSP (Laksanalamai et al. 2001).

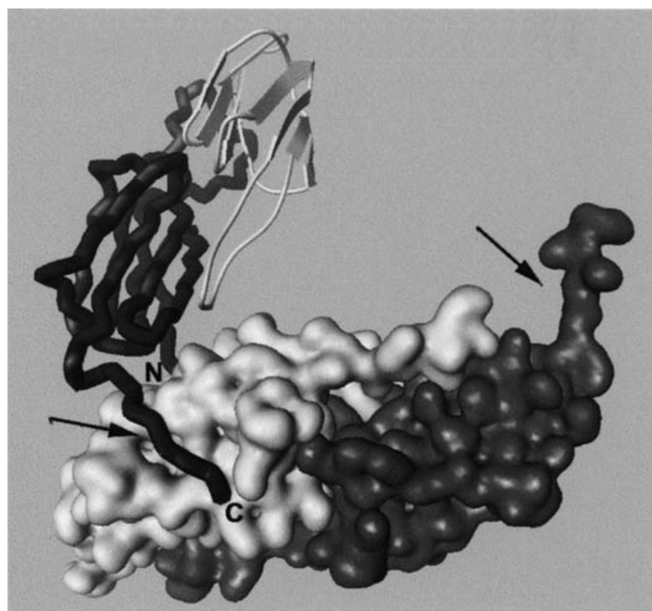

Fig. 1 Inter-dimeric interaction of Mj-sHSP. The *left arrow* indicates the inter-dimeric interaction via the carboxy terminal domain. The *right arrow* shows space filling image of the protruding carboxy terminus

Material and methods

Constructions and purification of the Pfu-sHSP mutants

Recombinant expression of the gene encoding Pfu-sHSP was previously described (Laksanalamai et al. 2001). The mutations of Pfu-sHSP were designed from Pfu-sHSP and Mj-sHSP amino acid sequence alignment. Molecular models (Fig. 1) were examined and graphics were generated using the free ViewerLite 4.2 program (Accelrys). The regions encoding Pfu-sHSP mutants were amplified from *P. furiosus* genomic DNA by using PCR primers as shown in Table 1. The amino terminal domain deletion was designated Pfu-sHSP^{N52} and the amino and carboxy domain double deletions were designated Pfu-sHSP$^{N/C}$. The process of cloning and overexpressing the Pfu-sHSP mutants was performed as described previously (Laksanalamai et al. 2001). The mutant proteins were subsequently purified by anion-exchange chromatography (MonoQ, Pharmacia Biotech, Uppsala, Sweden). The Pfu-sHSP$^{N/C}$ was eluted at 0.5 M NaCl, using a linear gradient of 0–1 M NaCl.

Structural analyses

Crosslinking was performed by adding 0.01% (W/V) glutaraldehyde to 0.4 μg/μl of the purified proteins in 25 mM sodium phosphate buffer. The crosslinking reactions were incubated at 30°C for 30 min and stopped by the addition of 10 μl of 1.5 M Tris-glycine buffer. The crosslinking reactions were subjected to 12% SDS-PAGE and visualized by silver staining (Sambrook et al. 1989). The molecular weight of Pfu-sHSP$^{N/C}$ was analyzed by size exclusion chromatography. Two hundred microliters of the purified Pfu-sHSP$^{N/C}$ was applied to a Superdex200 HR column previously equilibrated with phosphate buffer (Phamacia Biotech). Dynamic light scattering was performed with a DynaPro protein sizing instrument (Protein Solutions, Lakewood, NJ). The purified protein was analyzed at 50°C and the molecular size calculated.

Thermotolerance of *E. coli*

E. coli carrying different mutants of Pfu-sHSP was cultured in Luria–Bertani broth and induced by 0.1 M IPTG (isopropyl-1-thio-β-D-galactopyranoside) for 3 h as described previously (Laksanalamai et al. 2001). All cultures were diluted in the same medium to an A_{595} of 0.8. Two-hundred-μl aliquots of each culture were transferred to 13-mm glass tubes and shaken at 50°C. Samples were taken at 0, 30, 60, and 120 min and the optical density (A_{595}) was recorded.

