

Hsp26: a temperature-regulated chaperone

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Small heat shock proteins (sHsps) are a conserved protein family, with members found in all organisms analysed so far. Several sHsps have been shown to exhibit chaperone activity and protect proteins from irreversible aggregation *in vitro*. Here we show that Hsp26, an sHsp from *Saccharomyces cerevisiae*, is a temperature-regulated molecular chaperone. Like other sHsps, Hsp26 forms large oligomeric complexes. At heat shock temperatures, however, the 24mer chaperone complex dissociates. Interestingly, chaperone assays performed at different temperatures show that the dissociation of the Hsp26 complex at heat shock temperatures is a prerequisite for efficient chaperone activity. Binding of non-native proteins to dissociated Hsp26 produces large globular assemblies with a structure that appears to be completely reorganized relative to the original Hsp26 oligomers. In this complex one monomer of substrate is bound per Hsp26 dimer. The temperature-dependent dissociation of the large storage form of Hsp26 into a smaller, active species and the subsequent re-association to a defined large chaperone-substrate complex represents a novel mechanism for the functional activation of a molecular chaperone.

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Introduction

Cells increase the expression of several classes of proteins in response to environmental stresses such as heat shock (Lindquist and Craig, 1988). The major conserved families of these stress or, historically, heat shock proteins (Hsps), have been shown to be involved in protein folding as molecular chaperones (Morimoto *et al.*, 1994; Beissinger and Buchner, 1998). One of these chaperone classes consists of the small heat shock proteins (sHsps). sHsps have been found in all organisms studied so far. They

share conserved regions in the C-terminal part of the protein, while the N-terminal part is quite divergent in sequence and length, ranging from 12 to 40 kDa in different organisms (Arrigo and Landry, 1994; Ehrnsperger *et al.*, 1997a). The conserved C-terminal domain exhibits high sequence homology to the family of α -crystallins, which constitute a major part of the eye lens (de Jong *et al.*, 1993). In addition to the sequence homology, both sHsps and α -crystallins form large oligomeric complexes. The subunit stoichiometry varies substantially and complex sizes in the range of 150–800 kDa have been found for different members of the sHsp family (de Jong *et al.*, 1993). For Hsp16.5 from *Methanococcus jannaschii* and α -crystallin the three-dimensional structures have been described as hollow shells (Haley *et al.*, 1998; Kim *et al.*, 1998). While α -crystallin seems to be dynamic and exchanges subunits, Hsp16.5 is a precisely defined complex.

The interaction with non-native protein was first demonstrated for α -crystallin, murine Hsp25 and human Hsp27 (Horwitz, 1992; Jakob *et al.*, 1993; Merck *et al.*, 1993). Among others, these findings have been extended to sHsps from plants (Lee *et al.*, 1995), *Mycobacterium tuberculosis* (Chang *et al.*, 1996) and IbpB from *Escherichia coli* (Allen *et al.*, 1992; Veinger *et al.*, 1998). In contrast to other chaperone families, sHsps from plants and mammals bind several non-native proteins per oligomeric complex (Ehrnsperger *et al.*, 1997b; Lee *et al.*, 1997).

The binding and release of substrate requires neither ATP binding nor ATP hydrolysis (Jakob *et al.*, 1993). Release of substrate protein from the sHsp complex is rather inefficient (Lee *et al.*, 1995; Ehrnsperger *et al.*, 1997b), suggesting that the role of sHsps in the chaperone network of the cell is mainly to create a reservoir of non-native refoldable protein. In agreement with this view, it has recently been shown that proteins bound to sHsps can be refolded to the native state in cooperation with Hsp70 in an ATP-dependent reaction (Ehrnsperger *et al.*, 1997b). Experiments using cell extracts demonstrate that both the complex formation with a variety of unfolded proteins (Ehrnsperger *et al.*, 1997b) and the ATP-dependent release in concert with other factors (Lee *et al.*, 1997) also occur *in vivo*.

In mammalian cell lines increased thermotolerance was observed upon overexpression of sHsps (Landry *et al.*, 1989; Knauf *et al.*, 1992; van den IJssel *et al.*, 1994). However, deletion of Hsp26 in *Saccharomyces cerevisiae* did not result in a temperature-sensitive phenotype. Growth rate and survival at elevated temperatures were unaffected in this mutant under a variety of conditions (Petko and Lindquist, 1986).

Here we describe the structural and functional properties of Hsp26 and its complexes with non-native protein under heat shock conditions *in vitro* and *in vivo*. We show that

Hsp26 has chaperone properties and that Hsp26 complexes are functionally activated by dissociation upon heat shock.

Results

Hsp26 prevents the thermal aggregation of citrate synthase and slows down its inactivation kinetics

Hsp26 was purified from *S.cerevisiae* as a soluble, high molecular weight complex. To determine the size of this complex, size exclusion chromatography (SEC) was performed (see below). Hsp26 eluted as a single peak with an apparent molecular mass of 550 kDa in agreement with results from Bentley *et al.* (1992). Since the monomer has a molecular mass of 23 748 Da as calculated from the amino acid sequence (Bossier *et al.*, 1989; Susek and Lindquist, 1989), the complex appears to consist of 24 subunits. To test whether Hsp26 exhibits chaperone activity, as previously shown for its mammalian and plant homologues (cf. Ehrnsperger *et al.*, 1997a), we performed unfolding assays using citrate synthase (CS) as a model substrate (Buchner *et al.*, 1998). The dimeric enzyme is inactivated and aggregates irreversibly at temperatures $>40^{\circ}\text{C}$. As shown in Figure 1A, the spontaneous aggregation of CS as monitored by light scattering starts after 2 min at 43°C and reaches a maximum value after ~ 15 min. At a molar ratio of 2:1 (Hsp26 complex:CS dimer), CS aggregation could be almost completely suppressed. The chaperone function of Hsp26 is not restricted to CS, since Hsp26 could also suppress the aggregation of the model substrate insulin β -chain at 43°C (Figure 4) and α -glucosidase at 48°C (data not shown).

