Hsp70 displaces small heat shock proteins from aggregates to initiate protein refolding

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Abstract

Small heat shock proteins (sHsps) are an evolutionary conserved class of ATP-independent chaperones that protect cells against proteotoxic stress. sHsps form assemblies with aggregation-prone misfolded proteins, which facilitates subsequent substrate solubilization and refolding by ATP-dependent Hsp70 and Hsp100 chaperones. Substrate solubilization requires disruption of sHsp association with trapped misfolded proteins. Here, we unravel a specific interplay between Hsp70 and sHsps at the initial step of the solubilization process. We show that Hsp70 displaces surface-bound sHsps from sHsp–substrate assemblies. This Hsp70 activity is unique among chaperones and highly sensitive to alterations in Hsp70 concentrations. The Hsp70 activity is reflected in the organization of sHsp–substrate assemblies, including an outer dynamic sHsp shell that is removed by Hsp70 and a stable core comprised mainly of aggregated substrates. Binding of Hsp70 to the sHsp/substrate core protects the core from aggregation and directs sequestered substrates towards refolding pathway. The sHsp/Hsp70 interplay has major impact on protein homeostasis as it sensitizes substrate release towards cellular Hsp70 availability ensuring efficient refolding of damaged proteins under favourable folding conditions.

Keywords Hsp100 disaggregase; Hsp70; protein aggregation; protein refolding; sHsps

Subject Categories Protein Biosynthesis & Quality Control

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Introduction

Exposure of cells to stress leads to increased formation of misfolded, aggregation-prone proteins and results in the breakdown of protein homeostasis (Tyedmers *et al*, 2010; Hartl *et al*, 2011; Labbadia & Morimoto, 2015). Appearance of misfolded protein species in the cytosol not only results in a loss of active proteins, but also

promotes the trapping and aggregation of other proteins (Richter *et al*, 2010; Kim *et al*, 2013). The problem of protein aggregation in response to environmental stress is particularly severe in case of unicellular and sessile organisms. A conserved network of three molecular chaperone systems has evolved early in evolution to counteract protein aggregation in the cell. This system comprises small heat shock proteins (sHsps: IbpA and IbpB in *Escherichia coli* and Hsp26 or Hsp42 in *Saccharomyces cerevisiae*) and two ATP-driven chaperones: Hsp70 (DnaK in *E. coli* and Ssa1 in *S. cerevisiae*) and an Hsp100 disaggregase (ClpB in *E. coli* and Hsp104 in *S. cerevisiae*), which together efficiently rescue and refold polypeptides trapped in aggregates (Mogk *et al*, 2003a; Cashikar *et al*, 2005; Haslbeck *et al*, 2005; Liberek *et al*, 2008; Doyle *et al*, 2013). The refolding of proteins from aggregates is important for the cell since severe heat stress conditions impair both transcription and translation (Yost & Lindquist, 1986; Panniers, 1994). In agreement with a crucial function of sHsps as part of a chaperone triad, deletion of sHsps gene in *Synechocystis sp.* (Lee *et al*, 2000) and *Neurospora crassa* (Plesofsky-Vig & Brambl, 1995) results in temperature-sensitive growth phenotypes, while deletion of sHsps in *E. coli* decreases cell viability during prolonged growth at high temperature (Kuczynska-Wisnik *et al*, 2002) or under conditions of limiting DnaK levels (Mogk *et al*, 2003a). Similarly, deletion of Hsp26 or Hsp42 in yeast *S. cerevisiae* results in a dramatic change in cell morphology under heat shock conditions pointing to a general protective function of sHsps for proteome homeostasis in yeast (Haslbeck *et al*, 2004).

