Refolding of Substrates Bound to Small Hsps Relies on a Disaggregation Reaction Mediated Most Efficiently by ClpB/DnaK*

Axel Mogk†‡¶, Christian Schlieker‡, Kenneth L. Friedrich‡, Hans-Joachim Schönfeld**, Elizabeth Vierling, and Bernd Bukau‡ ‡‡

From the ‡ZMBH, Universität Heidelberg, Im Neuenheimer Feld 282, Heidelberg D-69120, Germany, the §Institut für Biochemie und Molekularbiologie, Hermann-Herder-Strasse 7, D-79104 Freiburg, the ¶Department of Biochemistry & Molecular Biophysics, University of Arizona, Tucson, Arizona 85721, and **Hoffmann-La Roche AG, 4070 Basel, Switzerland

Small heat shock proteins (sHsps) are ubiquitous molecular chaperones that bind denatured proteins in vitro, thereby facilitating their subsequent refolding by ATP-dependent chaperones. The mechanistic basis of this refolding process is poorly defined. We demonstrate that substrates complexed to sHsps from various sources are not released spontaneously. Dissociation and refolding of sHsp-bound substrates relies on a disaggregation reaction mediated by the DnaK system, or, more efficiently, by ClpB/DnaK. While the DnaK system alone works for small, soluble sHsp/substrate complexes, ClpB/DnaK-mediated protein refolding is fastest for large, insoluble protein aggregates with incorporated sHsps. Such conditions reflect the situation in vivo, where sHsps are usually associated with insoluble proteins during heat stress. We therefore propose that sHsp function in cellular protein quality control is to promote rapid resolubilization of aggregated proteins, formed upon severe heat stress, by DnaK or ClpB/DnaK.

The folding state of proteins within cells is monitored by an energy-dependent quality control network of ATP-dependent proteases and molecular chaperones, which operates under all growth conditions but becomes particularly important during stress (1–5). Different chaperones are documented to play complementary, and sometimes overlapping, roles in protection of proteins from stress. In Escherichia coli the DnaK (Hsp70) chaperone together with its co-chaperones DnaJ and GrpE (KJE) and the GroEL (Hsp60) chaperone with its GroES co-chaperone (ELS) are mainly responsible for preventing aggregation of misfolded proteins and for facilitating their subsequent refolding (4–6). The small heat shock proteins (sHsps) are proposed to bind unfolding proteins, providing a reservoir for subsequent refolding by the KJE and GroEL (Hsp60) chaperone together with its GroES co-chaperone (ELS). Thus, sHsps can prevent the formation of turbid and insoluble protein aggregates at an equivalent molar ratio of sHsp to substrate (16–18). While efficient in binding unfolded proteins, sHsps do not exhibit refolding activities (19, 20). In vitro substrate refolding from soluble sHsp/substrate complexes is dependent on Hsp70 chaperones (21–23). However, the basis of this dependence and the mechanism of substrate transfer to the refolding machinery are unknown. Furthermore, during stress in vivo sHsps are frequently found in association with denatured proteins in the insoluble fraction of cells, a state that cannot be efficiently acted on by Hsp70 chaperones, but is likely to be a substrate for ClpB. Interaction of ClpB in sHsp-refolding systems has not been investigated.

We show that refolding of substrates bound to sHsps is dependent on a disaggregation reaction mediated most efficiently by ClpB/KJE. Transfer of substrates to the refolding machinery appears to involve active extraction of sHsp-bound substrates by KJE or ClpB/KJE. By reducing protein aggregate size sHsps also expand the substrate spectrum that can be acted on by KJE alone. The presence of sHsps in insoluble protein aggregates, conditions, which mimic the in vivo situation, accelerates the ClpB/KJE-mediated disaggregation reaction. These in vitro findings firmly establish a role for sHsps in the protein quality control system of cells.

EXPERIMENTAL PROCEDURES

Proteins—Purifications of DnaK, DnaJ, GrpE, ClpB, GroEL, GroES, IbpB, Hsp16.6, and Hsp18.1 were performed as described previously (6, 10, 16, 24). α-Glucosidase, citrate synthase, and pyruvate kinase were purchased from Sigma and pig heart muscle malate dehydrogenase (MDH) and firefly luciferase from Roche Applied Science. Protein concentrations were determined with the Bio-Rad Bradford assay using bovine serum albumin as standard. Protein concentrations refer to the protomer.

Received for publication, April 7, 2003, and in revised form, June 2, 2003.

