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Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation

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Small heat shock proteins (sHsps) are a conserved and ubiquitous protein family. Their ability to convey thermoresistance suggests their participation in protecting the native conformation of proteins. However, the underlying functional principles of their protective properties and their role in concert with other chaperone families remain enigmatic. Here, we analysed the influence of Hsp25 on the inactivation and subsequent aggregation of a model protein, citrate synthase (CS), under heat shock conditions *in vitro*. We show that stable binding of several non-native CS molecules to one Hsp25 oligomer leads to an accumulation of CS unfolding intermediates, which are protected from irreversible aggregation. Furthermore, a number of different proteins which bind to Hsp25 can be isolated from heat-shocked extracts of cells. Under permissive folding conditions, CS can be released from Hsp25 and, in cooperation with Hsp70, an ATP-dependent chaperone, the native state can be restored. Taken together, our findings allow us to integrate sHsps functionally in the cellular chaperone system operating under heat shock conditions. The task of sHsps in this context is to efficiently trap a large number of unfolding proteins in a folding-competent state and thus create a reservoir of non-native proteins for an extended period of time, allowing refolding after restoration of physiological conditions in cooperation with other chaperones.

Keywords: chaperone/heat shock/Hsp25/Hsp70/protein folding

Introduction

Small heat shock proteins (sHsps) represent an abundant and ubiquitous family of stress proteins. They have been detected in virtually all types of organisms. While many different classes of sHsps co-exist in the cytosol as well as in the organelles of plants (Vierling, 1991), yeast and mammalian cells possess only one or two members of the sHsp family (cf. Arrigo and Landry, 1994). Interestingly, the eye lens protein α B-crystallin is also considered to be a member of the sHsp family, as it displays similar structural and functional properties (Ingolia and Craig, 1982; Klemenz *et al.*, 1991; Horwitz, 1992; Jakob *et al.*, 1993; Merck *et al.*, 1993). Although the overall homology between different sHsps is rather low, they are grouped together based on (i) conserved regions in the C-terminal half of the protein (Plesofsky-Vig *et al.*, 1992; de Jong *et al.*, 1993; Jakob and Buchner, 1994; Waters, 1995), (ii) their increased expression upon heat shock (Lindquist *et al.*, 1982; Welch, 1985; Landry *et al.*, 1989; Inaguma *et al.*, 1992; Klemenz *et al.*, 1993) and (iii) their monomeric size (15–30 kDa). Despite their low monomeric molecular masses, sHsps exist within the cell as large oligomeric complexes of 12–40 subunits with a mean M_r of 300–800 kDa (Chiesa *et al.*, 1990; Behlke *et al.*, 1991; Lee *et al.*, 1995; Waters *et al.*, 1996). Upon heat shock or under several other stress conditions, sHsps become phosphorylated and, in parallel, the oligomeric size undergoes significant dynamic changes leading to both smaller and larger complexes (Arrigo *et al.*, 1988; Collier *et al.*, 1988; Nover *et al.*, 1989; Kato *et al.*, 1994; Mehlen *et al.*, 1995).

The abundance of sHsps under physiological conditions varies significantly depending on the cell type and organism studied. Except in the eye lens, the highest levels of ~0.1% of the total protein are reached in heart muscle cells at physiological conditions (Bhat and Nagineni, 1989; Kato *et al.*, 1991). Furthermore, sHsp expression varies depending on development, growth cycle, differentiation and the oncogenic status of the cell (Bond and Schlessinger, 1987; Gaestel *et al.*, 1989; Crête and Landry, 1990; Pauli *et al.*, 1990; Ciocca *et al.*, 1993; Gernold *et al.*, 1993; Klemenz *et al.*, 1993). Under heat shock conditions, the level of sHsps increases drastically (10- to 20-fold), amounting to up to 1% of the total cellular protein (Arrigo and Landry, 1994), which suggests functional importance in supporting survival at higher temperatures (Tissières *et al.*, 1974). In mammalian cells, sHsps accumulate with kinetics similar to other Hsps but are synthesized for a longer time period after stress (Arrigo and Welch, 1987; Landry *et al.*, 1991; Klemenz *et al.*, 1993). Interestingly, overexpression of virtually all mammalian as well as plant sHsps was shown to convey a significant degree of thermoresistance to cells (Landry *et al.*, 1989; Knauf *et al.*, 1992; Aoyama *et al.*, 1993; Schirmer *et al.*, 1994; van den Ijssel *et al.*, 1994), indicating a general thermoprotective function of these proteins.

sHsps have been shown to function in a number of different, seemingly unrelated processes ranging from RNA stabilization (Nover *et al.*, 1989) to elastase inhibition (Voorter *et al.*, 1994) and interaction with the cytoskeleton (Miron *et al.*, 1991; Lavoie *et al.*, 1993; Benndorf *et al.*, 1994; Nicholl and Quinlan, 1994). Experiments using well established *in vitro* assays for chaperone function showed that sHsps possess chaperone properties similar to those of the model chaperone GroE (cf. Jakob and Buchner, 1994; Buchner, 1996). sHsps, including α-crystallin, which was previously thought to play only a structural role in

the eye lens, were shown to suppress aggregation of unfolding proteins and increase the yield of reactivation of denatured substrate proteins (Horwitz, 1992; Jakob *et al.*, 1993; Merck, *et al.*, 1993; Lee *et al.*, 1995). Interestingly, sHsps chaperone protein folding in an ATP-independent way (Horwitz, 1992; Jakob *et al.*, 1993; Merck *et al.*, 1993). This property is not altered significantly by sHsp phosphorylation (Knauf *et al.*, 1994). Due to their subunit molecular weight and their recent addition to the chaperone family, sHsps and α-crystallin have been termed 'junior chaperones' (Jaenicke and Creighton, 1993). However, little is known about the molecular mechanism of these chaperones and their integration into the chaperone system of the cell where only the coordinated function of several chaperone systems seems to guarantee survival under heat shock conditions.