Results and discussion

Designed, construction, and purification of the Pfu-sHSP mutants

The Pfu-sHSP mutants were constructed based on structural modeling, using the available crystal

Table 1 Primer sequences for constructing Pfu-sHSP mutants. The underline indicates the restriction site for cloning of truncated Pfu ORFs

Pfu-sHSP mutant	Primer sequence
Pfu-sHSP^{N52}	GCCATGGTAGGAGAAGTCTGGAGAGAGCCATTCGTTG
	ACTCGAGCTATTCAACTTTAACTTCGAATCCTTC
Pfu-sHSP$^{N/C}$	GCCATGGTAGGAGAAGTCTGGAGAGAGCCATTCGTTG
	ACTCGAGCTATGTTGGGTGCTTCTTTGGAACTCTGATCTCAAG

structures of the sHSPs from wheat (van Montfort et al. 2001) and *M. jannaschii* (Kim et al. 1998). Structural comparison revealed that the two proteins have a very similar basic dimeric form, although the quaternary structures are different. This implies that small heat shock proteins may acquire the same dimeric fold; however, functional assembly may vary depending on the species. From the structures of both sHSPs, it appears that the extended carboxy terminal is responsible for interaction between dimeric forms. Also, the amino terminal is disordered in the crystal structure of Mj-sHSP. We therefore used these observations to construct Pfu-sHSP mutants by deleting the amino and carboxy termini. The extent of deleted amino acid residues was determined from the amino acid sequence alignment of Pfu-sHSP (AF256212) and Mj-sHSP (AAB98273) (data not shown). There are 52 amino acid residues at the amino terminal and 12 amino acid residues at the carboxy terminal corresponding to the disordered and dimeric interaction regions in Mj-sHSP, respectively (Fig. 1). The predicted molecular weights of the Pfu-sHSP^{N52} and Pfu-sHSP$^{N/C}$ mutants are 15 and 12 kDa, respectively.

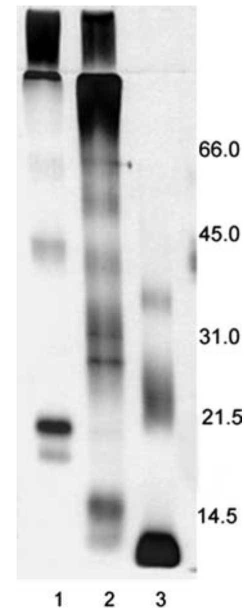

Fig. 2 SDS-12% PAGE of crosslinking products of Pfu-sHSP mutants. *Lanes 1*, *2*, and *3* represent wild-type Pfu-sHSP, Pfu-sHSP^{N52} and Pfu-sHSP$^{N/C}$, respectively

Analysis of the recombinant Pfu-sHSP mutants

Like most sHSPs and α-crystallin homologs, Pfu-sHSP is polydisperse, exhibiting a continuum of oligomeric structures. The sHSPs that have yielded crystal structures, therefore, appear to be exceptional. We have previously reported that the wild-type Pfu-sHSP is also polydisperse (Laksanalamai et al. 2001). The sHSP crystal structures show a disordered amino terminal domain, but a short carboxy terminal domain, which is in intimate contact with other dimers within the assembly and is predicted to be a major contributor to the stability of the polymeric form (see Fig. 1). Pfu-sHSP^{N52} should therefore exhibit polydisperse characteristics, whereas Pfu-sHSP$^{N/C}$ should not form large aggregates. Glutaraldehyde crosslinking of these mutants revealed that the Pfu-sHSP^{N52} was polydisperse, similar to the wild-type Pfu-sHSP (Fig. 2, lanes 2 and 1, respectively). Pfu-sHSP$^{N/C}$ did not exhibit large aggregates as found in the wild-type protein and Pfu-sHSP^{N52} (Fig. 2, lane3). These results indicate clearly that the carboxy terminal is involved in Pfu-sHSP subunit assembly.