Published, JBC Papers in Press, June 4, 2003, DOI 10.1074/jbc.M303587200

This paper is available on line at http://www.jbc.org

© 2003 by The American Society for Biochemistry and Molecular Biology, Inc.
Efficient Dissociation of sHsp/Substrate Complexes by ClpB/DnaK

RESULTS

ClpB Stimulates the DnaK-dependent Refolding of Heat-denatured Substrates from sHsp/Substrate Complexes—To obtain a generalized picture of the potential interaction of ClpB in refolding of substrates bound to sHsps, we chose to work with two different sHsps, Hsp16.6 from the cyanobacterium Synechocystis sp. PCC6803 and Hsp18.1 from Pisum sativum (pea). These sHsps have excellent properties for biochemical studies in that they are soluble, regular oligomers and will form complexes with diverse substrates (16, 24). Refolding of substrate from Hsp18.1 has also been demonstrated to function equally well with DnaK (prokaryotic) or GroEL (eukaryotic) refolding systems (23). We first established how the association of heat-denatured MDH with sHsps affected the efficiency by which this model substrate is refolded by the DnaK system (KJE) and the GroE system (ELS) alone and in combination. To generate sHsp/MDH complexes, MDH was subjected to a 30-min heat treatment at 47 °C in the presence of a 4–6-fold molar excess of sHsps. At the concentrations tested, both sHsps prevented MDH aggregation and formed soluble, non-turbid sHsp/MDH complexes (data not shown). These complexes alone did not support MDH refolding, but readily facilitated refolding by KJE (Fig. 1). In contrast, ELS did not support MDH reactivation, however it facilitated the KJE-dependent refolding and increased the refolding rate by 2.5-fold from Hsp16.6/MDH complexes (Fig. 1, Table I) and by 2.3-fold from Hsp18.1/MDH complexes (Fig. 1B). This cooperation of DnaK and GroELs in refolding from Hsp16.6 and Hsp18.1/MDH complexes is comparable to the action of these chaperones on complexes of denatured MDH with the E. coli sHsp IbpB (22), indicating that this synergy of chaperone action can be generalized to a range of different sHsps.

Having established the behavior of these sHsp/substrate complexes in the presence of KJE and ELS we then tested the additional effect of ClpB. Surprisingly ClpB also increased the rate of KJE-dependent MDH refolding by 2-fold from soluble Hsp16.6/MDH complexes and by 3.3-fold from soluble Hsp18.1 complexes, without supporting MDH refolding on its own (Fig. 1, A and B). The combination of ClpB and ELS did not reactivate MDH from either sHsp complex, but they exerted cooperative beneficial effects on MDH refolding in the additional presence of KJE. Their combined presence resulted in very fast KJE-dependent MDH refolding, with a refolding rate of 27.5 nM MDH/min from Hsp16.6 complexes (Table I), leading to completion of the refolding reaction within 60 min, and a refolding rate of 41.7 nM MDH/min from Hsp18.1 complexes, leading to completion within 45 min. Similar stimulatory effects of ClpB and ELS were also noticed for the KJE-dependent reactivation of MDH bound to E. coli sHsp IbpB (22).

The influence of ClpB on KJE-dependent MDH refolding was further investigated by determining the refolding rates in presence of various KJE concentrations. The dose response of KJE revealed that the presence of ClpB strongly reduced the

50 °C for 45 min, respectively. Protein refolding was started by diluting aggregated proteins or sHsp/protein complexes and chaperones 1:1 in buffer A at 30 °C. All assays were performed in the presence of an ATP-regenerating system (3 mM phosphoenolpyruvate; 20 μM pyruvate kinase; 2 mM ATP). Determination of enzymatic activities followed established protocols (10, 17, 25, 26). Refolding rates were calculated from the linear increase of substrate activities.

Spectroscopic Measurements—For light scattering measurements MDH, citrate synthase, and α-glucosidase were denatured in the absence or presence of various sHsp concentrations as described above. Turbidity was measured at an excitation and emission wavelength of 550 nm (PerkinElmer luminescence spectrometer LS50B). Decrease of light scattering was followed upon addition of the indicated chaperones in the presence of an ATP-regenerating system at 30 °C. Static light scattering measurements were performed at room temperature by use of a miniDawn instrument (Wyatt technologies), coupled to S200 HR10–30 (Amersham Biosciences) gel filtration runs in buffer A and molar masses were determined by Astra software (Wyatt technology). Dynamic light scattering measurements were done at 20 °C in buffer A by use of a DynaPro-MSTC instrument (Protein Solutions Ltd., High Wycombe, Bucks., UK). Dust was removed from samples by centrifugation (13,000 rpm, 5 min, 4 °C). Data were analyzed with the software DYNAMICS (version 5.26.60) supplied by the manufacturer.