Here we have investigated the interaction of Hsp25 with unfolding protein mimicking heat shock conditions *in vitro*. We show that Hsp25 forms complexes with unfolding intermediates and prevents their aggregation. These complexes are productive in that the bound proteins can be released and regain their native structure upon restoration of permissive folding conditions in cooperation with other ATP-dependent chaperones.

Results

Hsp25 suppresses aggregation of CS without changing the inactivation kinetics

To analyse the interaction of Hsp25 with unfolding proteins under heat shock conditions in detail, we chose to use citrate synthase (CS), a dimer of 50 kDa subunits, as a substrate protein since the unfolding behaviour of this protein at high temperature has been analysed in detail (Jakob *et al.*, 1995). This allows determination of the number of unfolding intermediates present at a given time during the inactivation process. Furthermore, a direct comparison of the mode of action of sHsps with that of Hsp90 is possible using this experimental system.

Upon incubation at elevated temperatures, CS is inactivated rapidly. After ~7 min at 43°C, CS activity is almost completely lost (Figure 1A). At this temperature, inactivation is accompanied by quantitative protein aggregation. Aggregation of CS starts 2 min after incubation at 43°C and reaches a plateau at ~15 min (Figure 1B). In the presence of high concentrations of a control protein such as IgG, the kinetics of aggregation were not altered significantly. However, Hsp25 is able to suppress aggregation of CS even in substoichiometric amounts. At a ratio of about one Hsp25 complex to two CS dimers, light scattering could be reduced to less than half the spontaneous value. Stoichiometric concentrations of Hsp25 led to maximum prevention of aggregation. Concentrations higher than a 2-fold molar excess of Hsp25 did not cause any further protection from aggregation. In contrast to the situation with GroEL, light scattering was not suppressed completely in the presence of Hsp25. A similar situation was found for Hsp90, another chaperone protein which was proposed to act independently of ATP (Jakob *et al.*, 1995).

Although Hsp25 prevents aggregation of the substrate protein very effectively, the kinetics of the inactivation reaction were not altered (Figure 1A). The loss of CS activity could not be prevented in the presence of either

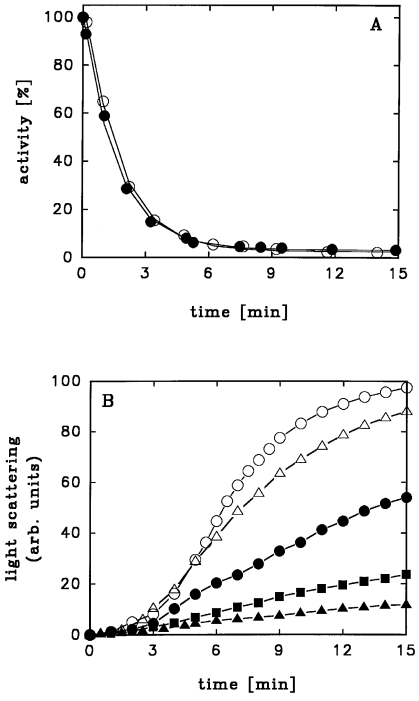

Fig. 1. Inactivation and aggregation kinetics of CS at 43°C in the presence and absence of Hsp25. (**A**) Influence of Hsp25 on the inactivation of CS at elevated temperatures. Inactivation of CS (0.075 μM) at 43°C in the presence of 0.3 μM Hsp25 (0.11 mg/ml) (●) or 0.11 mg/ml IgG (○). Inactivation was monitored by incubating the sample at 43°C and measuring the remaining CS activity at the time points indicated. (**B**) Influence of Hsp25 on the thermal aggregation of CS. CS (final concentration 0.075 μM) was diluted into a thermostatted solution of 0.03 μM (●) 0.06 μM (■), 0.2 μM Hsp25 (▲) and 0.5 mg/ml IgG (△). Open circles (○) represent the spontaneous aggregation of CS at 43°C. The kinetics of aggregation were determined by measuring the light scattering of the samples.

IgG or Hsp25. In both cases and in the absence of additional protein, apparent rate constants of ~1×10^{-2}/s were observed for the inactivation process. A further increase in the concentration of Hsp25 or IgG did not have a significant effect on the inactivation kinetics (data not shown). These findings are in contrast to data obtained for the effect of Hsp90 on the inactivation of CS (Jakob *et al.*, 1995). Hsp90 significantly decelerates the inactivation process of CS, indicating that by frequent binding and release of early folding intermediates of CS, Hsp90 shifts the equilibrium to the native enzyme. The data obtained for Hsp25 suggest that the chaperone forms a more long-lived complex with CS intermediates, thus preventing the refolding to the enzymatically active species.

Analysis of Hsp25–CS complexes

The data obtained in the inactivation experiments suggest that a stable complex is formed between Hsp25 and the unfolding substrate. To visualize and characterize this complex, HPLC size exclusion chromatography (SEC) was performed (Figure 2). Under our experimental conditions, Hsp25 eluted predominantly as an oligomer of ~400 kDa, corresponding to a complex composed of 16 Hsp25 subunits (Figure 2B). Furthermore, the elution profile of Hsp25 showed at least one more peak at ~100 kDa, indicating the presence of a tetramer of Hsp25. When CS