Small Hsps, protecting the cell from irreversible protein aggregation, are widely distributed in both prokaryotes and eukaryotes (Haslbeck & Vierling, 2015). Bacteria, Archaea and unicellular eukaryotes usually possess one or two genes coding for sHsps. The number of genes encoding sHsps is higher in multicellular eukaryotes, reaching 19 in *Arabidopsis thaliana* and 10 in human (Haslbeck & Vierling, 2015). Members of this family are characterized by low molecular mass (12–43 kDa) and a conserved β -sandwich α -crystallin domain of ~90 amino acid residues (Haslbeck & Vierling, 2015; Treweek *et al*, 2015). A striking feature of most sHsps is their ability to assemble into oligomers in which dynamic exchange of subunits takes place (Giese & Vierling, 2002; Lentze *et al*, 2004; Painter *et al*, 2008; Benesch *et al*, 2010; Aquilina *et al*, 2013;

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Delbecq & Klevit, 2013; Shi *et al*, 2013). The oligomeric forms possess limited ability to interact with substrates (Ecroyd *et al*, 2007; Ahmad *et al*, 2008; Peschek *et al*, 2013; Treweek *et al*, 2015). Many sHsp family members become activated by increase in temperature, which shifts the equilibrium from oligomeric forms towards smaller species, usually dimers, which readily bind partially misfolded proteins exposing hydrophobic patches (Cheng *et al*, 2008; Stengel *et al*, 2010; Basha *et al*, 2013). The interaction between a single sHsp dimer and a substrate is dynamic; however, the oligomeric sHsp–substrate assemblies are quite stable, suggesting that substrates are tethered at multiple sites (Mogk *et al*, 2003b; Cheng *et al*, 2008).

Small Hsps constitute the first line of defence against protein aggregation. Under heat stress, the cytoplasmic levels of sHsps rapidly increase. Heat-activated sHsps bind misfolded substrates in an ATP-independent manner to form assemblies containing both misfolded substrate proteins and sHsps (Ehrensperger *et al*, 1997; Lee *et al*, 1997; Ratajczak *et al*, 2009). These assemblies are not only morphologically different from protein aggregates formed in the absence of sHsps but also substantially smaller in size. Although these assemblies have decreased size, the molecular mass still typically exceeds 1 MDa (Ratajczak *et al*, 2009; Stengel *et al*, 2010, 2012). More importantly, trapped polypeptides are rescued from these assemblies more efficiently by the Hsp70–Hsp100-dependent disaggregation and refolding systems, which reduces the need for resynthesis of essential proteins required for stress recovery (Veinger *et al*, 1998; Mogk *et al*, 2003b; Matuszewska *et al*, 2005).

An intimate cooperation between Hsp70 and Hsp100 is needed for protein disaggregate and refolding (Glover & Lindquist, 1998). Hsp70 binds first to protein aggregates, recruiting and activating the cooperating Hsp100 hexamers in a second step through direct physical interactions (Miot *et al*, 2011; Seyffer *et al*, 2012; Lee *et al*, 2013; Rosenzweig *et al*, 2013). Activated Hsp100 extracts trapped polypeptides by threading the polypeptides through its central channel (Weibezahn *et al*, 2004). Hsp70 activity is regulated by J-proteins (DnaJ in *E. coli* and Ydj1/Sis1 in *S. cerevisiae*) and nucleotide exchange factors (NEFs; GrpE in *E. coli*, Sse1/Fes1 in *S. cerevisiae*). J-protein binding and concomitant interaction with the substrate trigger ATP hydrolysis in Hsp70, allowing stable substrate interaction. Substrate release from Hsp70 is facilitated by the action of NEFs, which release ADP, allowing the rebinding of ATP to complete the cycle (Langer *et al*, 1992; Schmid *et al*, 1994; Laufen *et al*, 1999; Kampinga & Craig, 2010).

Here, we analysed the mechanism of extraction and refolding of misfolded proteins from sHsp–substrate assemblies. The initial binding of sHsps to substrates is beneficial by preventing the formation of large aggregates and creating smaller assemblies possessing greater accessible surface for Hsp70–Hsp100 action. At the same time, sHsp–substrate interactions might also hamper subsequent Hsp70/Hsp100 association and have to be broken to allow for substrate refolding. How this Janus-faced sHsp feature is circumvented has not been addressed so far. Here, we demonstrate a step-by-step mechanism during which the sHsp–substrate assemblies are solubilized and the substrates reactivated. We show that sHsp–substrate assemblies are composed of two substructures, a stable core and a dynamic outer shell exclusively composed of sHsps. Substrate recovery starts with the displacement of sHsps from the assembly surface by Hsp70. Hsp70 outcompetes dynamic sHsp

molecules for rebinding to the assemblies. Hsp70–substrate interaction at this point preserves the beneficial properties of the assembly core and protects it from aggregation. Hsp70 next recruits Hsp100, ensuring efficient substrate solubilization and targeting to refolding pathways. The revealed mechanism explains the phenomenon of efficient refolding of aggregated proteins assembled with sHsps.

