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Detection of oligomerisation and substrate recognition sites of small heat shock proteins by peptide arrays

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Abstract

Small heat shock proteins (sHsps) form large oligomers that are characterised by their dynamic behaviour, e.g., complex disassembly/reassembly and extensive subunit exchange. These processes are interrelated with sHsp/substrate interaction. sHsps bind a broad spectrum of unrelated substrate proteins under denaturing conditions. Detailed knowledge about the binding process and regions critical for sHsp/substrate interaction is missing. In this study, we screened cellulose-bound peptide spot libraries derived from a bacterial sHsp and the model-substrate citrate synthase to detect oligomerisation and substrate interaction sites, respectively. In line with previous results, it was demonstrated that multiple contacts involving the N- and C-terminal extensions and the central α-crystallin domain are required for oligomerisation. Incubation of the citrate synthase membrane with sHsps revealed a putative substrate interaction site. A soluble peptide with the sequence RTKYWELIYEDCMDL ($CS_{191–205}$) corresponding to that site inhibited chaperone activity of sHsps, presumably by blocking their substrate-binding sites.

Keywords: Small heat shock protein; α-Crystallin; Chaperone; Protein–protein interaction; Oligomerisation; Protein folding

Small heat shock proteins (sHsps) are known to form large homo- and hetero-oligomers and exhibit a dynamic behaviour in terms of subunit exchange and/or temperature and concentration-dependent dissociation/re-association processes [1,2]. It is becoming more and more obvious that these features are strongly linked with sHsp/substrate interaction. Despite the formation of large sHsp oligomers, the functional substrate-binding units are thought to be smaller subparticles, most probably dimers [3–6]. Proper oligomerisation and subunit exchange are critical since perturbation of both processes by introducing mutations or N- and C-terminal truncations led to loss of chaperone activity [3,7,8]. After substrate-binding sHsps associate into large aggregates often exceeding a few MDa, depending on the substrate, sHsp/substrate ratio, protein concentration, and heating conditions [4,9–12]. Captured substrate proteins can be disaggregated and refolded by cooperative action of the ATP-dependent chaperone machineries ClpB, DnaK, and GroEL [9,13,14].

The formation of sHsp–substrate complexes seems to be an organised process. Visualisation of sHsp–substrate aggregates by electron microscopy revealed particles of defined size and shape. Exchange of sHsps but not of bound substrate between the aggregates was described. Interestingly, incorporation of different substrates in one and the same complex was allowed [10,15].

Despite the growing knowledge we are far away from a detailed picture of sHsp–substrate interaction. Unfortunately, the X-ray structures of two sHsps contribute little to this open question since the large homo-oligomeric complexes most probably do not represent the active units [5,16]. Putative substrate-binding sites in eukaryotic sHsps were revealed by the photo-incorporation of hydrophobic dyes such as bis-ANS or by

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cross-linking of artificial substrates like the hydrophobic peptide melittin. Pre-binding of either compound negatively effected sHsp chaperone activity pointing towards hydrophobic sHsp–substrate interactions [11,17,18]. According to these studies, substrates might be bound in a region that corresponds to β-strands three and four of *Methanococcus jannaschii* Hsp16.5 and wheat Hsp16.9.

Information concerning sHsps substrates is limited and mainly based on in vitro studies with model substrates. Until recently only very few in vivo substrates were known. *Synechocystis* Hsp16.6 was shown to interact with structurally and functionally unrelated substrate proteins. A common feature was that all these proteins were heat labile and found in the aggregated fraction after heat shock. Large proteins were significantly enriched in the sHsp-bound fraction most probably since multi-domain proteins are less heat stabile [19]. Similar observations were made for in vivo substrates of DnaK [20]. Generally, it is believed that sHsps mainly interact with exposed hydrophobic substrate patches but detailed studies are missing. In contrary, substrate-binding sites of other chaperones like *Escherichia coli* DnaK, its co-chaperone DnaJ, the translocation chaperone SecB, and trigger factor were investigated in some detail. Studies with cellulose-bound peptides derived from different substrates revealed that DnaK binds to sites that appear statistically every 30–40 amino acid in common proteins. The core of the binding motifs is enriched in aliphatic and aromatic residues and is flanked by basic amino acids. Substrate-binding sites of other chaperones were similar yet distinct [21–23].