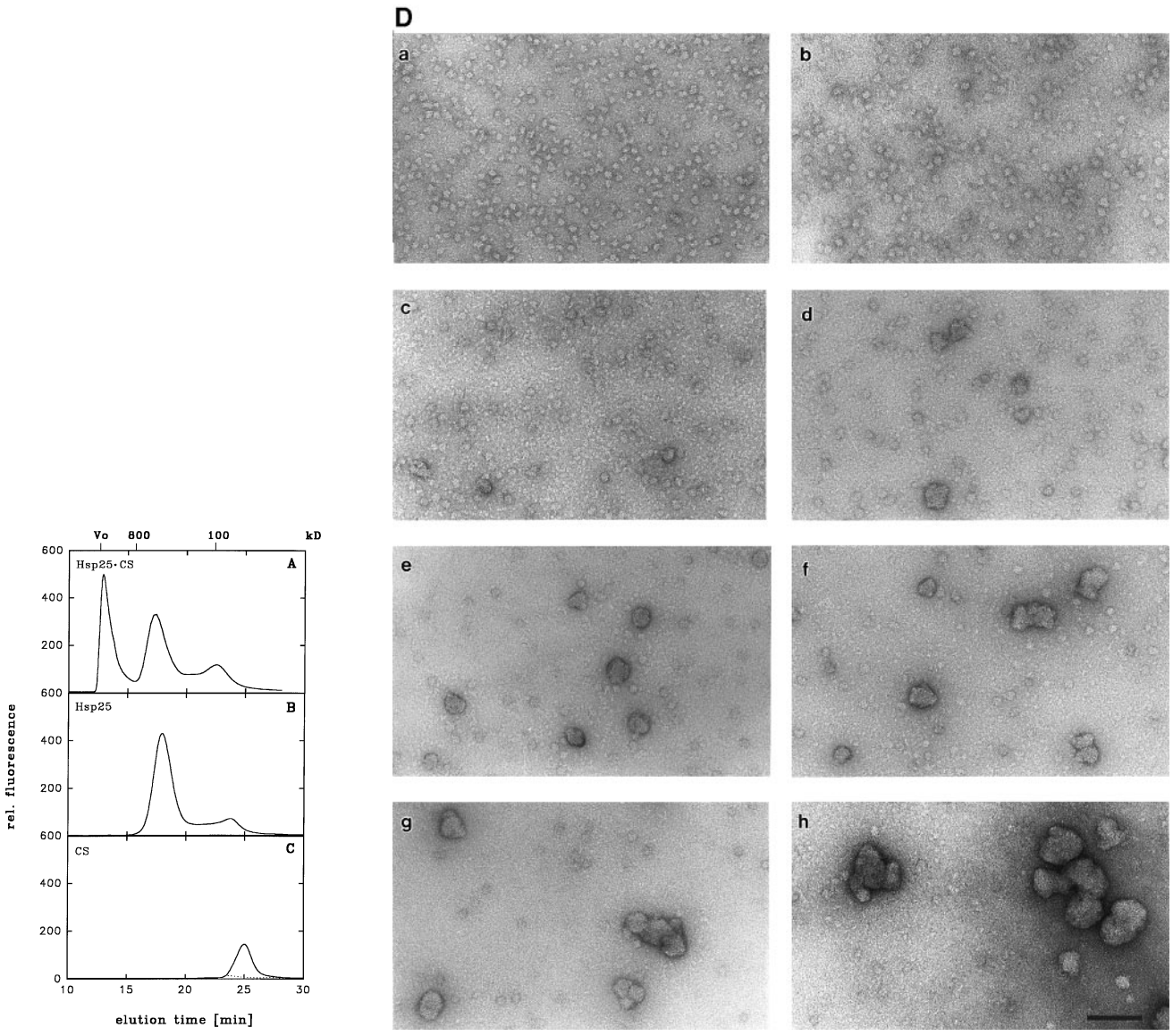

Fig. 2. Detection of Hsp25–CS complexes by SEC and electron microscopy. SEC was performed using a TosoHaas TSK G4000 SW column as described in Materials and methods. (**A**) Hsp25 (1 μM) and CS (3 μM) were incubated at 43°C for 15 min. The sample was centrifuged and then applied to the column. (**B**) Hsp25 (1 μM) without additional protein was treated as described above. (**C**) CS (3 μM) was either centrifuged directly and analysed by SEC (solid lines), or incubated at 43°C for 15 min before centrifugation and application (dotted line). (**D**) Analysis of the complex formation between Hsp25 and CS by electron microscopy. a and b: 0.1 μM Hsp25 after incubation on ice and at 43°C for 20 min, respectively. c–h: 0.1 μM Hsp25 and 0.3 μM CS after incubation at 43°C for 2, 5, 10, 20, 45 and 70 min, respectively. Scale bar, 100 nm.

was incubated at 43°C and centrifuged before application to the column, almost no protein could be detected (Figure 2C, dotted line), indicating that CS was quantitatively aggregated and insolubilized. This result was confirmed by electron microscopy of CS after incubation at 43°C. Here, only large amorphous aggregates were present (data not shown). The oligomeric state of Hsp25 did not change upon incubation at elevated temperatures. Furthermore, the peak areas and the peak distribution were comparable for Hsp25 at 25°C or after incubation at 43°C for up to 60 min (data not shown). Thus, elevated temperatures do not seem to lead to the aggregation of the chaperone. When Hsp25 was incubated with a 3-fold molar excess of CS, a new peak with an area of ~30% of the original peak areas of CS and Hsp25 appeared in the void volume of the column (Figure 2A). This indicates that the protein complex has an M_r of at least 7000 kDa. The peak observed consists of a complex between Hsp25 and unfolding CS, as confirmed by SDS–PAGE (data not shown).