Compared with aggregation, the loss of enzymatic activity is an early step in the unfolding process of CS (Buchner *et al.*, 1998). We were therefore interested in analysing whether Hsp26 exhibits any effect on the thermal inactivation of CS. At a molar ratio of 1:1 (Hsp26 complex:CS dimer), Hsp26 had little effect on the inactivation kinetics of CS at 43°C compared with spontaneous inactivation. However, higher concentrations of Hsp26 slowed down the inactivation reaction significantly (Figure 1B). The maximum effect could be seen at molar ratios of 4:1 to 8:1. A further increase in Hsp26 concentration did not decelerate the inactivation any further (Figure 1B, inset).

Taken together, these results suggest that the transient binding of CS by Hsp26 leads to an apparent stabilization.

Hsp26 forms large defined complexes when incubated with CS at elevated temperatures

Mammalian sHsps form complexes of >1000 kDa when incubated with substrate proteins under heat shock conditions (Ehrnsperger *et al.*, 1997b; Lee *et al.*, 1997). We were therefore interested in whether Hsp26 was also able to form large structures at elevated temperatures. Using SEC, negative-stain and cryo-electron microscopy (EM) we analysed changes in complex size in the presence of substrate protein.

For the SEC experiments, Hsp26 and CS were incubated together at 25 or 43°C for 45 min and then applied to a gel filtration column (Figure 2A). At a molar ratio of 1:2 (Hsp26 monomer:CS monomer), incubation of the two proteins at 25°C resulted in two clearly separated peaks. The high molecular weight peak corresponds to the Hsp26

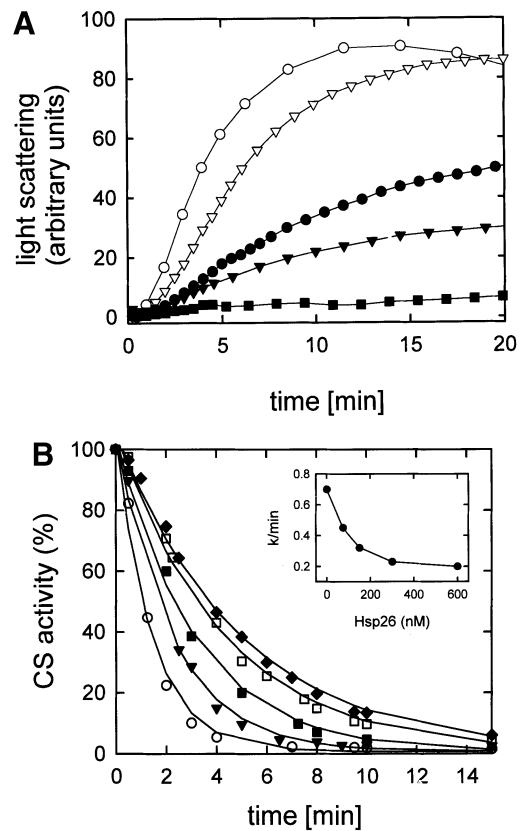


Fig. 1. Aggregation and inactivation kinetics of CS at 43°C in the presence and absence of Hsp26. (A) Influence of Hsp26 on the thermal aggregation of CS. CS (final concentration 75 nM) was diluted into a thermostatted solution of 37.5 nM (∇), 55 nM (\bullet), 75 nM (\blacktriangledown) and 150 nM Hsp26 complex (\blacksquare). Open circles (\circ) represent the spontaneous aggregation of CS at 43°C . The kinetics of aggregation were determined by measuring the light scattering of the samples. (B) Influence of Hsp26 on the inactivation of CS at elevated temperatures. Inactivation of CS (75 nM dimer) at 43°C in the absence (\circ) and in the presence of increasing concentrations of Hsp26 complex, 75 nM (\blacktriangledown), 150 nM (\blacksquare), 300 nM (\square) and 600 nM (\blacklozenge). Inactivation was monitored by incubating the sample at 43°C and measuring the remaining CS activity at the time points indicated. Inset, influence of Hsp26 concentration on the apparent rate constant of the thermal inactivation of CS at 43°C .

oligomer and the low molecular weight peak corresponds to the CS dimer (dotted line). Hsp26 could be shifted almost completely into a larger species when both proteins were incubated at 43°C (Figure 2A, solid line). SDS-PAGE analysis confirmed that the new species contained both Hsp26 and CS (Figure 2B). The peak eluted in the void volume of the column, indicating that binding of denatured CS to Hsp26 at heat shock temperatures resulted in the formation of particles >1500 kDa.

To determine the stoichiometry of substrate binding, Hsp26 was incubated with increasing amounts of CS and the complex peak in the void volume was collected, analysed on SDS-PAGE and the amounts of Hsp26 and CS were determined by densitometry (Figure 2B). Increasing the molar ratio of CS to Hsp26 resulted in a sigmoidal increase of CS in the complex. This clearly demonstrates that the formation of the large Hsp26-CS complex is a highly cooperative process. The maximum binding capacity is achieved at a molar ratio of one CS monomer per Hsp26 dimer.