Bacterial sHsps can be separated into two classes, A and B, according to their primary sequence [24]. Class A and B sHsps of the nitrogen-fixing soybean symbiont *Bradyrhizobium japonicum* prevent the thermally induced formation of insoluble aggregates of the model-substrate citrate synthase (CS) by forming stable sHsp–CS complexes [6,25]. To identify binding sites on peptide level, cellulose-bound peptide scans representing the entire CS sequence were screened. In an alternative approach, membranes carrying sHsp peptides were screened for oligomerisation regions. Both screens revealed potential interaction sites.

Materials and methods

Bacterial strains and plasmids. Escherichia coli DH5α, grown in LB (Luria–Bertani) medium, was used for recombinant DNA techniques, according to standard protocols [26]. The construction of the pET-derived plasmids for the production of *B. japonicum* $HspB_{His}$ (pRJ5304), $HspH_{His}$ (pRJ5307), $HspC_{His}$ (pRJ5305), and $HspF_{His}$ (pRJ5306) has been described recently [25].

Protein expression and purification. Escherichia coli BL21(DE3)-pLysS was freshly transformed with expression plasmids. Cultures were grown to $D_{600} = 0.6$ before protein expression was induced by the addition of isopropyl thio-β-D-galactoside (0.5 mM). After further 2 h at 30 °C, the cells were harvested and resuspended in binding buffer (500 mM KCl, 20 mM Tris–HCl, and 5 mM imidazole, pH 7.9) containing 1 mM phenylmethylsulphonyl fluoride and 10 μg ml^{-1} DNaseI. After lysis in a French pressure cell, soluble crude extracts were obtained by centrifugation at 12,000*g* for 30 min at 4 °C. Protein purification was carried out under native conditions by affinity chromatography using Ni–nitrilotriacetic acid resin (Ni–NTA, Qiagen) as described [8,25]. Purified proteins were used immediately or stored at −20 or −80 °C in elution buffer (500 mM KCl, 20 mM Tris–HCl, and 250 mM imidazole, pH 7.9).

Screening of cellulose-bound peptides. Peptide scans were obtained from Jerini AG (Berlin, Germany). The spotted peptides were derived from the sequence of HspB and from porcine heart citrate synthase, not including the mitochondrial transit peptide (M1-S27). 13-mers overlapping by 10 amino acids were covalently attached by their C-terminal end to cellulose membrane. His-tagged sHsps were purified as described and tested for chaperone activity prior to use. Peptide scans were rinsed with ethanol for 1 min and washed 3 × 10 min in sodium phosphate buffer (50 mM, pH 6.8) prior to incubation with sHsp in incubation buffer (50 mM sodium phosphate, 0.05% Tween 20, and 5% sucrose, pH 6.8) for 30 min. The sHsp concentration was between 200 and 600 nM. After incubation, unbound sHsps were removed with incubation buffer. Bound protein was electrotransferred onto four polyvinylidene fluoride (PVDF) membranes (Millipore) using a tank transfer method, in a fractionated manner (2 × 15, 1 × 30, and 1 × 60 min). Transferred sHsps were detected with tetra-His antibody (Qiagen) and visualised using a horseradish peroxidase conjugated second antibody (Bio-Rad) and a luminal-based chemiluminescence substrate (Super-Signal, Pierce).

Chaperone activity inhibition assay. The ability of HspH and HspB to prevent citrate synthase (CS) from thermal aggregation at 43 °C was monitored for over 40 min as described previously [25]. sHsp and CS concentration were 600 nM. pepCS1, pepHspB1, and the control peptide pepCS2 were added to the assay to final concentrations of 24 and 48 μM (40- and 80-fold molar excess over sHsps). sHsp alone or in combination with the peptides was pre-incubated in 50 mM sodium phosphate buffer (pH 6.8) at 43 °C for 10–15 min prior to addition of the CS. CS aggregation was measured by increased light scattering at 360 nm in an Ultrospec 3000 spectrophotometer (Amersham Pharmacia Biotech). Synthetic peptides were purchased from Jerini AG. The peptides were solved in the CS-assay buffer and stored at −20 °C. CS (Sigma) was dialysed against Tris/EDTA buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and stored at −20 °C before use.