Size Exclusion Chromatography—Labeling of MDH was performed by use of N-succinimidyl [2,3-3H]propionate (Amersham Biosciences) as described (27). Different 3H-labeled MDH species, occurring during MDH refolding from sHsp/MDH complexes, were separated by S200 HR10–30 (Amersham Biosciences) size exclusion chromatography in buffer A (+5% [v/v] glycerol at 4 °C and quantified by scintillation counting, or analyzed by SDS-PAGE, and silver staining.
amount of KJE needed for efficient refolding of MDH from Hsp16.6/MDH complexes (Fig. 1C). Interestingly, even increased KJE levels did not reach the high MDH refolding rates, determined in the presence of ClpB (4.2 nM MDH/min without ClpB compared with 8.5 nM MDH/min with ClpB). In the presence of ClpB, the KJE-dependent MDH refolding rates were similar for aggregated MDH and soluble sHsp/MDH complexes. In contrast, in the absence of ClpB, the presence of Hsp16.6 during substrate denaturation was a prerequisite for KJE-dependent refolding.

To test the generality of our findings further, other thermostable proteins were heat-inactivated in the absence of Hsp16.6, leading to the formation of protein aggregates, or in the presence of Hsp16.6, resulting in the formation of soluble sHsp/substrate complexes, and refolding was followed upon addition of KJE, ClpB and/or ELS. Substrate reactivation from sHsp/substrate complexes was again strictly dependent on the presence of KJE, while protein aggregates could not be refolded by KJE alone (Table I). Refolding rates of all substrates (aggregated or in sHsp complexes) were further stimulated to different extents upon addition of ClpB to the KJE reactions (Table I). Interestingly, in contrast to results with MDH, the addition of ClpB to KJE refolding reactions with α-glucosidase, citrate synthase, or luciferase complexed with sHsps led to higher refolding rates than when ClpB and KJE were used to refold the same proteins aggregated in the absence of sHsps (Table I). The extent of these rate increases differed for the three substrates, which along with the MDH results, indicates that substrates differ with respect to their dependence on ClpB and sHsps for optimal refolding.

ELS itself did not support refolding of any substrate tested, although ELS-dependent refolding was possible if ELS was present during substrate denaturation at high temperatures (data not shown). This latter finding shows that the chosen heat denatured proteins represent ELS substrates, however (data not shown). This latter finding shows that the chosen heat denatured proteins represent ELS substrates, however (data not shown). This latter finding shows that the chosen heat denatured proteins represent ELS substrates, however (data not shown).


tabular_data

| Substrate                | KJE    | ClpB  | ELS   | KJE/ELS | KJE/ClpB | ClpB/ELS | KJE/ClpB/ELS |
|-------------------------|--------|-------|-------|---------|----------|----------|-------------|
| sHsp/MDH                | 4.0    | 0     | 0     | 9.9     | 8.5      | 0        | 27.5        |
| Aggr. MDH               | 0      | 0     | 0     | 0.1     | 9.3      | 0        | 25.1        |
| sHsp/α-glucosidase      | 0.44   | 0     | 0     | 0.53    | 2.69     | 0        | 3.63        |
| Aggr. α-glucosidase     | 0      | 0     | 0     | 0       | 1.73     | 0        | 2.27        |
| sHsp/citrate synthase   | 0.12   | 0     | 0     | 0.22    | 0.4      | 0        | 0.63        |
| Aggr. citrate synthase  | 0      | 0     | 0     | 0       | 0.06     | 0        | 1.0         |
| sHsp/luciferase         | 0.17   | 0     | 0     | 0.16    | 0.48     | 0        | 0.5         |
| Aggr. luciferase        | 0      | 0     | 0     | 0.14    | 0.15     | 0        | 0.15        |

Refolding rates in presence of chaperones

KJE: 4 (0.5) M DnaK, 0.1 (0.05) M GroE; ELS: 4 M GroEL, 4 M GroES; ClpB: 1.5 (1) M ClpB; chaperone concentrations for refolding of luciferase are given in parentheses and refolding rates were determined as described under “Experimental Procedures.”
refolding reaction is performed. No significant exchange of MDH between sHsps was detectable, since added Hsp16.6 could not displace Hsp18.1 from existing Hsp18.1/MDH complexes (Fig. 3E). Consistently, Hsp18.1 was also not incorporated into complexes with MDH when added to preexisting Hsp16.6/MDH complexes (Fig. 3D). These results are consist-
ent with the interpretation that substrate is not released from sHsp complexes under the conditions and within the time frame at which significant substrate refolding can be catalyzed by KJE/ClpB.