Results

Dual role of Hsp70 in disaggregating IbpAB–substrate assemblies

The extraction of misfolded protein molecules from assemblies with sHsps requires accessibility of these substrates on the surface of the assemblies and an ability of the disaggregating chaperones to separate the sHsp–substrate associations. We therefore first analysed the size of sHsp–substrate assemblies, which determines the potential chaperone accessible surface. Thermal denaturation of substrates in the presence of sHsps results in formation of sHsp–substrate assemblies that are morphologically different from protein aggregates formed in their absence. Electron microscopy studies of thermolabile luciferase denatured in the presence of the *E. coli* sHsps IbpA and IbpB (IbpAB) revealed the formation of small, uniform and nearly globular structures that are distinct from luciferase aggregates formed in the absence of sHsps (Ratajczak *et al*, 2009). Size determination by dynamic light scattering (DLS) revealed that IbpAB–luciferase assemblies are 30 times smaller (average diameter: 58 nm) compared with luciferase aggregates (average diameter: 2,000 nm; Fig 1A). For additional characterization, we sedimented both the IbpAB–luciferase assemblies and luciferase aggregates in a glycerol gradient. The assemblies sedimented to the middle of the gradient in contrast to aggregates, which were found at the bottom following sedimentation (Fig EV1A). Based on SDS–PAGE followed by SYPRO Ruby staining, the assemblies isolated from the gradient showed an IbpA:IbpB:luciferase stoichiometry of ~1:0.8:1 ratio (Fig EV1A and B). DLS measurements estimated the molecular mass of the assemblies to be ~9 MDa. Based on molecular mass and stoichiometry estimation, each assembly on average contains ~90 luciferase molecules and a similar number of both IbpA and IbpB molecules.

As a consequence of sHsp/substrate assembly formation, the potentially accessible surface of the same amount of misfolded luciferase is much larger in IbpAB assemblies compared with its aggregated state. This may facilitate Hsp70–Hsp100-dependent disaggregation and refolding by offering more chaperone binding sites for initiating disaggregation. On the other hand, IbpAB present on the surface of the IbpAB–luciferase assemblies potentially also restricts chaperone binding to misfolded luciferase. Furthermore, substrate refolding requires disruption of sHsp–substrate interactions. To analyse how sHsp–substrate interactions influence subsequent Hsp70–Hsp100-dependent refolding, we compared the dependencies of luciferase reactivation from IbpAB–luciferase assemblies and luciferase aggregates on varying Hsp70 and Hsp100 chaperone concentrations (Fig 1B).

We started with an unexpected observation: when luciferase was denatured in the presence of IbpAB to form smaller assemblies, its reactivation by Hsp70–Hsp100 was less efficient compared with luciferase aggregates when using limiting concentrations of the

Figure 1. IbpAB-luciferase assemblies differ in biophysical and biochemical properties from luciferase aggregates.

- A** Dynamic light scattering graphs depicting the size distribution of the following entities: native luciferase (green), IbpAB (grey), luciferase aggregates (red), IbpAB-luciferase assemblies (blue), shown as volume distribution of analysed species. Luciferase was present at 1.5 μM, IbpA at 3 μM, IbpB at 7 μM concentration.
- B** Scheme of the disaggregation experiment.
- C, D** Renaturation kinetics for luciferase denatured in the presence or absence of IbpAB. Luciferase (1.5 μM) was denatured in the presence of IbpA (3 μM) and IbpB (7 μM), diluted to 40 nM and refolded at limited (0.3 μM; C) or elevated (2.4 μM; D) KJE machinery concentrations and standard (1.5 μM) ClpB concentration. The KJE machinery concentration is given with respect to DnaK with a constant DnaK:DnaJ:GrpE ratio 1:0.3:0.3. Data are the mean ± SD of three independent experiments.
- E** A comparison of renaturation rates for luciferase denatured in the presence or absence of IbpAB in changing KJE and constant ClpB concentrations. The KJE machinery concentration is given with respect to DnaK with a constant DnaK:DnaJ:GrpE ratio 1:0.3:0.3.
- F** A comparison of renaturation rates for luciferase denatured in the presence or absence of IbpAB in changing ClpB and constant KJE concentrations. The dashed line indicates the theoretical scenario that luciferase refolding from aggregates and sHsp assemblies proceeds with identical refolding rates.

compared to luciferase aggregates (Fig 1D). Thus, IbpAB plays an inhibitory role in substrate disaggregation and refolding at the limiting KJE concentration, while it becomes beneficial at elevated KJE level.