In addition to the SEC analysis, we examined complexes

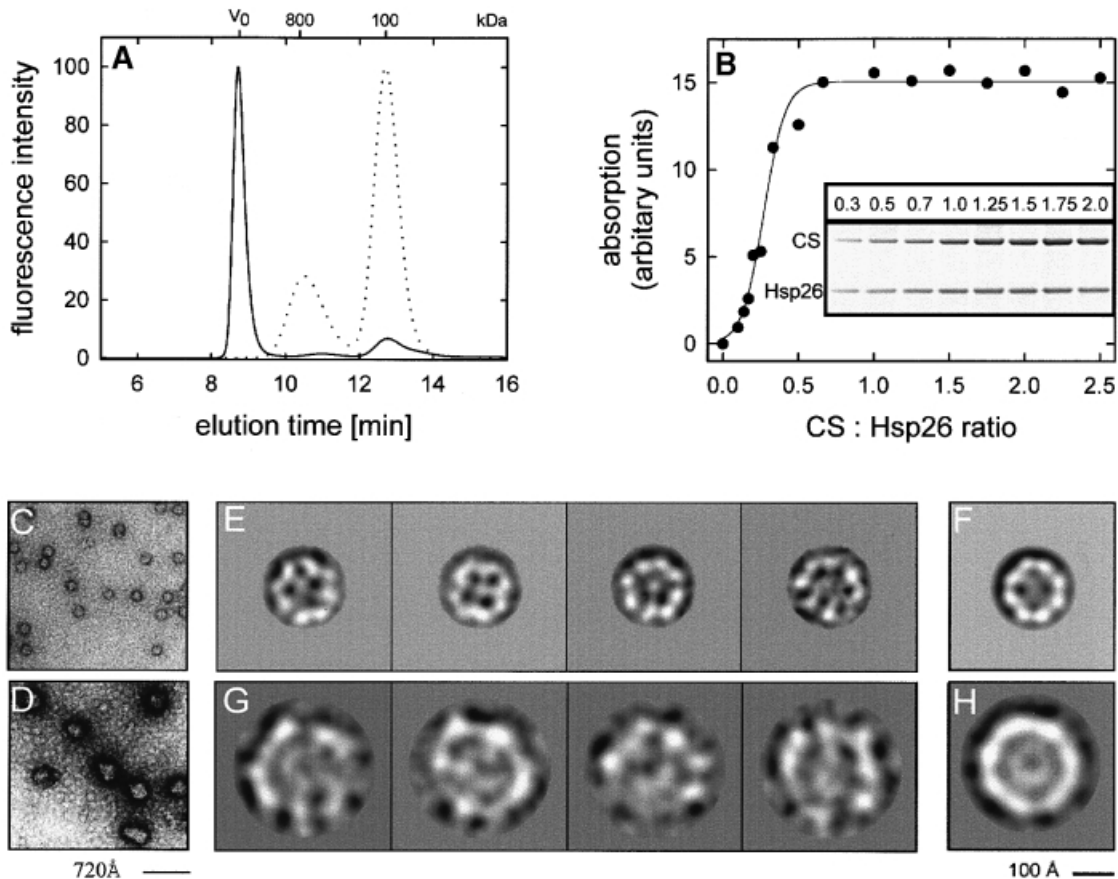


Fig. 2. Complex formation between Hsp26 and CS. (A) Detection of Hsp26–CS complexes by SEC. Hsp26 (3 μ M) was incubated in the presence of CS (6 μ M) for 45 min at 25°C (.....) or at 43°C (—). The complexes were then applied to a TSK 4000 PW column. V_0 , void volume of the column determined by Blue Dextran (8.69 min). The column was calibrated using LMW and HMW gel filtration calibration kits (Amersham-Pharmacia). (B) Denitometric analysis of CS and Hsp26 in Hsp26–CS complexes. Hsp26 (3 μ M) was incubated with increasing amounts of CS (1–15 μ M) for 45 min at 43°C and applied to a TSK 4000 PW column. The resulting Hsp26–CS complex peak was analysed as described in Materials and methods. The amounts of CS found in the complexes compared with the molar ratio of CS:Hsp26 in the mixture, show a sigmoidal increase of CS bound in the Hsp26–CS complex. Inset, representative section of a Coomassie-stained SDS–PAGE used in the analysis. (C) Negative-stained electron microscopic image of Hsp26 (0.13 μ M) incubated for 15 min at 25°C. (D) Negative-stained electron microscopic image of Hsp26–CS complexes. Hsp26 (0.13 μ M) was incubated in the presence of CS (0.4 μ M) for 15 min at 43°C. The scale bar for (C) and (D) represents 720 Å. (E) Class averages of cryo-EM images of Hsp26. (F) Average of all aligned Hsp26 images. (G) Class averages of the Hsp26–CS complexes. (H) Average of all aligned Hsp26–CS complex images. The class averages contain ~100 images each and the total averages each contain ~3000 images. For the complexes, Hsp26 (1.3 μ M) was incubated in the presence of CS (2.14 μ M) at 43°C for 40 min. The scale bar for (E–H) represents 100 Å.

by EM. The negative-stain electron micrographs of Hsp26 showed regular, spherical complexes at 25°C (Figure 2C). The particles seemed to be uniform in size and structure with a diameter of ~15 nm, which is in the same size range as found by Bentley *et al.* (1992) for Hsp26 overexpressed in *E. coli*. Figure 2D shows a representative part of an electron micrograph of negative-stained Hsp26–CS complexes. The particles are of globular shape and up to twice the size of Hsp26 complexes at 25°C. When CS is incubated in the absence of Hsp26, large amorphous protein aggregates are detected (Ehrnsperger *et al.*, 1997b; data not shown). These aggregates were completely missing when Hsp26 was present, underlining the ability of Hsp26 to suppress the thermal aggregation of CS (cf. Figure 1A).

To analyse further the structure of Hsp26 and the Hsp26–CS complexes, cryo-EM images were analysed (Figure 2E–H). They confirmed that the Hsp26 preparation consists of uniformly sized particles with diameters of ~150 Å (Figure 2E). The averaged images of Hsp26 demonstrate that the complex forms a hollow shell

(Figure 2F), as has been seen for α B-crystallin (Haley *et al.*, 1998) and an archaeobacterial sHsp (Kim *et al.*, 1998).