Furthermore, the time course of complex formation between Hsp25 and a 3-fold molar excess of CS at 43°C was analysed by electron microscopy (Figure 2D, a–h). To establish that changes in the Hsp25 oligomer are due to the association with non-native CS and not just to changes in the Hsp25 complex itself at higher temperatures, we compared the appearance of the Hsp25 oligomer after incubation on ice or at 43°C by electron microscopy. As shown in Figure 2D, a and b, the complex seems to increase slightly in size at higher temperature. Since the elution profile in SEC experiments does not change (data not shown), it is reasonable to assume that this apparent

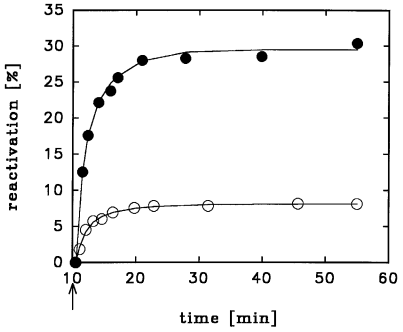

Fig. 3. Influence of Hsp25 on the OAA-induced reactivation of CS after inactivation at 43°C. CS (0.075 μM) was inactivated for 10 min in the presence of 0.3 μM Hsp25 (●) or with 0.11 mg/ml IgG (○) at 43°C. At the time point indicated by an arrow, reactivation was started by lowering the temperature to 25°C and adding OAA to a final concentration of 1 mM. The kinetics of reactivation were determined by measuring CS activity as described in Materials and methods. The remaining activity at the beginning of reactivation was subtracted from the refolding kinetics.

increase in size reflects a reorientation of subunits in the complex rather than a specific association of additional subunits.

Upon incubation of CS in the presence of Hsp25 at heat shock temperatures, regularly shaped round complexes appear, which increase in size considerably during a time period of ~60 min. Together with the results of the SEC experiments, this argues for the binding of several CS molecules on the outside of the complex. Furthermore, prolonged incubation at 43°C leads to the association of Hsp25–CS complexes, which is indicative of an interaction of different complexes via bound CS molecules.

Hsp25 traps unfolding intermediates of CS in a folding-competent state

Having established that Hsp25 forms specific and defined complexes with unfolding CS, we were interested in analysing the conformational state of the non-native protein bound to Hsp25 and, importantly, in determining whether the complexes we observed were productive or dead-end intermediates of cellular protein folding. If they were productive, this would imply that the bound non-native protein should be able to dissociate from the complex and fold to the native state. On the other hand, a dead-end complex would not allow the release of protein and the regaining of enzymatic activity.

In order to differentiate between the two possibilities, which is crucial for understanding the contribution of sHsps to the chaperone power of eukaryotic cells, we employed two potential approaches to trigger refolding of proteins. First, we used oxaloacetic acid (OAA), a substrate and ligand of CS. We had shown previously that unfolding intermediates of CS can be shifted back to the native state by the addition of OAA (Jakob *et al.*, 1995) which stabilizes the native enzyme (Srere, 1966) (Figure 3). Another method we used to try to reactivate CS was a temperature shift to 25°C after previous incubation at 43°C. This creates physiological or 'permissive' conditions for refolding of CS.

In all the reactivation experiments, CS was incubated for 10 min at 43°C to achieve complete inactivation. After that time, reactivation was initiated establishing permissive folding conditions either by shifting the temperature to 25°C and adding OAA (Figure 3) or by transferring the sample to 25°C (see below). Addition of equivalent concentrations of IgG was used as a control for unspecific protein effects. In the absence (not shown) and presence of the control protein, ~8% of the starting activity of CS was regained after addition of OAA. However, in the presence of a 4-fold molar excess of Hsp25, the reactivation yield increased substantially to ~30% (Figure 3). When reactivation was triggered by a shift in temperature from 43 to 25°C in the absence of OAA, surprisingly not more than 5% of CS activity could be recovered in the presence of Hsp25, compared with $<$3% reactivation with IgG as a control protein (not shown). This indicates that lowering of the temperature, which mimics the recovery period after heat stress, is not sufficient to release bound substrate from Hsp25. Other factors seem to be necessary to support refolding.

Unfolding intermediates of CS are stably accumulated in the presence of Hsp25

As shown above, Hsp25 is able to immobilize unfolding CS intermediates and suppress their irreversible denaturation during thermal inactivation. To establish the efficiency and kinetics of this protective action, we investigated the formation of intermediates during the unfolding of CS in the presence of Hsp25 (Figure 4). We determined the number of reactivatable intermediates at a given time point during inactivation by adding OAA. Reactivation of CS was triggered at various time points during inactivation. When incubated with IgG (to exclude unspecific protein effects), not more than 17% of CS could be refolded (Figure 4A). The occurrence of intermediates was restricted to the first 10 min of inactivation. After that time, CS is completely lost to irreversible unfolding and aggregation reactions (cf. Figure 1). The maximum amount of intermediates that can be reactivated was observed after 4 min of incubation at 43°C. However, in the presence of Hsp25, the time range in which CS intermediates could be observed was increased to ~15 min and the maximum amount of detectable folding-competent species was increased reproducibly $>$2-fold to ~40% (Figure 4B). This is in contrast to findings with Hsp90, where the formation of unfolding intermediates is reduced and inactivation is decelerated by transient complex formation (Jakob *et al.*, 1995). In the case of Hsp25, the apparent inactivation process of CS is not influenced, but intermediates formed during the inactivation process are trapped on the chaperone. Thus the bound substrate remains in a folding-competent state and, upon addition of release factor, can dissociate from the chaperone and refold to its native state.