Results

Subunit interaction involves all three sHsp regions

Recently, it was demonstrated that oligomerisation of *B. japonicum* sHsps involves N- and C-terminal parts as well as the α-crystallin domain. Especially a highly conserved I-x-I motif in the C-terminal extension was critical for oligomerisation [7]. To identify sHsp interaction sites on the peptide level, cellulose-bound peptides derived from the sequence of HspB were incubated with purified HspB or HspH, another class A protein. Both experiments revealed similar interaction patterns with sHsp-binding sites in all three regions of HspB. The N-terminal region produced rather weak, yet distinct, signals from peptides 3 to 5 and 7 to 8 after incubation with HspB (Fig. 1A) and from peptides 2 to 6 after

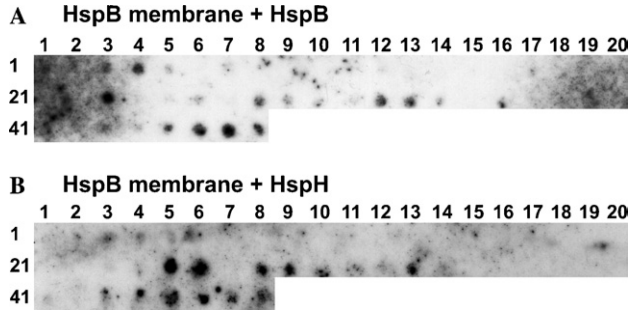


Fig. 1. sHsp binding to cellulose-bound HspB peptide scans. A peptide scan derived from the sequence of HspB was screened for binding of the class A proteins HspB (A) and HspH (B). The sequence of HspB was covered by 48 peptides, each consisting of 13 amino acids with a 10 amino acid overlap. The number of the first peptide in each row is indicated on the left, the first peptide in each column is numbered on top of the membranes. Peptide spots 1–11 represent the N-terminal region, spots 12–38 the α-crystallin domain, and spots 39–48 the C-terminal extension of HspB.

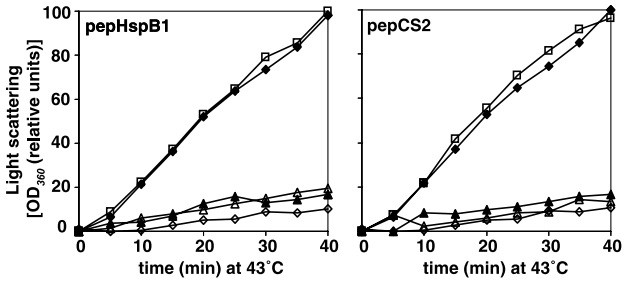


Fig. 3. The C-terminal HspB peptide pepHspB1 has no effect on chaperone activity of HspB. Thermal aggregation of CS at 43 °C in the presence of HspB and the peptides pepHspB1 (left panel, 100 units = 0.221) or pepCS2 (right panel, 100 units = 0.209) was recorded at 360 nm. The chaperone assay was performed with 600 nM CS alone (♦), with CS and 600 nM HspB (◊), with CS and peptides at 48 μM (□), and in the presence of HspB plus pepHspB1 or pepCS2 at concentrations of 24 μM (△) or 48 μM (▲).

incubation with HspH (Fig. 1B). A second interacting region was present in the C-terminal half of the α-crystallin domain, represented by signals at spots 23, 25, and 26, 28–30, 32–34, and 36 for interaction with HspB, and 24–26 and 28–33 for interaction with HspH. Peptides 43–48 covering the C-terminal extension of HspB reacted strongly in both screens. The different interaction sites are marked in the HspB amino acid sequence in Fig. 2.