Compared with the Hsp26 oligomer, Hsp26–CS complexes (Figure 2G) formed by heat treatment were greatly increased in size, with diameters of ~230 Å. This represents a 3.6 \times increase in particle volume. The particles are more irregular, with angular features in the outer shell, but still relatively uniform in size. The averaged images hint at an internal shell of density, in addition to the enlarged and thickened outer shell (Figure 2H). Other sHsp–substrate complexes that have been examined by EM are significantly more irregular in shape (Ehrnsperger *et al.*, 1997b; Lee *et al.*, 1997).

Hsp26 dissociates at elevated temperatures

Fluorescence spectroscopy, circular dichroism and light scattering studies (data not shown) gave rise to the idea that the structure of Hsp26 might change at heat shock temperatures. Therefore, we performed SEC experiments at different temperatures, and at Hsp26 concentrations ranging from 0.04 to 4 μ M. At 25°C, Hsp26 eluted as a

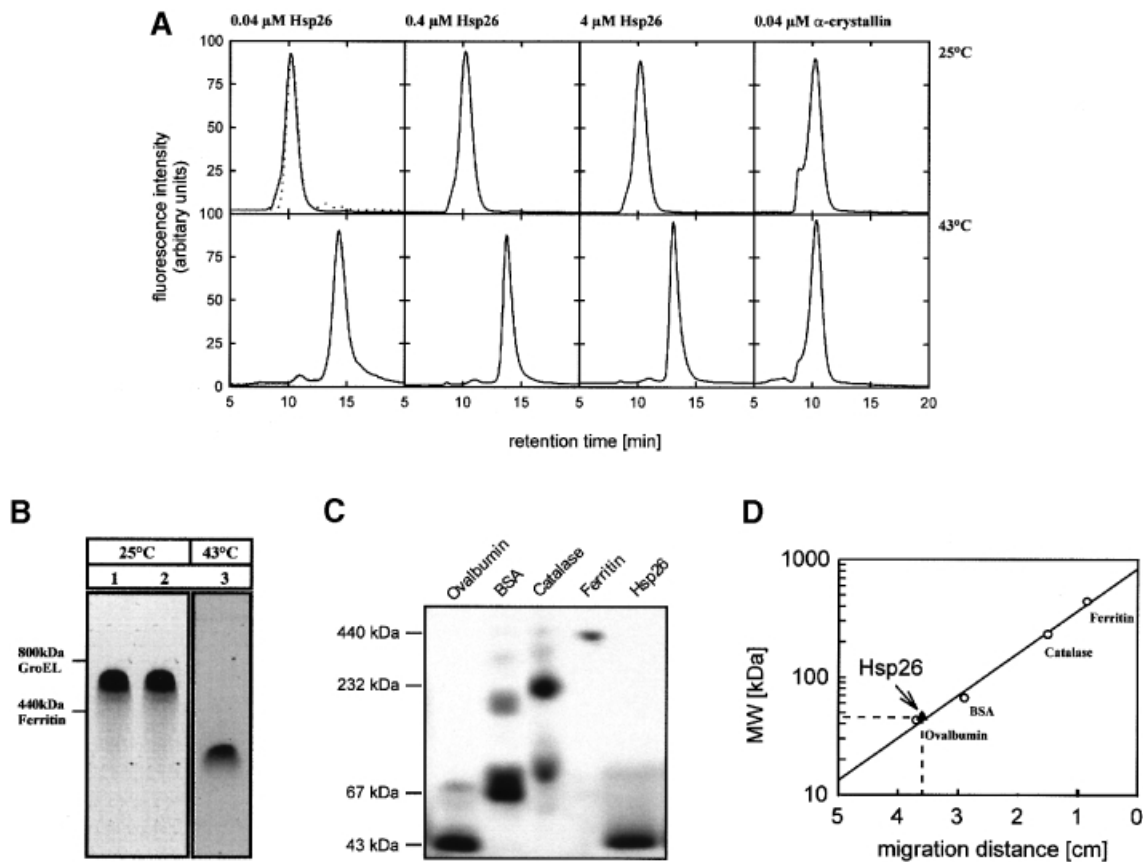


Fig. 3. Influence of elevated temperature on Hsp26 quaternary structure. **(A)** Size exclusion chromatography. HPLC was performed using a TosoHaas TSK 4000 PW column as described in Materials and methods. Hsp26 and α -crystallin were incubated for 20 min at the concentrations and temperatures indicated before they were applied to the column, which was kept at the same temperatures. The signal intensity was normalized (dotted line). To investigate the reversibility of Hsp26 dissociation, the protein (0.04 μ M) was incubated at 43°C for 20 min, then kept for 2 h at 25°C before application to the column. **(B)** Native PAGE. Bands were visualized by Coomassie staining. Hsp26 (5 μ g) was incubated for 30 min at 25°C (lane 1) and 30 min at 43°C (lane 2), and separated at 25°C, compared with Hsp26 (5 μ g) that was incubated for 30 min at 43°C and separated at 43°C (lane 3). The bars represent the native PAGE position of GroEL and ferritin. **(C)** Blue native PAGE. All proteins (5 μ g) were incubated for 30 min at 43°C and separated at 43°C. **(D)** Molecular mass calibration curve for blue native PAGE. The arrow indicates the complex size of Hsp26 at 43°C.

single distinct peak (Figure 3A, solid lines) with an apparent molecular mass of 550 kDa at all concentrations investigated. As this peak remained constant over a concentration range of two orders of magnitude, this experiment confirms that at room temperature there is only one specific oligomeric state of Hsp26. Addition of salt (up to 1 M NaCl) did not result in changes in complex size (data not shown). However, when Hsp26 was incubated at 43°C for 20 min and applied to a gel filtration column equilibrated at the same temperature, the 550 kDa complex disappeared almost completely and a smaller species was predominantly detected (Figure 3A). This dissociation process of the Hsp26 oligomer started at temperatures $>35^{\circ}\text{C}$, where dissociation is slow and an equilibrium of the Hsp26 oligomer, several intermediate species and the smaller species was observed. At higher temperatures the dissociation became faster and the equilibrium was shifted to the smaller species, until at $>40^{\circ}\text{C}$ only the smaller species was observed. At 43°C the Hsp26 oligomer remains completely dissociated up to 60 min (data not shown), implying that the small species is a stable end product of dissociation. To determine whether the temperature-dependent dissociation behaviour of Hsp26 is reversible, Hsp26 was incubated at 43°C for 20 min to achieve complete dissociation. Then the protein

was transferred to 25°C for 2 h before SEC was performed at 25°C. The dotted line in Figure 3A shows that Hsp26 was completely re-associated and, according to the peak areas, virtually no loss of protein was observed during the dissociation–re-association process. This distinct behaviour seems to be specific for Hsp26 since the related α -crystallin did not change quaternary structure between 25 and 43°C (Figure 3A).