Next we were interested in determining how long the complex between CS and Hsp25 is maintained under recovery conditions. To this end, we formed Hsp25–CS complexes, then lowered the temperature to 25°C and determined the amount of bound CS intermediates at various time points over a period of 2 h (Figure 5). Interestingly, the amount of intermediates which can be reactivated does not decrease over this time span. When reactivation was triggered within 1 h after the temperature shift, we could even observe a steady increase in refoldable CS from ~21 to 32%. This might be due to rearrangements

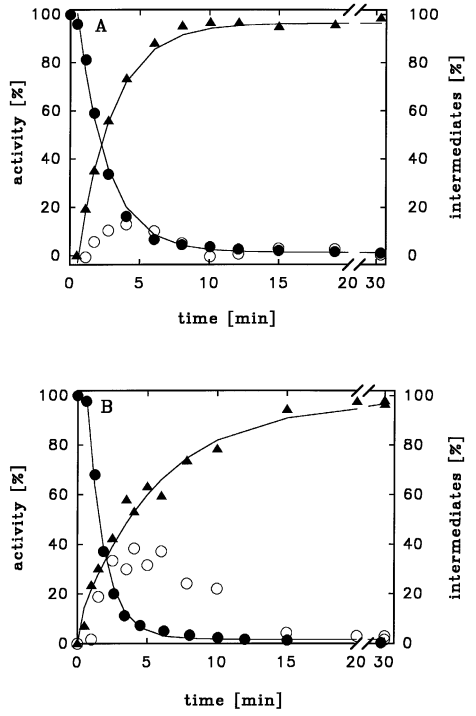

Fig. 4. Dissection of the unfolding pathway of CS. CS (0.075 μM) was incubated at 43°C. Inactivation (●) was monitored by activity measurements at the time points indicated. In a subsequent experiment, reactivation was started with OAA (1 mM) at the same time points. The amount of intermediate (○) present at a given time was calculated by subtracting the activity at the beginning of reactivation from the activity 60 min after addition of OAA. The irreversibly denatured CS (▲) represents the difference between the native protein at the beginning of the reaction and the sum of native CS and intermediates at a given time point. (**A**) Formation of unfolding intermediates in the presence of 0.11 mg/ml IgG. (**B**) Formation of unfolding intermediates in the presence of 0.3 μM Hsp25. The maximum deviation of the data points was <5%.

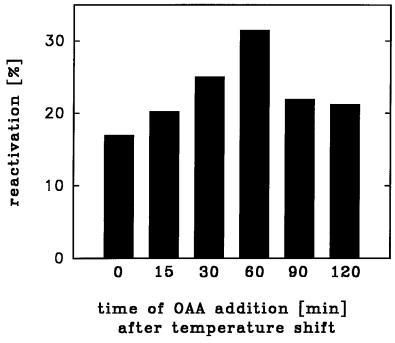

Fig. 5. Stability of the Hsp25–CS complex. CS (0.075 μM) was incubated at 43°C for 20 min in the presence of 0.3 μM Hsp25. After this inactivation process, the sample was shifted to 25°C. To initiate reactivation, OAA (to a concentration of 1 mM) was added at the times indicated after temperature shift. The endpoints of the reactivation kinetics are shown in the figure.

in the folding pattern of CS intermediates while bound to Hsp25 or during binding–release cycles.

Hsp70 cooperates with Hsp25 in the folding of trapped unfolding intermediates

As very little reactivation occurs when Hsp25–CS complexes are shifted to lower temperatures after heat treatment, we were looking for physiological factors which

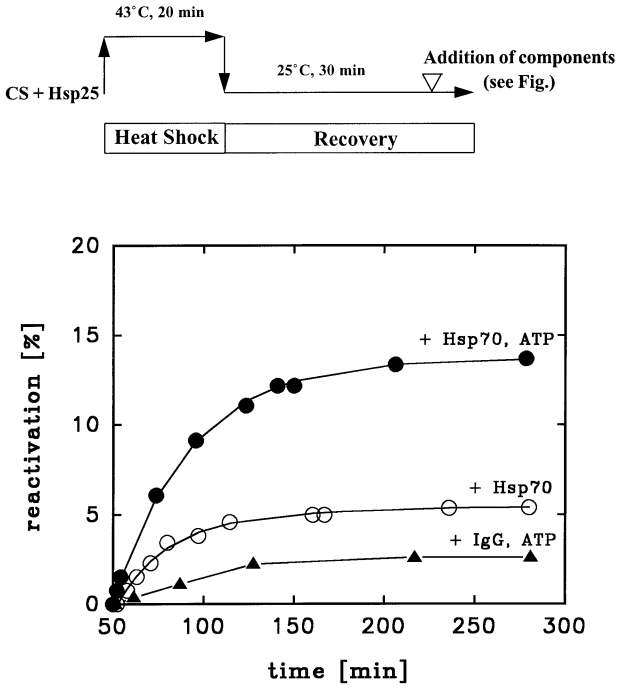

Fig. 6. Release of CS from Hsp25 in the presence of Hsp70. CS was incubated in the presence of Hsp25 for 20 min at 43°C to form complexes. The samples were then shifted to 25°C and incubated for another 30 min to simulate recovery conditions. At 50 min after the start of the experiment, 10 μM $MgCl_2$ and KCl, and either 0.5 μM Hsp70 (○), 1 μM ATP and 0.5 μM Hsp70 (●) or 1 μM ATP and 0.25 μM IgG (▲) were added. The time course of the experiment is depicted in the schematic diagram. Note that the data presented in the figure represent the time course of reactivation after the addition of components such as Hsp70, IgG and ATP (indicated in the diagram with an open triangle). A 30 min temperature shift to 25°C after Hsp25·CS complex formation did lead to only minor CS reactivation of 4–5%. The figure is corrected for these starting values.

could play a role in the cell similar to that of the artificial release factor OAA. Hsp70 is known to act as an ATP-dependent chaperone and is present in the cytosol at high concentrations after heat shock; therefore, it seemed to be a likely partner protein for Hsp25. To test this, we formed Hsp25–CS complexes as described previously and added Hsp70 30 min after a shift to 25°C (to simulate a recovery situation) (Figure 6). Whereas in the presence of IgG and ATP only a little reactivation of CS could be observed, in the presence of Hsp70 and ATP, CS reactivation was increased almost 5-fold. Interestingly, when Hsp70 was added to the Hsp25–CS complexes in the absence of ATP, the amount of regained CS activity was only slightly higher than the IgG control. These experiments illustrate that Hsp70 is able to trigger release of CS folding intermediates from Hsp25 and support subsequent refolding in an ATP-dependent manner.