We next compared the initial rates of luciferase refolding from either IbpAB-luciferase assemblies or luciferase aggregates over a wide range of both KJE and ClpB concentrations. In this set-up, the level of KJE system was varied, while the Hsp100 chaperone (e.g. ClpB) was kept at saturating concentration. Again, we observed that the relative refolding rate of IbpAB-bound luciferase was highly dependent on KJE concentrations (Fig 1E). Increasing KJE concentrations allowed for superior refolding of luciferase from IbpAB-luciferase assemblies. In contrast, at saturating KJE concentration, ClpB, under all concentrations tested, stimulated luciferase reactivation from IbpAB-luciferase assemblies ~eightfold as compared to luciferase aggregates (Fig 1F).

These findings reveal that IbpAB-luciferase assemblies require much higher concentration of the Hsp70 chaperone system for efficient disaggregation and refolding than luciferase aggregates. At the same time, Hsp100 results in a similar degree of stimulation of the disaggregation of assemblies in comparison to aggregates over a range of Hsp104 concentrations used. This suggests that the Hsp70 chaperone system, in addition to its role in Hsp100 activation and docking to aggregates, has a novel function critical to sHsp-substrate assembly dissociation.

Hsp70-Hsp100-dependent disaggregation and refolding of aggregated proteins are preceded by a delay time (Zietkiewicz *et al*, 2004). We noticed that the delay time during luciferase reactivation from IbpAB-luciferase assemblies is significantly longer than the delay observed under analogous conditions for luciferase aggregates (Appendix Fig S1A and B). This observation, together with the higher demand for Hsp70 in luciferase rescuing from IbpAB-luciferase assemblies, led us to perform order of addition experiments to analyse which step of the disaggregation and refolding process requires elevated Hsp70 concentration. IbpAB-luciferase assemblies were initially incubated with specific components of the KJE-ClpB

Hsp70 system (KJE: 0.3 μM DnaK, 0.1 μM DnaJ and 0.1 μM GrpE; Fig 1C). While the yield of luciferase refolding was low under such conditions, significant and reproducible differences in refolding kinetics were determined for both substrate types: the assemblies were refolded at a twofold lower rate than the aggregates (Fig 1C). In contrast, eight times higher KJE concentrations (2.4 μM DnaK, 0.8 μM DnaJ and 0.8 μM GrpE) allowed for 10 times more efficient and faster protein refolding from IbpAB-luciferase assemblies as

Figure 2. KJE acts prior to the disaggregation step of IbpAB–luciferase assemblies.

An order of addition experiment. Luciferase (1.5 μ M) was denatured in the presence of IbpA (3 μ M) and IbpB (7 μ M), diluted to 40 nM and incubated first for 20 min with the components listed in the legend and then complemented with the missing components of the functional ClpB–KJE bichaperone system. The DnaK, DnaJ, GrpE, ClpB concentrations used were as follows: 1, 0.3, 0.3 and 1.5 μ M. Data are the mean $\pm$ SD of three independent experiments.

bichaperone machinery and the missing components were added at a later step to initiate disaggregation. The final yield of refolded luciferase was almost identical for all reactions. When IbpAB–luciferase assemblies were first incubated with KJE and ATP, the refolding reaction started earlier, as compared to the reactions in which these assemblies were initially incubated with either KJE in the absence of ATP or ClpB and ATP or without any chaperones (Fig 2). Additionally, initial incubation with KJE and ATP resulted in a 1.6-fold increased refolding rate, underlining an ClpB-independent activity of DnaK towards IbpAB–luciferase assemblies.

To generalize our kinetics observations, we used thermolabile malate dehydrogenase (MDH) as an alternative substrate. MDH was denatured in the presence of IbpAB, and Hsp70–Hsp100-dependent MDH refolding was performed. As observed earlier for IbpAB–luciferase assemblies, initial incubation of IbpAB–MDH with KJE (+ATP) resulted in a faster initiation of MDH reactivation but did neither influence the rate of MDH reactivation nor the final refolding yield (Fig EV2).