In a second approach, the change in quaternary structure of Hsp26 at elevated temperatures was examined by native PAGE. Figure 3B shows Coomassie-stained native gels of Hsp26 incubated for 30 min at 25°C and separated at 25°C in comparison with Hsp26 incubated for 30 min at 43°C and separated at 43°C. The band representing the native 24mer (Figure 3B, lane 1) can be distinguished clearly from the lower molecular weight species formed at higher temperatures (Figure 3B, lane 3). When Hsp26 was incubated for 30 min at 43°C and then shifted to 25°C, only the 24mer could be detected, confirming that the disassembly of the 24mer is reversible (Figure 3B, lane 2). Furthermore, quantitation of the Coomassie-stained bands revealed that during disassembly and reassembly no protein was lost (data not shown).

To determine the oligomeric state of the smaller Hsp26 species, blue native PAGE was employed (Schägger and

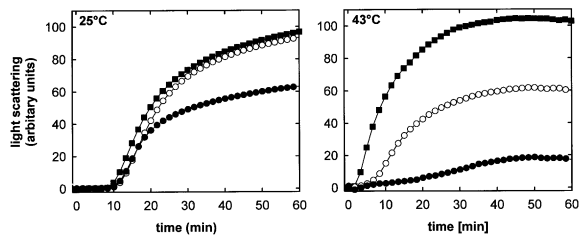


Fig. 4. Temperature dependence of the influence of Hsp26 on the aggregation of insulin. Insulin (final concentration 45 μ M) was incubated at the temperatures indicated in the absence (■) and presence of 0.02 μ M (○) and 0.45 μ M (●) Hsp26 (oligomer). Aggregation of insulin was initiated by addition of DTT (final concentration 15 mM). The kinetics of insulin aggregation were monitored at 400 nm.

von Jagow, 1991; Schägger *et al.*, 1994). For the calibration of the system, proteins known to separate according to their molecular weights were used (Schägger *et al.*, 1994; Figure 3C–D). According to the standard curve (Figure 3D), Hsp26 dissociates into a 45 kDa species, which seems to represent an Hsp26 dimer. At 25°C, blue native PAGE analysis confirmed that Hsp26 is a high molecular weight oligomer (data not shown).

Activation of the chaperone function of Hsp26 requires complex dissociation

The data reported above clearly show that significant structural rearrangements of Hsp26 occur in the heat shock temperature range. It was therefore important to investigate whether the chaperone activity of Hsp26 is affected by these structural changes.

To characterize the apparent temperature dependence of the chaperone activity, we used insulin as a model substrate, since the β -chain of insulin aggregates upon reduction of the disulfide bond between the α - and β -chain over a wide temperature range (Sanger, 1949; Das and Surewicz, 1995; Scheibel *et al.*, 1998). As expected, the aggregation kinetics of insulin were influenced only to a small extent by temperature, with the aggregation reaction being faster at higher temperatures. Nevertheless, the final signal as judged by the absorbance increase at 400 nm was almost independent of the reaction temperature (Figure 4). Thus, the assay itself is essentially temperature-independent and allowed the analysis of the temperature dependence of Hsp26 function. Using substoichiometric concentrations of Hsp26, a concentration-dependent influence on the aggregation of insulin at 25°C could be observed (Figure 4). Most interestingly, when the insulin assay was performed at 43°C, Hsp26 was much more active in suppressing insulin aggregation (Figure 4). This suggests that the 24mer, the predominant species at 25°C, is much less active than the heat shock form of Hsp26, confirming that complex dissociation is required for full chaperone activity.

Complex dissociation is a prerequisite for the *in vivo* function of Hsp26

To test whether the temperature-dependent dissociation of the oligomeric form of Hsp26 is also necessary for *in vivo* chaperone activity, we analysed the substrate binding properties of Hsp26 in cellular lysates. Yeast cytosol was incubated for 30 min at physiological or heat shock temperatures and applied to native PAGE. To exclude association or dissociation processes during the separation

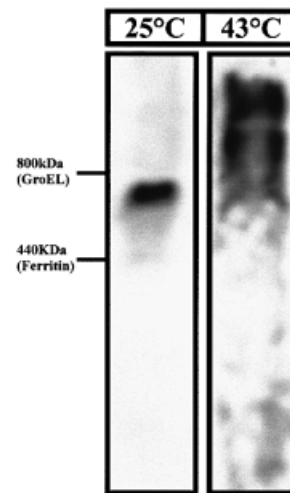


Fig. 5. Temperature dependence of the complex formation between Hsp26 and *in vivo* substrates. Detection of Hsp26 by immunoblotting after native PAGE. Hsp26 (10 μ g) was added to yeast wild-type cell lysate (15 mg/ml protein), incubated for 30 min at 25°C and separated by native PAGE at 25°C, compared with the same experiment at 43°C. The bars represent the native PAGE positions of GroEL and ferritin.

procedure, the native PAGE was performed at the temperatures indicated. The resulting gels were analysed by immunoblotting. Because of the reversibility of the dissociation-activation of the Hsp26 complex, the outcome was independent of whether yeasts were grown at physiological or heat shock conditions before lysate preparation (data not shown). Therefore, the same lysate from yeast grown at 30°C was used for experiments at all incubation temperatures.