A variety of proteins bind to Hsp25 under heat shock conditions

Based on the previous results, we suggest that the function of Hsp25 is to bind a variety of unfolding cellular proteins stably under heat shock conditions. To test our hypothesis further, we mimicked heat shock *in vivo* by incubating an Ehrlich ascites tumour (EAT) cell lysate for 30 min at 45°C and analysed the proteins bound to Hsp25 before and after heat treatment. To isolate the endogenous Hsp25

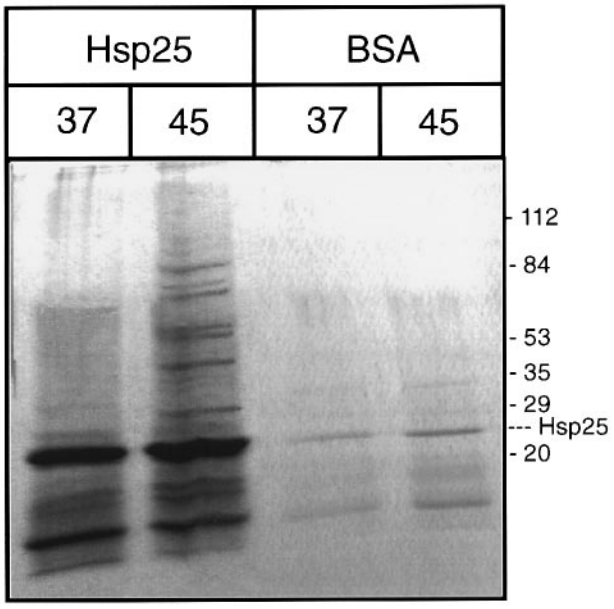

Fig. 7. Binding of cellular proteins to Hsp25 after heat shock treatment of cell extracts. Lysates from Ehrlich ascites tumor cells were incubated at 37°C (control) and 45°C (heat shock conditions) for 30 min. Endogenous Hsp25 and associated proteins from the control and heat-shocked lysates were isolated by their ability to bind to Hsp25–Sepharose (Hsp25). As a negative control, lysates were also allowed to bind to BSA–Sepharose (BSA) to analyse non-specific binding. The bound proteins were eluted under acidic conditions and analysed by SDS–PAGE. The positions of marker proteins (M_r in kDa) and of endogenous Hsp25 are indicated.

and its binding partners from the lysate, we used Hsp25 or Hsp27 covalently coupled to Sepharose. Due to the complex-forming properties of sHsps, the endogenous Hsp25 complexes bind efficiently by oligomerizing with the resin-bound Hsp25/27 and could only be eluted under strong acidic conditions. Leakage of the coupled sHsps from the resin did not occur, since in the eluate of the Hsp27–Sepharose only Hsp25 but no Hsp27 was detectable (not shown). Apart from some proteins with a molecular mass smaller than Hsp25 (~10–12 kDa), which also bind to the controls with bovine serum albumin (BSA)–Sepharose, there is no non-specific binding to the BSA–Sepharose detectable at 37 and 45°C (Figure 7). Interestingly, there is a significant increase in proteins bound to the Hsp25 oligomers from the cell lysate incubated at 45°C (Figure 7) compared with the analysis carried out under physiological conditions, indicating the ability of Hsp25 to interact specifically with unfolding proteins under heat shock in the cell. The proteins interacting with Hsp25 at elevated temperatures have M_rs between 25 and 100 kDa.

Discussion

Heat shock proteins are synthesized in response to increased growth temperatures and other stress factors in virtually all organisms. The analysis of the molecular mechanism of Hsps has so far been focused mostly on the ATP-dependent Hsp70 and GroE families, whereas the function of the members of the ATP-independent group of small Hsps is still poorly understood.

While the expression of Hsps is ubiquitous and a similar set of proteins is produced from prokaryotes to mammals, the importance and apparent function of the different Hsps seem to vary in different organisms (Parsell and Lindquist, 1994). In yeast, thermotolerance is conveyed predominantly by Hsp104, whereas in *Drosophila* Hsp70 seems to be the important factor. Finally, GroE is essential for protein folding in prokaryotes and organelles; however, a functional counterpart seems to be lacking in the eukaryotic cytosol, since the structurally related TCP-1 complex appears to be restricted to actin and tubulin folding (Lewis *et al.*, 1996). Others, such as Hsp70, show promiscuous binding properties and are involved in many cellular events under physiological as well as under stress conditions (Becker and Craig, 1994). The question arises as to why the cell expends a considerable amount of energy on the expression of several distinct classes of Hsps under heat shock conditions. In this context, it is reasonable to assume that functional interactions between different classes of chaperones are necessary to form a defined 'chaperone machinery' which provides optimal conditions for protein protection (Gething, 1991).

Here we have analysed the function of sHsps as molecular chaperones under heat shock conditions. In contrast to the situation with Hsp90 or Hsp70, where inactivation of substrate proteins is slowed down in the presence of the chaperone (Stege *et al.*, 1994; Jakob *et al.*, 1995), Hsp25 does not alter the inactivation kinetics of CS, indicating that Hsp25 binds to folding intermediates more stably than does Hsp90. CS stays bound to Hsp25 for several hours even under recovery conditions and thus provides a pool of folding-competent protein. The ability of Hsp25 to form complexes with unfolding proteins was confirmed further by experiments using heat-shocked extracts of cells. Here, stable complexes with a number of cellular proteins ranging in size from 25 to 100 kDa could be isolated after but not before heat shock.