Release of substrate from sHsp complexes would also predict that addition of more sHsp to preformed complexes would alter the size of the sHsp substrate complexes, because the apparent size of sHsp/substrate complexes decreases as the ratio of sHsp to substrate increases (16). To test this prediction we first determined by static light scattering measurements coupled to size exclusion chromatography the molecular masses of Hsp16.6/MDH complexes formed upon 47 °C incubation of Hsp16.6 and MDH at two different molar ratios (Fig. 4). The simultaneous determination of the light intensity scattered by a protein sample, and of the protein sample concentration (by measuring the refractive index of the sample) allowed the calculation of absolute molar masses. As expected, the sizes of Hsp16.6/MDH complexes generated during incubation for 30 min at 47 °C decreased with increasing sHsp to MDH ratios. When Hsp16.6 was present at a 2-fold molar excess over MDH (relative to their monomers), all Hsp16.6 molecules were recovered in complexes with MDH. These complexes varied in size from 2300 to 4000 kDa, with the most abundant complex having a mass of 3500 kDa. When MDH was denatured in the presence of a 4-fold molar excess of Hsp16.6, free Hsp16.6 molecules could be detected, indicating that the sHsp levels were saturating during substrate denaturation. Under these conditions the sizes of Hsp16.6/MDH complexes were reduced, with the most abundant complex having a mass of 2100 kDa. To test if the larger sHsp/substrate complexes generated at lower ratios of sHsp to substrate could be converted to smaller complexes by increasing the sHsp to substrate ratio, MDH was first denatured in presence of a 2-fold molar excess of Hsp16.6 to form the larger complexes. Subsequently, an excess of Hsp16.6 was added and the mixture was incubated at 30 °C for 2 h or subjected to a second cycle of heat denaturation at 47 °C for 30 min. A conversion of the initial large complexes to smaller complexes was not observed, even after heat treatment (Fig. 4). These findings indicate that, within the time frame of the experiment (2 h), no significant sHsp/substrate complex reorganization occurs consistent with a lack of substrate release and rebinding to added sHsp.

The final prediction we tested is that if substrates are in an equilibrium between free and sHsp bound states, it should be possible to trap the free state through binding to other chaperones. For this experiment we used the GroEL-D87K mutant, which is deficient in ATP hydrolysis and consequently does not release bound substrates (29). Several results demonstrated the suitability of EL-D87K for such trap experiments. The EL-D87K trap mutant is capable of rapid and stable association with heat denatured MDH, as evidenced by its ability to efficiently suppress MDH aggregation at high temperatures and to form stable [3H]-labeled MDH-EL-D87K complexes (data not shown). Also, when MDH was denatured in the presence of equimolar concentrations of Hsp16.6 dimers and EL-D87K heptamers, more than 80% of the substrate associated with EL-D87K indicating that EL-D87K outcompetes Hsp16.6 with respect to binding heat denatured MDH (Fig. 5A). To test for spontaneous substrate release from sHsp/substrate complexes, the EL-D87K trap was added at 30 °C to preformed Hsp16.6/[3H]MDH complexes. No substantial transfer of [3H]MDH to EL-D87K was observed within the time frame of the experiment (120 min), even if the mixture was subjected to an additional denaturation cycle at 47 °C (Fig. 5A) or if 10-fold higher levels of EL-D87K were added to the Hsp16.6/[3H]MDH complexes (data not shown). Identical results were obtained with...
other sHsps (IbpB, Hsp18.1) and substrates (α-glucosidase), demonstrating that the observed stability of the sHsp/substrate complexes is not restricted to specific substrates or sHsp classes (data not shown). Transfer of Hsp16.6 bound MDH molecules to EL-D87K was only observed when the refolding reaction was initiated with the addition of KJE (Fig. 5B). This indicates that GroEL can interact with unfolded MDH, but only after it has been extracted from the Hsp16.6 complex by KJE. These results, along with the above documented stable organization of sHsp complexes suggest that KJE is required to actively release substrate from sHsps for efficient refolding to occur.