Summarized, we observed that assembly formation between sHsps and substrates causes a delay in substrate refolding and at low Hsp70 concentrations negatively affects substrate refolding. Both effects are overcome by initially treating sHsp–substrate assemblies with higher Hsp70 levels, suggesting that Hsp70 is playing an additional, so far non-recognized role upstream of Hsp70–Hsp100-mediated protein disaggregation.

Hsp70 releases sHsp-dependent inhibition of disaggregation

Our kinetic experiments (Fig 2) suggested that the role of Hsp70 in processing of sHsp–substrate assemblies is not limited to recruitment and activation of Hsp100 during protein disaggregation, but that Hsp70 possesses an additional activity at the initial step of the

process. However, it is problematic to directly investigate an independent Hsp70 activity in the processing of sHsp–substrate assemblies, since substrate disaggregation and reactivation require both Hsp70 and Hsp100. To divide this process into discrete steps depending exclusively on one of these chaperone systems, we took advantage of the properties of a hyperactive D484K variant of the yeast Hsp104 disaggregase (Lipinska *et al*, 2013). This variant is able to recover aggregated substrates in the absence of Hsp70 as it does not require recruitment and activation by the Hsp70 partner chaperone.

We speculated that the determined Hsp70 activity is related to the release of an inhibitory sHsp impact on the disaggregation process. In order to investigate this hypothesis, we used aggregated GFP as model substrate. GFP was initially aggregated at 85°C and the preformed GFP aggregates were subsequently incubated at 48°C with IbpAB to trigger sHsp binding to the aggregates (Appendix Fig S2A). As a result, the size of GFP aggregates and GFP aggregates coated with IbpAB (IbpAB–GFP assemblies) is similar (Appendix Fig S2B). Any observable changes in GFP reactivation by chaperones can therefore be directly related to IbpAB association with the aggregate surface. Similar to the findings obtained for IbpAB–luciferase assemblies, rescuing of GFP from IbpAB–GFP assemblies by KJE–ClpB proceeded at a slower rate compared with aggregated GFP when KJE was used at low concentration (Appendix Fig S2C). This inhibitory effect of IbpAB was again overcome by increasing KJE concentration (Appendix Fig S2D).

The comparison of GFP refolding efficiencies from IbpAB–GFP assemblies and GFP aggregates by Hsp104–D484K revealed that IbpAB presence strongly inhibits disaggregase activity (Fig 3A). In a control experiment, GFP aggregates and IbpAB were incubated separately at 48°C and mixed after cooling. This treatment does not allow for IbpAB binding to the surface of GFP aggregates. Under these conditions, no inhibition of Hsp104–D484K-mediated GFP refolding was observed (Fig 3A), demonstrating that the association of IbpAB with GFP aggregates but not the mere presence of IbpAB inhibits the hyperactive Hsp104 variant.

We speculated that IbpAB association restricts Hsp104–D484K access to the binding sites at the aggregate surface, thereby reducing its disaggregation activity. This model predicts that the additional role of KJE in substrate reactivation from IbpAB–substrate assemblies observed in the kinetic and order of addition experiments (Figs 1C and 2) is required to overcome this inhibitory IbpAB effect. To test this, we added KJE chaperones to the reactivation reaction containing IbpAB–GFP assemblies and Hsp104–D484K. It is important to note that the bacterial Hsp70 system does not cooperate with the yeast Hsp104 disaggregase (Glover & Lindquist, 1998; Krzewska *et al*, 2001), neither does it efficiently refold denatured GFP on its own (Appendix Fig S3). Therefore, any changes in GFP reactivation noticed upon KJE addition can be attributed to its activity at the initial step of disaggregation. We observed a substantial increase in GFP reactivation from the IbpAB–GFP assemblies by Hsp104–D484K in the presence of KJE (Fig 3B). This means that an Hsp100-independent activity of KJE resulted in the release of an IbpAB-dependent inhibition of GFP reactivation. In a control experiment, we showed that the addition of KJE chaperones to a reaction containing Hsp104–D484K and GFP aggregates lacking IbpAB did not stimulate, but partially inhibited GFP disaggregation (Fig 3B), excluding that the beneficial effect of KJE observed in case

Figure 3. Hsp70, independently of Hsp100, releases IbpAB-dependent inhibition of disaggregation.