When the experiment was done at 25°C, Hsp26 could be detected as a single band representing the Hsp26 oligomer (Figure 5). However, when the same cellular lysate was incubated at 43°C, Hsp26 was found in a broad smear in the high molecular weight range (Figure 5). A defined oligomer band was missing. This smear seems to represent Hsp26-substrate complexes formed at 43°C (Figure 5). The broad smear is indicative of the variety of substrate proteins Hsp26 is able to bind.

Taken together, these experiments demonstrate that structural changes in the heat shock temperature range are required for functional activation of Hsp26 *in vitro* and *in vivo*.

Discussion

The experiments described above characterize the molecular chaperone activity of Hsp26 from *S.cerevisiae*. These findings extend previous results, demonstrating chaperone properties for sHsps from several different species (Ehrnsperger *et al.*, 1998; Prévaille *et al.*, 1999). Furthermore, for the first time the formation of Hsp26-substrate complexes was detected in cellular lysates under *in vivo* conditions, demonstrating that binding of non-native protein is an *in vivo* property of Hsp26. Because of the broad size distribution of these complexes a wide spectrum of substrate proteins is likely.

Differences seem to exist between individual sHsps in their mode of interaction with non-native proteins as exemplified by the influence of Hsp26 on the inactivation kinetics of CS. Hsp26 significantly decelerated the inacti-

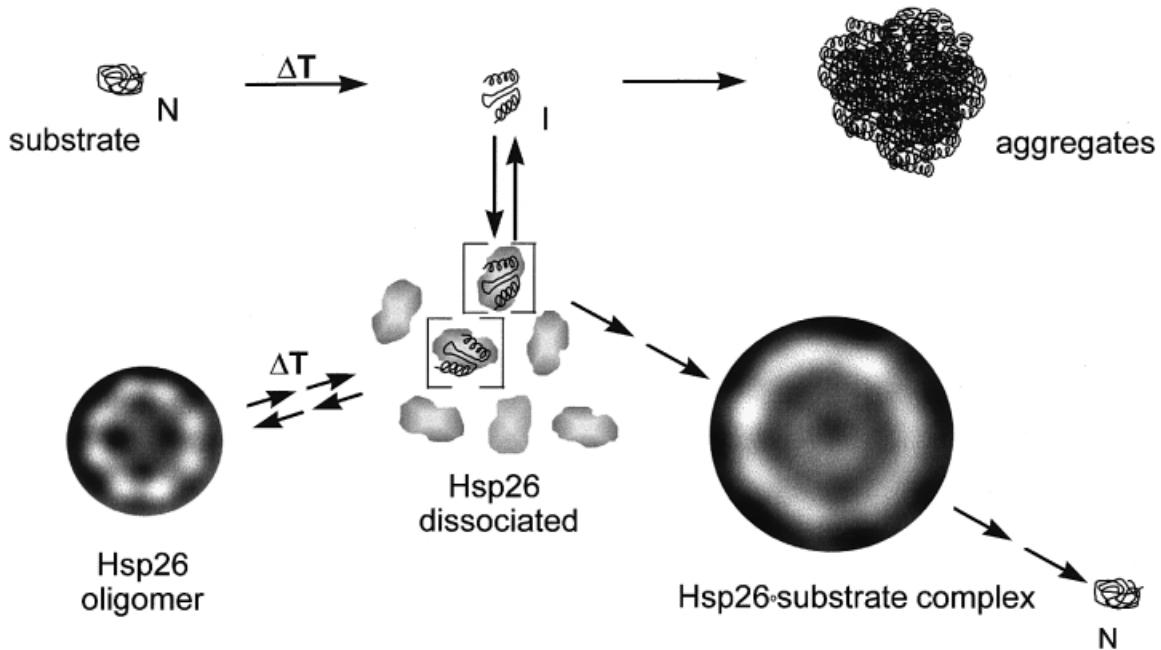


Fig. 6. Model of the chaperone function of Hsp26. Under physiological conditions Hsp26 exists in an inactive oligomeric storage form. Upon heat shock Hsp26 dissociates into dimers, which are able to bind non-native proteins, thus preventing their aggregation. Well defined Hsp26–non-native protein complexes subsequently assemble from the dissociated species. After release of the substrate protein, Hsp26 re-associates to the inactive storage form under physiological conditions. The Hsp26 dimer–CS complex is shown in parentheses to indicate that this species was not directly detected because of the highly cooperative association to the large Hsp26–CS complex.

vation reaction, while Hsp25 from mouse does not change the inactivation kinetics at all (Ehrnsperger *et al.*, 1997b). This suggests that Hsp25 binds denatured CS more tightly than Hsp26 at heat shock temperatures. In this respect, two plant sHsps (Hsp17.7 and Hsp18.1) behave similarly to Hsp26 (Lee *et al.*, 1995). Thus, while the general chaperone properties are conserved among sHsps, variations in the interaction with non-native proteins seem to exist.

The overall structure of the oligomeric complex also seems to be conserved among sHsps. The averaged cryo-EM images show that Hsp26, like mammalian α -crystallin (Haley *et al.*, 1998) and *Methanococcus* sHsp (Kim *et al.*, 1998), consists of a hollow shell of subunits. In this context the regular, defined structure of Hsp26 resembles Hsp16.5 from *M.jannaschii* more than the more flexible structure of α -crystallin.