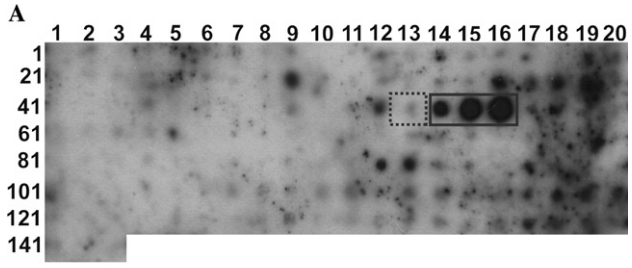
A peptide interacting with HspB might inhibit chaperone activity by disturbing proper oligomerisation. To test this assumption, a peptide corresponding to the reactive C-terminal extension of HspB was synthesised. The sequence of the peptide pepHspB1 is underlined in the HspB sequence in Fig. 2. Addition of up to 40- and 80-fold molar excess of pepHspB1 over HspB to the CS protection assay did not reduce HspB activity (Fig. 3, left panel). Similarly, the control peptide pepCS2 (see below) did not have an influence on the HspB chaperone activity (Fig. 3, right panel), demonstrating that neither peptide interfered with the oligomerisation/dissociation process and substrate-binding of the chaperone.

Detection of a putative CS interaction site for sHsps

Mitochondrial citrate synthase from pig heart is a widely used model substrate for sHsp chaperone activity assays [27]. Formation of large sHsp–CS complexes was repeatedly observed with sHsps from various organisms. To elucidate the CS sites interacting with sHsps, a peptide scan was screened with *B. japonicum* class A and B proteins, which resulted in partially overlapping binding patterns. The strongest signal derived from an experiment with HspH covered spots 54–56 corresponding to amino acids E187-GIHRTKYWELIYEDCMD-L205 of the CS (Fig. 4A). Weaker signals corresponding to the same site were also observed in screens with the class B proteins HspF and HspC (data not shown). The core sequence of this putative chaperone recognition site is KYWELIY (Fig. 4B).

A synthetic peptide corresponding to this site (pepCS1: RTKYWELIYEDCMDL) was tested for its influence on chaperone activity. Relatively low concentrations around 2.4–4.8 μM (4- to 8-fold excess over sHsps) had no effect (data not shown). However, addition of up to an 80-fold molar excess of pepCS1 over sHsp inhibited HspH and HspB activity (Figs. 5A and B, left panels). A control peptide (pepCS2: ARAYAEGIHRTKYWE) corresponding to spot Nr. 53,

Fig. 2. Putative oligomerisation sites of HspB. N- and C-terminal regions and α-crystallin domain are indicated. Interaction sites derived from peptide scans are shaded in black for overlapping HspB and HspH binding sites, in grey for HspB, and in dark grey for HspH. The sequence of peptide pepHspB1 is underlined.



B

Nr. 54:	EGIHRTKYWELIY
Nr. 55:	HRTKYWELIYEDC
Nr. 56:	KYWELIYEDCMDL
Core region:	KYWELIY
pepCS1:	RTKYWELIYEDCMDL

Fig. 4. Binding of HspH to cellulose-bound peptide scans of CS. A peptide scan derived from the sequence of CS was screened for sHsp/substrate interaction sites with the class A protein HspH (A). The CS sequence is covered by 143 peptides consisting of 13mers. The peptides overlap by 10 amino acids. The number of the first peptide in each row is indicated on the left. The first peptide in each column is numbered on the top. The best binding spots (Nr. 54–56) and the non-binding spot Nr. 53 (control peptide pepCS2, see below) are indicated on the membrane by solid and dotted squares, respectively. (B) The amino acid sequences of the binding spots and the derived core sequence as well as of the putative interaction peptide pepCS1 are given.