sHsps Prepare Protein Aggregates for Faster Resolubilization

Current models suggest that ClpB can act on larger protein aggregates than can KJE (30), leading to the hypothesis that the relative importance of KJE and ClpB in the refolding reaction may depend on aggregate size. Since in vivo the majority of sHsp/substrate complexes is insoluble (31), we tested whether the size of sHsp/substrate complexes, and in particular the incorporation of sHsps into insoluble protein aggregates can affect substrate refolding by KJE or ClpB/KJE. We prepared differently sized protein aggregates by altering the ratio of sHsp to substrate. MDH was heat denatured in the presence of varying Hsp16.6 concentrations, from sub-stoichiometric to a molar excess of Hsp16.6 relative to substrate, and resulting complexes were characterized with respect to turbidity, solubility and size (Table II). The turbidity of the heat denatured MDH complexes decreased even in the presence of substoichiometric Hsp16.6 concentrations. Solubility of such complexes was still poor and all Hsp16.6 molecules were found associated with aggregated MDH (data not shown). Increasing Hsp16.6/MDH ratios decreased turbidity and increased solubility further. These physical properties are directly correlated to the sizes of the Hsp16.6/substrate complexes: incubation of MDH in the absence of Hsp16.6 concentrations reduced the diameter of the most abundant aggregates from 45 nm in the absence of Hsp16.6 to 14 nm at 4:1 ratio (Hsp16.6: MDH), as determined by dynamic light scattering (Table II). The complexes formed at any Hsp16.6:MDH ratio showed some degree of heterogeneity which, however, decreased at higher Hsp16.6 ratios. In agreement with these measurements the masses of the corresponding complexes decreased continuously from 24,000 kDa (average size) at 0.25:1 Hsp16.6 to MDH ratio to 2100 kDa at 4:1 Hsp16.6 to MDH ratio, as determined by static light scattering. It is important to note that in the case of MDH aggregates lacking Hsp16.6, and of poorly soluble sHsp/MDH complexes, the determined complex sizes and masses reflect only a subpopulation of the complexes and represent an underestimation. Taken together, varying the levels of Hsp16.6 during MDH denaturation, allowed the formation of sHsp/substrate complexes of variable size.

Efficient Dissociation of sHsp/Substrate Complexes by ClpB/DnaK

FIG. 4. Once formed sHsp/substrate complexes exhibit a stable size distribution. 2 μM MDH was heat-denatured in presence of 4 or 8 μM Hsp16.6. Alternatively MDH was firstly denatured in presence of a 2-fold excess of Hsp16.6 and 4 μM Hsp16.6 was added afterward, followed by further incubation at 30 °C for 2 h or at 47 °C for 30 min. Proteins were separated by size exclusion chromatography (S200 HR10–30) and molar masses of complexes were determined by static light scattering measurements. Molecular weights (data points, left axis) and the UV signals (lines, right axis) of complexes were plotted versus the elution volume. Molar masses of the most populated sHsp/MDH complex (peak size) are given.
In striking contrast, in the presence of KJE and ClpB, a clear size dependence was no longer observed. For all the differently sized sHsp16.6/substrate complexes tested, identical MDH refolding rates (9 nM/min) were obtained (Fig. 6B). Moreover, an ∼30 min lag phase observed for the KJE-mediated refolding reaction (Fig. 6A) was reduced upon addition of ClpB. We

**FIG. 5.** Transfer of unfolded MDH from sHsp/MDH complexes to GroEL relies on DnaK-dependent complex dissociation. **A,** 3H-labeled MDH (2 μM) was heat-denatured either in presence of 14 μM GroEL-D87K or 4 μM Hsp16.6 and 14 μM GroEL-D87K. Alternatively, MDH (2 μM) was firstly denatured in presence of a 2-fold excess of Hsp16.6, forming soluble sHsp/MDH complexes. GroEL-D87K (14 μM) was added afterward, followed by further incubation at 30 °C for 2 h or at 47 °C for 30 min. Generated [3H]MDH-containing complexes were separated by size exclusion chromatography (S200 HR10–30) and quantified by scintillation counting. **B,** soluble sHsp/MDH complexes were generated by denaturing 2 μM MDH in presence of 4 μM Hsp16.6. MDH refolding was initiated at 30 °C by addition of the DnaK system (KJE: 1 μM DnaK, 0.2 μM DnaJ, 0.1 μM GrpE) and an ATP-regenerating system. For some reactions GroEL-D87K (14 μM) was added after 55 min. Generated [3H]MDH species were separated at the indicated time points by size exclusion chromatography (S200 HR10–30) and quantified by scintillation counting.