Characteristics of the Pfu-sHSP double deletion mutant

Since the N- and C-terminal deletion mutant (Pfu-sHSP$^{N/C}$) was not capable of large complex formation, it served as a control. In addition, due to the polydisperse character of the intact protein, structural analyses by crystallography or NMR would be impossible, whereas an assembly-deficient mutant will allow us to drive the structure of the dimeric form. We therefore analyzed the

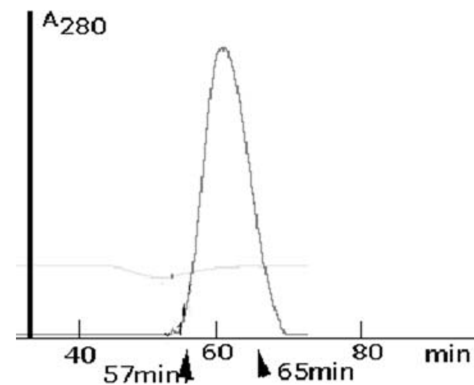

Fig. 3 Fractionation of Pfu-sHSP$^{N/C}$ by size exclusion chromatography. The Pfu-sHSP$^{N/C}$ was eluted as a single peak at 60 min, corresponding to 24 kDa

subunit assembly of the purified Pfu-sHSP$^{N/C}$ by size exclusion chromatography and dynamic light scattering. The Pfu-sHSP$^{N/C}$ eluted as a single peak at the position corresponding to the dimeric form, approximately 24 kDa (Fig. 3). Dynamic light scattering also revealed that Pfu-sHSP$^{N/C}$ is monodisperse and assembled into a dimeric form, whereas the wild-type Pfu-sHSP forms very large complexes with a wide range of sizes (Fig. 4). The molecular weight calculated from dynamic light scattering also revealed the dimeric molecular weight of the Pfu-sHSP$^{N/C}$ similar to the size exclusion result. These results confirm that the carboxy terminal domain is involved in complex formation. The deletion of the carboxy terminal residues therefore renders the Pfu-sHSP$^{N/C}$ mutant monodisperse, while retaining its ability to form dimers.

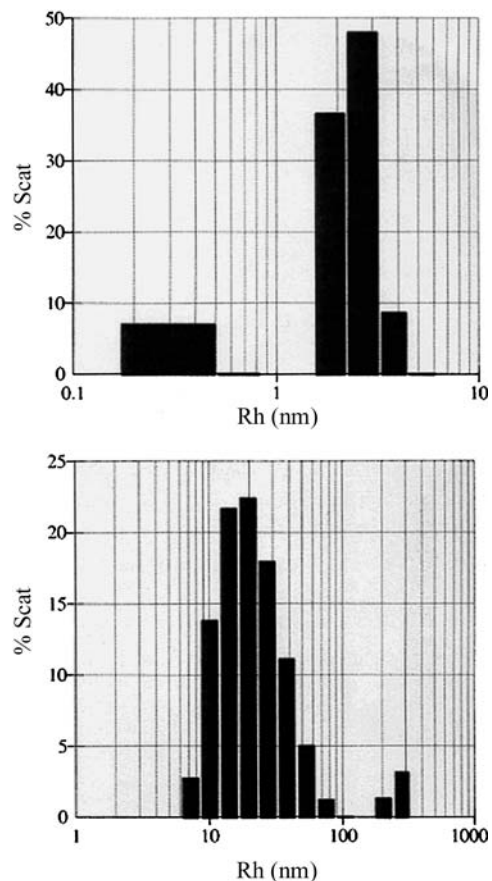

Fig. 4 Dynamic light scattering profile of purified Pfu-sHSP$^{N/C}$ (**A**) and wild-type Pfu-sHSP (**B**)