Again, similarly to other sHsps (Lee *et al.*, 1995, 1997; Ehrnsperger *et al.*, 1997a,b), Hsp26 forms large complexes with non-native substrate proteins at elevated temperatures. However, in contrast to previously examined sHsp substrate complexes, the Hsp26 complexes have a well defined structure (Figure 2G). Moreover, this structure appears to be a completely new assembly, in which the original Hsp26 shell has been replaced by a larger diameter and thicker outer shell, accompanied by an additional, internal shell of density. The complex cannot be explained by the addition of a shell of substrate to a pre-existing shell of Hsp26. The particle diameter increases from 150 Å for Hsp26 to 230 Å for the Hsp26–CS complex. Neither layer resembles the original Hsp26 shell. In these complexes, the stoichiometry of binding is one substrate monomer per Hsp26 dimer, which is similar to the binding ratios reported for other sHsps (Lee *et al.*, 1997).

Dissociation of the chaperone complex at heat shock

temperatures precedes the formation of the Hsp26–substrate complex. The change in the quaternary structure occurs hand in hand with a change of conformation. The dissociated species seems to expose hydrophobic regions that may represent the binding site for non-native protein. These structural changes are of functional importance because Hsp26, unlike other sHsps, is an efficient chaperone only at elevated temperatures. We therefore conclude that Hsp26 requires dissociation at heat shock temperatures to become activated. Thus, Hsp26 is an intrinsic temperature sensor for detecting heat shock conditions. We propose the following model illustrating how this novel mechanism of chaperone regulation may work (Figure 6): large oligomeric Hsp26 complexes consisting of 24 monomers exist as an inactive storage form under physiological conditions. Heat shock has two consequences: (i) it leads to the unfolding and subsequent aggregation of a large number of cellular proteins; and (ii) at the same time, Hsp26 is activated directly through temperature-induced dissociation of the 24mers into dimers. This species specifically recognizes and binds one non-native polypeptide chain. Then, substrate dimer complexes assemble into large, well defined Hsp26–substrate complexes in a cooperative process. The non-native protein will either dissociate from the complex spontaneously or with the help of other chaperones such as Hsp70, and will then fold to the native state (Ehrnsperger *et al.*, 1997b; Lee *et al.*, 1997). Upon restoration of physiological temperatures, the large and inactive storage form of Hsp26 will be formed again.

A dissociation–activation mechanism has also been reported for BiP, an endoplasmic reticulum-located member of the Hsp70 family, which forms inactive dimers that dissociate into active monomers upon contact with denatured substrate protein (Blond-Elguindi *et al.*, 1993).

This could be seen as a variation of the proposed model, except that the denatured substrate proteins itself, rather than increased temperature, could induce the activation of the chaperone. For Hsp33, a recently discovered cytosolic chaperone of *E.coli*, chaperone activity was demonstrated to be redox-regulated (Jakob *et al.*, 1999). This provides a rapid first defence mechanism against the toxic effects of oxidative stress, and closes a gap in the timescale of the stress response (Ruddock and Klappa, 1999). Thus, the specific activation of chaperones by stress may be a more widespread phenomenon than previously thought.

It is tempting to speculate that the heat-induced dissociation and activation of sHsps represents an early and simple mechanism, which developed into a more sophisticated system of regulation during the evolution of higher eukaryotes. Instead of a direct temperature-sensing system, additional signals could thus be integrated into the activation process of the respective sHsp.

Materials and methods

Materials

Mitochondrial CS from pig heart (EC 4.1.3.7), acetyl-CoA, bovine serum albumin (BSA), ferritin, catalase and chymotrypsin were obtained from Roche Biochemicals. Bovine insulin was from Sigma. The concentration of CS was determined using the extinction coefficient of 1.78 for a 1 mg/ml solution at 280 nm and a 1 cm pathlength. CS was stored in 50 mM Tris-HCl, 2 mM EDTA pH 8.0. The concentrations for CS and Hsp26 given in the text refer to a dimer and a 24mer, respectively, unless otherwise indicated. α -crystallin concentrations correspond to an 800 kDa oligomeric complex. Purified α -glucosidase (maltase, EC 3.2.1.20) from yeast, murine Hsp25 and bovine α -crystallin were kind gifts of Dr A.Grossmann (Roche Biochemicals), Dr M.Gaestel (Martin-Luther Universität, Halle-Wittenberg) and Dr J.Horwitz (UCLA Medical School), respectively. *Escherichia coli* GroEL was purified as described previously (Schmidt *et al.*, 1994). A polyclonal antiserum against Hsp26 was raised in rabbits by standard procedures. The Hsp26 overexpressing yeast strain was a kind gift of Dr S.Lindquist (University of Chicago).

Expression and purification of Hsp26

Hsp26 was purified from the *S.cerevisiae* strain JT(DIP) GPD26(A), which had been transformed with the 2 μ plasmid pJV517. This plasmid carries the Hsp26 gene under the control of a GPD-promoter (Bitter and Egan, 1984; Mumberg *et al.*, 1995). Cells were grown in minimal medium to late logarithmic phase, harvested, washed once with buffer A [40 mM HEPES-KOH pH 7.5, 50 mM NaCl, 1 mM 1,4-dithioerythritol (DTE), 1 mM EDTA] and lysed with glass beads. The cell lysate was centrifuged (46 000 g, 45 min, 4°C) and the soluble extract was applied to a DE52 ion exchange column equilibrated in buffer A and eluted with an NaCl gradient. Hsp26-containing fractions were pooled, dialysed against buffer A and loaded onto a Resource-Q ion exchange column. The NaCl eluate was further purified on a Superdex S200 pg column equilibrated in buffer B (40 mM HEPES-KOH pH 7.5, 200 mM NaCl, 1 mM DTE, 1 mM EDTA). Hsp26-containing fractions were pooled, dialysed against buffer A and concentrated using an Amicon cell and Centricon Microconcentrators (cut-off 30 kDa). The Hsp26 purity was >98% as judged by densitometry of Coomassie-stained polyacrylamide gels.