**TABLE II**

**Characterization of sHsp/MDH complexes**

2 μM MDH was heat-denatured in the absence or presence of various Hsp16.6 concentrations, indicated as ratios of sHsps to MDH. Turbidity of formed sHsp/MDH complexes was determined and set at 100% for MDH aggregates formed in the absence of Hsp16.6. Soluble and insoluble complexes were separated by centrifugation (13,000 rpm, 30 min, 4 °C) and analyzed by SDS-PAGE. Solubility of native MDH was set 100%. Complex radii were calculated based on diffusion coefficients, determined by dynamic light scattering measurements. Molecular weights of the complexes were determined by static light scattering measurements, coupled to size exclusion chromatography (S200 HR10–30). Distribution of molar masses and the molecular weights of the most populated complexes are given.

| Ratio sHsp/MDH | Turbidity | Solubility | Calculated radius | Mass distribution | Mass of most populated complex |
|----------------|-----------|------------|-------------------|-------------------|-------------------------------|
| 0              | 100       | <10        | 45 ± 15           | ND                | ND                            |
| 0.25           | 68        | <10        | 33.7 ± 12.5       | 1.8E ± 07 − 7.0E + 07 | 2.4E ± 07                |
| 0.5            | 37        | 18         | 31.5 ± 9          | 1.8E ± 07 − 7.0E + 07 | 2.4E ± 07                |
| 1              | 0         | 57         | 24 ± 6            | 5.6E + 06 − 1.5E + 07 | 7.9E ± 06                |
| 2              | 0         | 84         | 19 ± 5            | 2.3E + 06 − 4.0E + 06 | 3.5E ± 06                |
| 4              | 0         | 92         | 14 ± 5            | 1.5E + 06 − 3.1E + 06 | 2.1E + 06                |

a ND, not determined.

In striking contrast, in the presence of KJE and ClpB, a clear size dependence was no longer observed. For all the differently sized sHsp16.6/substrate complexes tested, identical MDH refolding rates (9 nM/min) were obtained (Fig. 6B). Moreover, an ∼30 min lag phase observed for the KJE-mediated refolding reaction (Fig. 6A) was reduced upon addition of ClpB. We
FIG. 6. Presence of sHsps in protein aggregates facilitates protein disaggregation by ClpB/DnaK. A–B, 2 μM MDH was denatured in absence (filled squares) or presence of 0.5 μM (filled circles), 1 μM (filled triangles), 2 μM (open squares), 4 μM (open circles), and 8 μM (open triangles) Hsp16.6. MDH refolding was started at 30 °C by addition of the DnaK system (1 μM DnaK, 0.2 μM DnaJ, 0.1 μM GrpE) in the absence (A) or presence of 1.5 μM ClpB (B). MDH activities were determined at the indicated time points. The enzymatic activity of native MDH was set at 100%. C, 2 μM MDH was denatured in absence (circles) and presence of 1 μM (squares) or 8 μM (triangles) Hsp16.6. MDH refolding was initiated at 30 °C by addition of the DnaK system (see A) in the presence of 0.15 μM ClpB. MDH activities were determined at the indicated time points. The enzymatic activity of native MDH was set at 100%. D, 2 μM MDH was denatured in the absence or presence of 1 μM Hsp16.6. Disaggregation of MDH aggregates (open symbols) and insoluble sHsp/MDH complexes (filled symbols) was determined by following the change in the light scattering signal at 30 °C in the presence of the DnaK system and 0.15 μM (squares) or 1.5 μM (circles) ClpB. Turbidity of MDH aggregates generated in the absence of sHsps was set at 100%. E–F, 1 μM α-glucosidase (E) or 1 μM citrate synthase (F) were denatured in the absence (open symbols) or presence of 0.5 μM (filled symbols) Hsp16.6. Protein disaggregation was started at 30 °C in the presence of the DnaK system (see A) and 1.5 μM ClpB. Resolubilization of aggregated proteins was followed by measuring the decrease in turbidity. Turbidity of protein aggregates generated in the absence of sHsps was set at 100%.
further noticed that the first time point of detectable MDH activity was earlier if the KJE/ClpB-mediated refolding reaction was started from insoluble Hsp16.6/MDH complexes compared with aggregated MDH and soluble Hsp16.6/MDH complexes (Fig. 6B). This indicates faster resolubilization of MDH aggregates containing Hsp16.6. The difference in the refolding process was even more striking when the ClpB concentration was reduced from 1.5 to 0.15 μM during MDH refolding (Fig. 6C). Thus a reduced disaggregation potential sensitizes the bi-chaperone system toward the aggregate state.