A IbpAB association with GFP aggregates blocks substrate translocation through the central Hsp100 pore. Aggregated GFP (1.5 μ M) was incubated for 10 min at 48°C (marked "T" in the Figure) alone (blue) or with IbpA and IbpB (3 μ M and 7 μ M, respectively; sky blue) or aggregated GFP and IbpAB were incubated separately at 48°C and then mixed (orange). Hsp104 D484K hyperactive variant (1 μ M) was added, and GFP reactivation was monitored.

B KJE machinery allows to overcome the IbpAB-dependent inhibition of IbpAB–GFP disaggregation by hyperactive Hsp104 D484K. Aggregated GFP was incubated alone at 48°C (blue and orange) or with IbpAB at 48°C (sky blue and black). After addition of the Hsp104 D484K hyperactive mutant (all plots) and KJE machinery (black and magenta), GFP reactivation was monitored. DnaK, DnaJ and GrpE were present at 1 μ M, 0.3 μ M and 0.3 μ M. Aggregated GFP, IbpAB and Hsp104 D484K were in concentrations as in (A).

of IbpAB–GFP assemblies stems from cooperation with Hsp104–D484K. The noticed inhibition can be explained by competition between KJE and Hsp104–D484K for binding to GFP aggregates. We conclude that KJE releases the IbpAB-dependent inhibitory effect in a process not involving cooperation with the Hsp100 disaggregase. This Hsp70 activity is only required when IbpAB is present in substrate assemblies but not for protein aggregates lacking sHsps.

Hsp70 is required for dissociation of sHsps from assemblies

The exact nature of KJE activity in releasing the sHsp-dependent inhibition of disaggregation is unknown. KJE activity may result in rearrangement of sHsp–substrate assemblies facilitating subsequent refolding reactions. Alternatively, KJE might cause the release of sHsps from the substrate assembly. To distinguish between these two possibilities, we isolated IbpAB–luciferase assemblies by sedimentation in a glycerol gradient. The assemblies were incubated for different periods of time in the presence of varying chaperone combinations and tested for integrity by subjecting them to a second round of sedimentation (Fig 4A). Assemblies incubated without chaperones remained stable. Cosedimentation of IbpAB and luciferase in the middle of the gradient, indicative for assembly presence, was confirmed by Western blot (Fig 4B). Incubation of isolated IbpAB–luciferase assemblies with KJE for 30 min resulted in the release of over 40% of IbpAB from the assemblies in an ATP-dependent manner (Figs 4B and EV3A). The released IbpAB molecules were found in the top fractions of the gradient. IbpAB dissociation from the assemblies was further increased to 80% upon longer incubation (180 min) with KJE chaperones. Notably, a small fraction of IbpAB remained associated with luciferase and could not be displaced (Fig 4B). These findings define the existence of two distinct IbpAB populations in their assemblies with substrates. We hypothesize that the majority of IbpAB molecules is surface-exposed and can be displaced by KJE activity, whereas a second, smaller population forms an integral part of the assembly. The KJE-mediated release of IbpAB from the assemblies was not linked to a regain of luciferase activity. Accordingly, luciferase species migrated to nearly the same position in the glycerol gradient showing that no major change in sedimentation properties of assemblies took place following KJE-mediated release of IbpAB.

Next, we carried out a set of analogous sedimentation experiments additionally including ClpB. Incubation of the assemblies with both KJE and ClpB for 30 min resulted in a release of 70% of IbpAB species and also led to the appearance of monomeric, active luciferase in the top of the gradient (Fig 4C). Prolonged incubation for 180 min substantially enriched the top fractions of the gradient with native luciferase and resulted in dissociation of all IbpAB molecules from the assemblies (Fig 4C). It is worth noticing that the release of IbpAB from assemblies (30 min) preceded the formation of substantial amounts of active luciferase (180 min).

To verify whether the determined, KJE-dependent release of sHsps from sHsp–substrate assemblies is not related to one particular substrate, luciferase, similar experiments were performed using IbpAB–MDH as alternative assemblies. As observed for IbpAB–luciferase assemblies, the majority of IbpAB but not MDH was released following incubation with KJE and ATP (Fig EV4A and B). Native MDH only appeared in the gradient top fractions when ClpB was additionally present (Fig EV4B). These findings confirm that KJE releases surface-located sHsps without affecting the integrity of the core of the sHsp–substrate assembly.