Thermal aggregation of CS

Light scattering was used to examine the influence of Hsp26 on the thermal aggregation of CS. For this, 15 μ M CS was diluted 1:200 in 40 mM HEPES-KOH pH 7.5 and equilibrated at 43°C in the presence and absence of Hsp26. To determine the aggregation kinetics, light scattering was measured in a Perkin Elmer MPF44A luminescence spectrophotometer in a stirred and thermostatted quartz cell. Both the excitation and emission wavelengths were set to 500 nm with a spectral bandwidth of 2 nm. The oligomeric Hsp26 complex does not contribute to the light scattering signal at 500 nm, where only larger aggregates are detected.

Thermal inactivation of CS

Inactivation of CS was performed as described previously (Buchner *et al.*, 1998). CS was incubated in the absence or presence of Hsp26 at

43°C in 40 mM HEPES-KOH pH 7.5. To determine CS activity, aliquots were taken at the time points indicated and the activity was measured according to Srere (1966). The activity measurements were carried out at 25°C in 50 mM Tris-HCl, 2 mM EDTA pH 8.0.

Aggregation of insulin under reducing conditions

The insulin aggregation assay was performed in 40 mM HEPES-KOH pH 7.5. Aggregation was monitored at 400 nm in micro-cuvettes (100 μ l) with a pathlength of 10 mm. Insulin (0.45 mg/ml) and Hsp26 (concentration as indicated in the figure legend) were pre-incubated at the respective temperatures and the aggregation reaction was started by the addition of dithiothreitol (DTT) to a final concentration of 15 mM.

Electron microscopy

To determine changes in oligomeric size, Hsp26 (0.4 μ M) was incubated at various temperatures for 15 min. Complexes between Hsp26 and CS were formed by incubating Hsp26 (0.13 μ M) and CS (0.4 μ M) for 40 min at 43°C. Aliquots were applied to glow-discharged, carbon-coated copper grids. After an incubation time of ~5 s, proteins were negatively stained with 3% uranyl acetate. Electron micrographs were recorded at a nominal magnification of 60 000 \times using a Philips CM12 electron microscope operated at 120 kV.

Cryo-EM images of Hsp26 alone and in complex with heat-denatured CS were collected on a JEOL 2010 HC TEM at 200 kV and 30 000 \times with an Oxford Instruments cryotransfer stage. Negatives were digitized on a Leafscan 45 densitometer (Ilford Ltd, Cheshire, UK) with a 10 μ m step size (3.3 \AA /pixel). Three thousand views were selected of each sample and iteratively aligned to their average to bring them all to a common centre. Using Imagic software (van Heel *et al.*, 1996), the centred images were classified by multivariate statistical analysis to produce class averages. A set of class averages showing good signal to noise and clear features was selected for iterative multi-reference alignment of the data set to yield improved averages.

Size exclusion chromatography

Size exclusion HPLC was performed using a TosoHaas TSK 4000 PW column (separation range 10–1500 kDa). The buffer was 100 mM HEPES-KOH pH 7.5, 150 mM KCl, and a flow rate of 0.75 ml/min was used. The column was thermostatted at 25 or 43°C. Hsp26 and complexes with CS were detected by fluorescence at an excitation wavelength of 280 nm and an emission wavelength of 323 nm using a Jasco FP 920 fluorescence detector. Detection of α -crystallin (Figure 3A) was carried out at 280 and 330 nm.

To analyse temperature-induced changes in Hsp26 complex size, Hsp26 was incubated at different concentrations (0.04, 0.4 and 4 μ M) for 20 min in 40 mM HEPES-KOH pH 7.5 at the respective temperatures and subsequently applied to the thermostatted HPLC column. α -crystallin samples were prepared identically. As fluorescence decreases with temperature, the connecting tube between column and detector was cooled on ice to obtain better signals. To compare elution profiles directly at different temperatures the maximum signals were normalized.

To investigate whether temperature-induced dissociation of Hsp26 was reversible, 0.04 μ M Hsp 26 was incubated at 43°C for 20 min to achieve complete dissociation and then kept at 25°C for another 2 h before application to the column.

Complexes between Hsp26 (3 μ M) and CS (6 μ M) were formed by incubation at 43°C for 45 min and were subsequently separated by SEC operated at 25°C. Molar ratios of 2:1 (CS monomer:Hsp26 monomer) were used.

Determination of the binding stoichiometry

Hsp26-CS complexes were formed at 43°C for 45 min using 3 μ M Hsp26 monomers and varying amounts of CS (1–15 μ M monomers). They were then shifted to 25°C, and after SEC the Hsp26-CS complex peak was collected and precipitated with sodium deoxycholate/trichloroacetic acid. Precipitates were resuspended in SDS sample buffer (Laemmli, 1970), separated by SDS-PAGE on 10–20% precast Tricine gradient gels (Novex, San Diego, CA) and visualized by Coomassie Blue staining. The scanned gels were analysed using the ImageMaster-1D software (Amersham-Pharmacia).

Native PAGE and immunoblotting

Proteins were separated by native PAGE using 4–12% precast gradient gels (Novex, San Diego), and transferred to PVDF membranes. The immunodetection was performed using affinity-purified polyclonal rabbit antiserum raised against purified Hsp26. For detection, a horseradish peroxidase-linked secondary conjugate (Sigma) was used, and reactive bands were visualized by Enhanced Chemiluminescence (ECL) Detection Reagents (Amersham-Pharmacia). Cellular lysates for *in vivo* immuno-

blotting were obtained by breaking up logarithmic phase cultures of *S. cerevisiae* in a cell-homogenizer at 2.5 kbar (Constant Systems). After separation of insoluble cellular material by centrifugation for 10 min and 13 000 g at 4°C, the cleared lysate was used for further experiments.

Blue native PAGE

Blue native PAGE was performed according to Schagger *et al.* (1994). Chymotrypsin, BSA, catalase and ferritin were used as standards (Schagger and von Jagow, 1991). All proteins were incubated for 30 min at 43°C and separated at 43°C.

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