In order to demonstrate directly that incorporation of sHsps into turbid protein aggregates can accelerate the solubilization process by ClpB/KJE we directly followed the disaggregation reaction by measuring the decrease of aggregate turbidity. Indeed, ClpB/KJE were able to disaggregate the insoluble, turbid sHsp/MDH complexes more rapidly than MDH aggregates (Fig. 6D). The degree of this stimulation was higher in presence of 0.15 μM ClpB (2.5-fold) compared with higher ClpB concentrations (1.5-fold stimulation in presence of 1.5 μM ClpB). These data demonstrate that the presence of Hsp16.6 within MDH aggregates, conditions, which reflect the in vivo situation, facilitates the solubilization of MDH by ClpB/KJE in vitro. To investigate whether this effect of Hsp16.6 can be generalized to other substrates we compared the disaggregation rates of aggregated α-glucosidase and citrate synthase and of insoluble, turbid complexes of sHsps with the same substrates. As for MDH, faster solubilization of aggregates of these substrates was detected when Hsp16.6 was incorporated. Such complexes were disaggregated 3-fold faster in case of α-glucosidase and 17-fold faster in the case of citrate synthase, which was nearly resistant to solubilization in absence of sHsps (Fig. 6, E–F). The incorporation of sHsps into protein aggregates therefore acts generally to permit the faster solubilization of substrates by the ClpB/KJE bi-chaperone system.

**DISCUSSION**

This study provides new insight into the interaction of sHsps with the KJE/ClpB chaperone system and into the refolding mechanism of substrates bound to sHsps. By examining the rate of refolding from differently sized sHsp/substrate aggregates we demonstrated directly that sHsps increasingly facilitate KJE-mediated refolding which correlated with decreasing protein aggregate size. In contrast, excess ClpB enabled KJE to refold all sized protein aggregates of MDH and, furthermore, under limiting ClpB, incorporation of substoichiometric amounts of sHsps increased the rate of ClpB-dependent disaggregation for MDH, α-glucosidase, and citrate synthase. These effects are highly relevant to the conditions in vivo, where a high percentage of sHsps are found in the insoluble cellular fraction as large aggregates that would not be accessible to KJE without the action of ClpB. Thus, our data provide the first biochemical demonstration of the connection between the ClpB and sHsp chaperones, and as well a direct biochemical evidence that the combination of KJE, ClpB and sHsps form a robust system that can provide protection of cellular proteins under a wide range of substrate and available chaperone concentrations.

Our observations that sHsp/substrate complexes are stable protein aggregates that do not spontaneously release substrate has implications for the mechanism of substrate transfer from sHsps to the refolding chaperones. Using three different asays, we failed to find evidence for spontaneous substrate release from sHsp/substrate complexes. First, MDH prebound to either Hsp16.6 or Hsp18.1 was not found to transfer to added free sHsp under conditions permissive for refolding (Fig. 3). Second, the size of preformed sHsp/MDH aggregates could not be reduced by addition of more sHsp, although the additional sHsp would have been sufficient to form reduced sized aggregates during complex formation (Fig. 4). This same lack of detectable substrate transfer between sHsps and stable behavior of sHsp/substrate complexes has been observed with other sHsps and other substrates.3 We do not know whether this high stability can be generalized for all sHsp-substrate complexes, but consistent with our findings Buchner and colleagues demonstrated for several other sHsp/substrate complexes that substrate release is slow (12, 21). Third, no transfer of sHsp-bound substrates to a GroEL D87K trap mutant was detectable (Fig. 5). As an additional assay we also used a ClpB protein with mutations in the Walker B motifs of both nucleotide binding domains (E279A/E678A) as an alternative chaperone trap. In the presence of ATP this ClpB variant exhibits strongly stabilized interactions with protein substrates and can protect MDH from heat-induced aggregation.4 Importantly, ClpB E279A/E678A blocked the KJE-dependent dissociation of sHsp/MDH complexes by outcompeting DnaK for substrate binding. However, as for GroEL D87K, no transfer of MDH from sHsp/MDH complexes to the ClpB trap was observed after 120 min incubation at 30 °C.4 Altogether these results indicate that substrates are stably associated with sHsps. Therefore substrate refolding relies on an active complex dissociation through the ATP-dependent activity of KJE. This explains mechanistically the dependence of substrate refolding from sHsp/substrate complexes on Hsp70 chaperones and extends existing models (7, 21, 22). Furthermore, these findings also explain why ELS, which is unable to disaggregate proteins (6), cannot substitute for KJE in the refolding of sHsp-bound substrates (Fig. 1 and Table I). Instead, the beneficial effects of ELS in this process rely on the ability of this chaperone to associate with a partially folded monomeric intermediate of dissociated MDH and to assist its folding to the native dimeric state (Figs. 1 and 2).