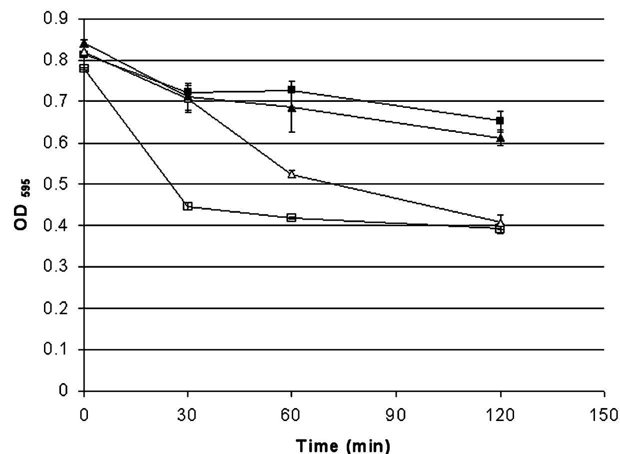

Fig. 5 Effects of recombinant expression of different Pfu-sHSP mutants on *E. coli* cultures at 50°C. The cell density of cultures is shown as A_{595} of wild-type Pfu-sHSP ■, Pfu-sHSP^{N52} ▲, Pfu-sHSP$^{N/C}$ Δ; and pET19b (control) ❑

Biological aspects of the terminal deletion mutants of Pfu-sHSP

The biological functions of sHSP and α-crystallin homologs may depend on the oligomeric status and vary among species. For instance, the mammalian HSP20 was found in two forms, dimeric and multimeric complexes, depending on the protein concentration of the protein. It was suggested that the dimeric form has less efficient chaperone activity (van de Klundert et al. 1998). During stress conditions, HSP20 concentration will increase, leading the protein to form multimeric complexes, whereas during normal conditions, the lower concentration of HSP20 maintains the majority of the HSP20 in the dimeric form. However, the HSP26 from yeast, *Saccharomyces cerevisiae*, appeared to dissociate during exposure to elevated temperature. The functional form may be a smaller complex (Haslbeck et al. 1999). Since a genetic system is currently not available in *Pyrococcus furiosus*, we have examined the biological roles of both Pfu-sHSP^{N52} and Pfu-sHSP$^{N/C}$ in *E. coli*. Our results confirm that, upon exposure to lethal temperature, i.e. 50°C, *E. coli* will undergo autolysis, as reported by Soto et al. (1999). The expression of wild-type sHSP and Pfu-sHSP^{N52} significantly reduced the amount of cell lysis relative to the control, whereas the expression of Pfu-sHSP$^{N/C}$ resulted in cells that were not protected (Fig. 5). Since only the Pfu-sHSP and Pfu-sHSP^{N52} could form oligomeric structures, this suggested that large aggregates of sHSP may be required to stabilize cells in vivo.

Our results indicate that the extended carboxy terminal domain is involved in subunit assembly, whereas the amino terminal is dispensable. This is consistent with both sHSP crystal structures, which show that the carboxy terminal is involved in inter-dimeric interactions (see Fig. 1). Since oligomerization of α-crystallin homologs varies among organisms, there may be more than one mechanism of action. Several studies indicate that bacterial and archaeal HSPs associate with cytoplasmic membranes (Garduno et al. 1998; Trent 2000). The sHSP from *Mycobacterium tuberculosis* was isolated and purified from a membrane preparation (Lee et al. 1992). These findings suggest possible alternative functions of HSPs that may contribute to the stabilization of cellular structures.

Large aggregates are characteristic of most sHSPs and α-crystallins. Our results suggest that the formation of extended polymers may be critical for the cell protection function of the sHSP. Although purified recombinant Mj-sHSP appears to be less heterogeneous than other sHSPs, Mj-sHSP may increase in the degree of polydispersity at the growth temperature of the organism (Koteiche and McHaourab 2002). The polymerization of the Pfu-sHSP may therefore represent an important adaptive mechanism that occurs when hyperthermophiles are exposed to extremely high temperature.

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