This is reminiscent of the finding that the prokaryotic homologues of sHsps are found tightly bound to insolubilized recombinant protein in overexpressing cells and thus were called inclusion body proteins (Allen *et al.*, 1992). Furthermore, our results are consistent with data presented by Kampinga *et al.* (1995) and Stege *et al.* (1995) where recovery of marker proteins after thermal stress was investigated in the nucleus of HeLa S3 cells. Whereas overexpression of Hsp70 led to a deceleration of the inactivation of target proteins, in the presence of Hsp27 the inactivation of nuclear proteins was not influenced and both the 'substrate' and the chaperone were found to be insolubilized. However, when the cells were transferred to permissive conditions, both proteins became soluble again and the cells recovered much faster than in the absence of Hsp27. Under stress conditions and during the subsequent recovery phase, sHsps are present in high concentrations as they are synthesized for a longer period of time than other Hsps (Klemenz *et al.*, 1993). Therefore, many denaturing proteins can be bound and protected from irreversible aggregation. In the light of our results, it seems likely that sHsps generally act as traps for unfolding proteins, preventing them from irreversible aggregation and keeping them in a folding-competent state until permissive conditions for refolding are re-established (Figure 8).

For plant sHsps, it was proposed that non-native protein

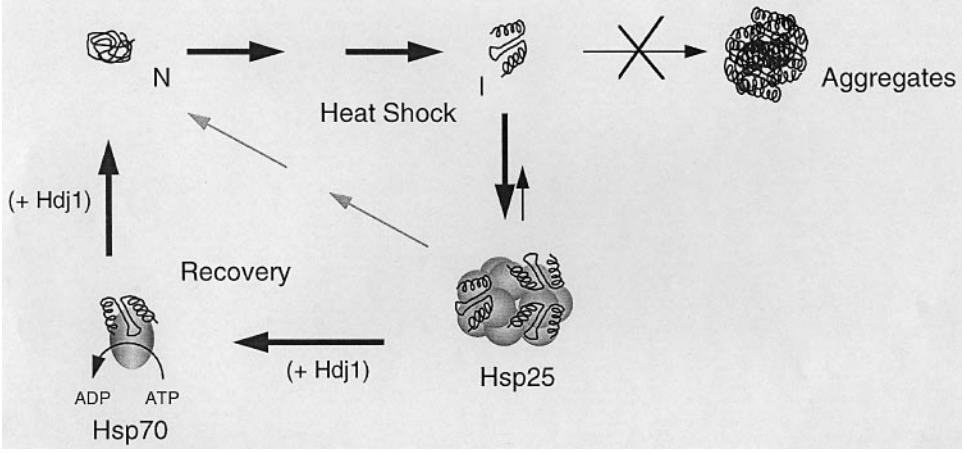

Fig. 8. Model for the chaperone function of sHsps under heat shock conditions. The unfolding of native proteins under heat shock conditions leads to the formation of non-native intermediates which are sensitive to irreversible reactions such as aggregation. Hsp25 is able to bind tightly to those intermediates and thus prevents aggregation and insolubilization. Even when the temperature is lowered to permissive conditions, only a small part of bound substrate is released and can refold to its native state. However, when Hsp70 is present, during the recovery phase the non-native protein is released from Hsp25 and reactivation occurs in an ATP-dependent manner. The amount of refolding observed in the presence of Hsp70 does not account for all the substrate that was initially bound to Hsp25. Thus it is likely that other presently unidentified factors in the cell, such as Hdj-1 or Hip, are necessary for optimal function of Hsp70. N, native state; I, non-native intermediate.

stays passively bound until it can be degraded (Lee *et al.*, 1995). Furthermore, in the eye lens, folding of non-native lenticular proteins to α-crystallin, a member of the sHsp family, seems to be sufficient to prevent cataract formation (Horwitz, 1992). However, we could show here that mammalian cytosolic sHsps seem to be involved in more active processes of protein protection. Hsp25 holds the model substrate CS in a folding-competent state until conditions permissive for folding are restored (Figure 8). One has to bear in mind that under heat stress the energy level in the cell decreases, due to the disruption of several metabolic processes, such as respiration and oxidative phosphorylation (Patriarca and Maresca, 1990). In agreement with these findings, it seems likely that the abundant energy-independent sHsps trap non-native proteins, thus protecting them from irreversible aggregation until more active, ATP-dependent chaperones such as Hsp70 or GroE can complete the refolding of cellular proteins after restoration of physiological conditions. In this context, we could show that Hsp70 in the presence of ATP is able to trigger the release of bound CS and to support refolding of the protein. Similar data have been reported for the cooperation of Hsp90, Hsp70 and Hdj-1 in the refolding of β-galactosidase *in vitro* (Freeman and Morimoto, 1996). As only half of the bound CS intermediates could be refolded in our experiments in the presence of Hsp70, it is likely that in the cellular environment co-chaperones such as Hdj-1 or HiP (Höhfeld *et al.*, 1995), which regulate the chaperone activity of Hsp70 and interact with non-native proteins, participate in this process and help to increase the efficiency of synergistic folding (Figure 8).

Taken together, our results allow us to define the role of sHsps in concert with Hsp70 in preventing irreversible damage of cellular proteins under heat shock conditions.

Materials and methods

Proteins and materials

Recombinant murine Hsp25 and Hsp27 were expressed and purified as previously described (Gaestel *et al.*, 1989; Jakob *et al.*, 1993). Hsp70 was purified from bovine tissue as described (Wiech *et al.*, 1993). Mitochondrial CS from porcine heart (EC4.1.3.7.), acetyl-CoA and MAK 33 IgG were from Boehringer Mannheim GmbH and OAA was from Sigma. The concentrations of CS and Hsp25 were determined using the extinction coefficients of 1.78 and 1.87, respectively, for a 1 mg/ml solution at 280 nm. CS was stored in 50 mM Tris, 2 mM EDTA, pH 8.0 and Hsp25 in 40 mM HEPES–KOH, pH 7.5. The concentrations for CS and Hsp25 given in the text refer to a dimer and a hexadecamer, respectively.