The fact that incorporation of sHsps into protein aggregates increasingly enabled KJE to interact productively with the formed complexes, indicates that sHsps extend the substrate spectrum of the KJE system. In this sense sHsps act as a type of co-chaperone for the DnaK system. Direct cooperation of Hsp70 and sHsp systems is also implied by the presence of both genes in a single operon in Thermotoga maritima (32) and by their specific HspR-dependent co-regulation in Mycobacterium tuberculosis (33).

It is of interest to consider how the incorporation of sHsps into protein aggregates could facilitate the disaggregation reaction by KJE. Since substrates, bound to sHsps from different organisms, were all refolded efficiently by the E. coli KJE system, it is unlikely that direct and specific contacts between KJE and sHsps provide the basis for the observed cooperativity in protein disaggregation. The same conclusion was reached previously in studies showing refolding from Hsp18.1 was equally efficient not only with KJE, but also with Hsp70 proteins from different organisms (23). We consider it as more likely that sHsp intercalation into aggregates of substrates increases the accessibility of DnaK binding sites in substrates, and/or decreases the number of hydrophobic contacts between substrate molecules and hence the tightness of the complexes. In disfavor of an increased exposure of DnaK binding sites, aggregated MDH and soluble sHsp/MDH complexes show no differences in their ability to stimulate the ATPase activity of DnaK in single turnover experiments or to bind the hydrophobic probe, bio-ANS (data not shown). We therefore suggest that intercalation of sHsps into protein

3. K. Friedrich and E. Vierling, manuscript in preparation.
4. Weibezahn, J., Schlieker, C., Bukau, B., and Mogk, A. J. (2003) J. Biol. Chem., in press.
aggregates reduces the force needed for the extraction of incorporated substrates. The aggregate-specific chaperone ClpB strongly accelerates the KJE-dependent sHsp/substrate complex dissociation and additionally reduces the need for high KJE levels for this reaction. ClpB furthermore renders the dissociation reaction largely independent of the size of the complexes. These findings can be likely explained by ClpB acting first with a stronger force on sHsp/MDH complexes. Importantly, the physiologically relevant large sHsp/substrate complexes, which scatter light and can be pelleted by centrifugation, are dissociated 2–3-fold faster by ClpB/KJE than soluble sHsp/substrate complexes and substrate aggregates lacking sHsp. If such beneficial effects of incorporated sHsps in vitro are physiologically relevant, then similar effects should be observable in vivo. 

Indeed, consistent with our in vitro findings, E. coli ΔibpAB mutant cells exhibit a delay in resolubilization of heat-aggregated proteins. Similarly, IbpaB remain longer in the insoluble cell fraction during recovery from heat stress in E. coli ΔclpB mutant cells, compared with wild-type cells. Comparably results were obtained for Arabidopsis plants that are mutant for the eukaryotic ClpB protein, Hsp101, compared with wild-type plants. We can therefore conclude that the importance of sHsps is not restricted to prevent the formation of insoluble protein aggregates as previously assumed, but rather that they have an equally important function in insoluble aggregates, priming them for faster resolubilization by ClpB/KJE during the recovery phase of stressed cells. The incorporation of sHsps into protein aggregates thereby represents a second line of defense, allowing for faster protein disaggregation by ClpB/KJE. By influencing different aspects of the disaggregation reaction sHsps and ClpB work independently but also cooperatively with the DnaK system. Consistent with this suggestion, E. coli ΔibpAB ΔclpB double mutants exhibited strongly increased protein aggregation compared with the single knockout strains. Deletion of ClpB in an sHsp null background also increased the temperature sensitivity of Synechocystis cells (24). Most importantly sHsp function can even become essential for the viability of E. coli cells at regular and heat shock temperatures if the DnaK levels are low. Such conditions lead to an increased protein aggregation and a reduced disaggregation potential of cells at the same time. These findings underline the important contributions of sHsps to the survival of cells during severe stress by accelerating the solubilization of aggregated proteins.

---

5 E. Basha and E. Vierling, unpublished results.
Refolding of Substrates Bound to Small Hsps Relies on a Disaggregation Reaction Mediated Most Efficiently by ClpB/DnaK

Axel Mogk, Christian Schlieker, Kenneth L. Friedrich, Hans-Joachim Schönfeld, Elizabeth Vierling and Bernd Bukau

*J. Biol. Chem.* 2003, 278:31033-31042.

doi: 10.1074/jbc.M303587200 originally published online June 4, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M303587200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 20 of which can be accessed free at http://www.jbc.org/content/278/33/31033.full.html#ref-list-1