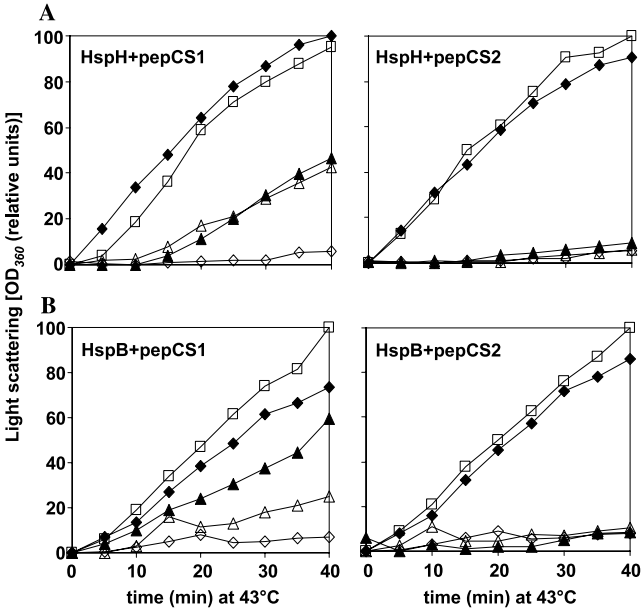


Fig. 5. Inhibition of HspH and HspB chaperone activity by pepCS1. Thermal aggregation of CS at 43 °C in the presence of HspH (A) or HspB (B) and the peptides pepCS1 (left panels, 100 units = 0.24 (A) or 0.19 (B)) or pepCS2 (right panels, 100 units = 0.26 (A) or 0.16 (B)) was recorded at 360 nm. The chaperone assay was performed with 600 nM CS alone (♦), with CS and 600 nM HspB or HspH (◊), with CS and peptides at 48 μM (□), and in the presence of HspB or HspH plus pepCS1 or pepCS2 at concentrations of 24 μM (△) or 48 μM (▲).

which did not interact on the peptide scan, did also not influence HspH and HspB activity (Figs. 5A and B, right panels).

Discussion

sHsps oligomerise via an extended interaction surface

Oligomerisation into large complexes is a hallmark feature of sHsps. The contribution of N- and C-terminal regions and the central α-crystallin domain to this process is not completely understood [2]. Recently, it was shown by mutagenesis studies that all three regions of *B. japonicum* sHsps are involved in oligomerisation [7,8]. These results were clearly supported by our epitope mapping experiments.

The finding of interaction sites in the N-terminus is in agreement with previous studies. Removal of the first 20 N-terminal residues in α-crystallin did not affect multimerisation, whereas derivatives with more extended truncations formed significantly smaller complexes [28]. Similarly, deletion of the first nine (but not three) residues of the relatively short N-terminal region of the *B. japonicum* class A protein HspH led to a significant decrease of the oligomer size [7]. In wheat Hsp16.9, only every second N-terminus was found to be structured and to contribute to the oligomerisation via α-helical interactions. In contrast, in each *M. jannaschii* Hsp16.5 subunit the first 32 amino acids were unstructured and not solved in the crystal structure. Most probably they point into the central cavity and contribute to complex stability [5,16].

Despite their effect on oligomerisation, N- and C-terminal truncations did not impair the dimerisation of *B. japonicum* sHsps. In contrast, two mutations, HspH(F94A) and HspB(G116A) within the central domain, led to interruption or destabilisation of the dimerisation interface, respectively [6,8]. These observations argue for the α-crystallin domain as the main dimerisation region. Crystallographic data from Hsp16.5 and Hsp16.9, spin labelling studies with αA-crystallin and Hsp27 as well as the finding that αB-crystallin comprising only the α-crystallin domain formed a dimer point in the same direction [5,16,29,30]. Consistently, our peptide scans revealed binding sites within the α-crystallin domain. The signals cover a region that corresponds to the β6 dimerisation-loop of both known sHsp structures. A prerequisite for binding to these sites in the epitope-mapping experiments would be the dissociation of the dimer into monomers. Subunit exchange experiments with two plant sHsps by nanoESI-MS suggested that indeed single units might be exchanged between large complexes although dimers were the main subspecies [31].

The presence of signals representing the C-terminal end fully supports the importance of this region in the oligomerisation process. Elimination of the highly conserved I-x-I motif within this extension prevented the formation of complexes exceeding dimers [7]. In both

solved sHsp structures, the C-terminal stretch extends from the dimer and forms contacts with β-strands of neighbouring subunits [5,16].

At present, it cannot be excluded that some of the sHsp spots in our screen represent substrate-binding rather than oligomerisation sites. However, such peptides should interfere with the chaperone activity by blocking substrate interaction sites (such as pepCS1, see below). At least in the case of pepHspB1 no such effect was observed in our sHsp competition assay, suggesting that pepHspB1 does not display features characteristic of sHsp substrates. Moreover, peptides corresponding to a putative substrate-binding site at the beginning of the α-crystallin domain did not reveal any signals in our scans. Thus, oligomerisation sites appear to be distinct from substrate interaction sites.