Aggregation assay

CS (15 μM) was diluted 1:200 in 40 mM HEPES–KOH, pH 7.5 equilibrated at 43°C, in the presence and absence of Hsp25. To monitor the kinetics of thermal aggregation, light scattering was measured in a Perkin Elmer MPF44A luminescence spectrophotometer in stirred and thermostatted quartz cells. During the measurements, both the excitation and emission wavelengths were set to 500 nm with a spectral bandwidth of 2 nm.

Inactivation/reactivation

Inactivation of CS was achieved by incubating the native protein at 43°C. First, CS (7.5 μM) was diluted 1:100 into 40 mM HEPES, pH 7.5, in the presence and absence of additional proteins at 25°C. Inactivation was started by shifting the sample to 43°C. To determine the inactivation and reactivation kinetics, aliquots were taken at the time points indicated and CS activity was measured according to Srere *et al.* (1966), with the modification that 40 mM HEPES, pH 7.5 was used instead of Tris buffer. The activity measurements were carried out at 25°C.

To initiate reactivation of CS, the stabilizing ligand OAA was diluted 1:100 into the inactivation reaction, to a final concentration of 1 mM (Jakob *et al.*, 1995). After starting the reactivation reaction, incubation at 43°C was continued (Figure 4) or the temperature was lowered to 25°C (Figure 3) and the kinetics of reactivation were determined as described above.

To simulate recovery conditions after incubation at elevated temperatures and to check for reactivation of Hsp25-bound CS upon a downshift of the temperature, temperature shift experiments were performed. After incubation at 43°C for 10 or 20 min, the samples containing CS and Hsp25 were transferred to 25°C and changes in CS activity were monitored. To determine the stability of Hsp25–CS complexes, CS (0.075 μM) was inactivated in the presence of 0.3 μM Hsp25 as described above. After 20 min of incubation at 43°C, the incubation temperature was lowered to 25°C. CS intermediates still present were detected by adding OAA to a final concentration of 1 mM to the samples at various time points after the temperature shift.

All inactivation/reactivation experiments were performed in 40 mM HEPES, pH 7.5.

Analysis of Hsp25–CS complexes

Size-exclusion chromatography. Hsp25 (1 μM) was incubated with and without CS at the given temperatures. To analyse complex formation between Hsp25 and CS, SEC was performed using a TosoHaas TSK G4000 SW column. Chromatography was carried out in 100 mM HEPES, pH 7.5, with a flow rate of 0.5 ml/min and a sample size of 100 μl. All samples were prepared in the chromatography buffer and centrifuged for 10 min at 14 000 *g* before application. Proteins were detected by fluorescence at an excitation wavelength of 280 nm and an emission wavelength of 330 nm using a Merck Hitachi fluorescence detector.

Electron microscopy. Hsp25 (0.1 μM) or CS and Hsp25 at concentrations of 0.3 and 0.1 μM, respectively were incubated at 43°C. At various time points during the incubation, 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 using a Phillips CM12 electron microscope operated at 120 kV.

Dissociation of Hsp25–CS complexes in the presence of Hsp70 and ATP

Complexes were formed by incubating CS (0.075 μM) in the presence of 0.3 μM Hsp25 for 20 min at 43°C. The samples were then shifted to 25°C for another 30 min. Then $MgCl_2$ and KCl (10 μM each), ATP and Hsp70 (1 and 0.5 μM, respectively) were added. The kinetics of reactivation were followed as described above. To check for the influence of ATP and for non-specific protein effects, the same experiments were performed with Hsp70, without ATP or in the presence of IgG (0.25 μM) and ATP.

Binding of proteins from cell lysates to Hsp25 under heat shock conditions

Cell lysates were prepared from 6×10^8 mouse EAT cells after three washes with ice-cold 154 mM NaCl/10 mM HEPES pH 7.2, using 3 ml of lysis buffer containing 10 mM HEPES, pH 7.2, 10 mM potassium acetate, 1.5 mM magnesium acetate, 0.1 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% Triton X-100. After 15 min on ice, nucleic acid was precipitated by adding 150 μl of 2 M potassium acetate/50 mM magnesium acetate. After centrifugation at 18 000 *g* for 10 min at 4°C, the supernatant was used as lysate for the following experiments. Aliquots (500 μl) of lysate were incubated at 37°C (control) or 45°C (heat shock) for 30 min in the presence of 200 μl of Hsp25– or Hsp27–Sepharose CL-4B (4.5 mg of Hsp25 or 5.5 mg of Hsp27/ml gel) and, as a control, in the presence of BSA–Sepharose CL-4B (5 mg BSA/ml gel) under constant shaking and subsequently transferred to 4°C. The agarose gel was sedimented by centrifugation for 4 min at 13 000 *g* at 4°C and washed three times with 1 ml of ice-cold phosphate-buffered saline (PBS) followed by a wash with 500 μl of 0.1 M ammonium acetate, pH 7.4. Bound proteins were eluted twice with 200 μl of 16 mM ammonium acetate/4.2% formate, pH 2.0. Both eluate fractions were pooled and concentrated by lyophilization in a speedvac. Lyophilized proteins were re-dissolved in 30 μl of 20 mM Tris–HCl, pH 7.4, 10 μl of 4× SDS loading buffer was added, samples were heated for 3 min at 95°C and applied to reducing SDS–PAGE using an acrylamide gradient from 7.5 to 22%. Separated proteins were stained with Coomassie R250.

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