The CS peptide pepCS1 might be a major recognition determinant for sHsps

sHsps most probably interact with unfolded stretches of substrate proteins. Hence, such sites might be continuous rather than discontinuous as in many other protein/protein interactions. Cellulose-bound peptides have been very useful to study chaperone/substrate interaction sites. Good binding sites for DnaK occurred statistically every 84 residues. Most of the sites are buried in the native fold. The binding motif consists of a hydrophobic core enriched in Leu, Ile, Val, Phe, and Thr. The flanking regions are enriched in basic residues whereas acidic residues are excluded from both sites. The DnaK co-chaperone DnaJ binds to very similar motifs [21,22]. Despite the fact that TF mainly binds to motives enriched in basic and aromatic residues about 80% of its binding peptides also associated with DnaK [21,32].

We identified three partially overlapping peptide spots interacting with HspB. The stretch covers $CS_{187–205}$ corresponding to the N-terminal part of helix nine of mitochondrial pig heart CS [33] (Fig. 6). The fact that three consecutive spots reacted suggests that a true recognition site has been revealed. The common core region of this stretch (KYWELIY) mainly contains aromatic and hydrophobic amino acids that are hidden inside the native CS dimer and display low solvent accessibility. Although $CS_{187–205}$ clearly gave the strongest signal in our experiment additional putative interaction sites were detected. The presence of several binding sites within one and the same substrate as observed for other chaperones is reasonable since substrate promiscuity of sHsps is high and demands binding motives that are not highly conserved [19]. To allow predictions on binding motives, many sHsp substrate-binding sites are required. Unfortunately, only one substrate peptide of sHsps was described so far. The yeast ADH peptide with the sequence -YSGVCHTDLHAWHGDWPLPVK- binds to α-crystallin during chaperone action [34]. There is no obvious similarity with the interaction site in CS and the ADH peptide did not inhibit chaperone activity of *B. japonicum* sHsps (data not shown).

The fact that pepCS1 had an inhibitory effect on the sHsps chaperone activity is an indication that it competes with the partially unfolded CS for substrate-binding sites on sHsps. Peptide pepCS2 did not have an inhibitory effect in spite of the six C-terminal amino acids that it shares with pepCS1. This indicates that these amino acids are not crucial for the sHsp/peptide interaction. The high, ≈40- to 80-fold molar excess of pepCS1 that was needed suggests that the binding affinity might not be very high. Much lower amounts (≈12-fold molar excess) of the hydrophobic peptide melittin were necessary to completely prevent α-crystallin from protecting temperature-induced ADH aggregation [18]. In solution the affinity of TF to a substrate peptide detected in a screen with in-mobilised peptides was very low with a K_D of approx. 120 μM. This low affinity can be explained by the fact that TF rather acts in its ribosomal-bound

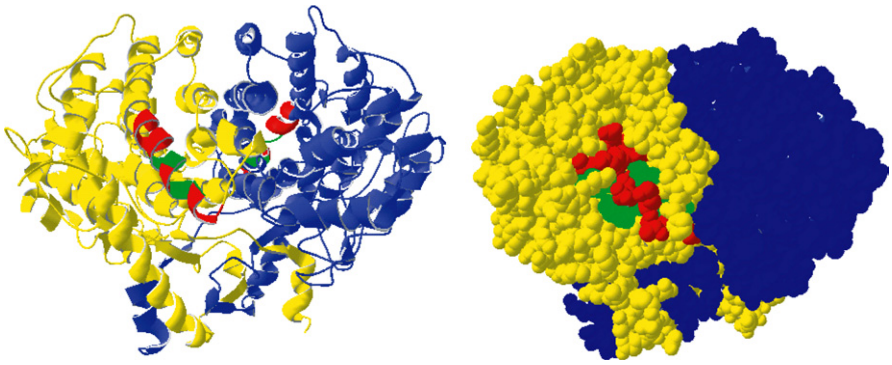


Fig. 6. Ribbon and space filling representation of the CS structure. The dimer subunits are given in yellow and blue. The putative sHsp-binding site is indicated in red. Hydrophobic residues are depicted in green.

state than in solution [21]. In contrary, peptides that displayed affinity to DnaK when bound to cellulose also had high affinity when tested in solution. The K_D of such peptides was shown to be in the range of 0.1–7 μM [22].

Acknowledgments

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