Hsp70 binding displaces dynamic sHsps from the assembly

We considered two possible modes of KJE-mediated release of sHsps from the assemblies. The first mode, defined as an active one, involves remodelling of sHsp–substrate assemblies by Hsp70 in

Figure 4. Hsp70 releases sHsps from sHsp-substrate assemblies *in vitro*.

A Experimental scheme. Purified IbpAB-luciferase assemblies were incubated with indicated components for the indicated time and resubjected to glycerol gradient sedimentation.

B KJE releases IbpAB from IbpAB-luciferase assemblies. Fractions were collected from the top of the gradient, and luciferase and IbpA were visualized by Western blot following SDS-PAGE. The band intensities of luciferase and IbpA retained in the core assembly (fractions 5–12) or released from the assembly (fractions 1–3) were quantified and plotted on the graphs on the left. IbpA-IbpB-luciferase assemblies (approximate stoichiometry 1:0.8:1), DnaK, DnaJ and GrpE were present at 100 nM (calculated for luciferase monomer), 1 μ M, 0.3 μ M and 0.3 μ M concentrations, respectively.

C Concerted action of ClpB-KJE results in release of IbpAB and luciferase from IbpAB-luciferase assemblies. Experiments were performed and analysed as in (B). ClpB was present at 1.5 μ M concentration.

Source data are available online for this figure.

cycles of ATP hydrolysis leading to IbpAB release. The second mode suggests that binding of Hsp70 to the sHsp-substrate assembly is sufficient to displace sHsp molecules. This latter mode of DnaK action in releasing sHsp molecules from assemblies demands for dynamic interactions between sHsps and substrates. Constant dissociation and re-association of sHsps with sHsp-substrate assemblies has indeed been reported (Friedrich *et al*, 2004).

To test for spontaneous dissociation of IbpAB from assemblies, we performed an experiment in which we blocked the ability of dissociated IbpA molecules to re-associate with substrate assemblies. This was achieved by adding an excess of the peptide pC (PEAKPRRRIEIN), which corresponds to the twelve C-terminal residues of IbpA. Peptide pC efficiently inhibits IbpA ability to bind to substrates (Strozecka *et al*, 2012). As control, the inactive peptide variant pC-AEA was used, in which the two conserved isoleucines were replaced with alanines. Isolated IbpAB-luciferase assemblies were incubated with pC or pC-AEA peptide and tested for integrity

via sedimentation. The presence of pC but not pC-AEA peptide resulted in partial release of IbpAB from the assemblies as evident from appearance of IbpAB in the top fractions of the gradient (Fig 5). These results are in line with dynamic dissociation and re-association of sHsps from sHsp-substrate assemblies, with the rebinding step being inhibited by peptide pC.

Notably, incubation of IbpAB-luciferase assemblies with peptide pC also strongly increased the size of assemblies, which were now found in the bottom fraction of the gradient (Fig 5). This shows that the pC-mediated displacement of surface-located IbpAB causes aggregation of the core assemblies, including luciferase and some IbpAB. Core assembly aggregation is not observed upon IbpAB dissociation by Hsp70 (Fig 4), indicating that Hsp70 preserves the size of the sHsp-substrate assembly core. This suggests stable association of Hsp70 with the IbpAB-luciferase core assemblies following IbpAB displacement. To analyse this, IbpAB-luciferase assemblies were incubated with the KJE chaperone system and,

Figure 5. sHsps spontaneously dissociate from and re-associate with sHsp-substrate assemblies.

Surface-exposed IbpAB are dynamically binding and dissociating from the assemblies. Purified IbpAB-luciferase assemblies (100 nM; calculated for luciferase monomer; approximate stoichiometry 1:0.8:1) were incubated with the indicated peptides (1 mM each) for 60 min and subjected to glycerol gradient sedimentation. Fractions were collected from the top of the gradient, and luciferase and IbpA were visualized by Western blot following SDS-PAGE.

Source data are available online for this figure.

following sedimentation, the association of DnaK with IbpAB-luciferase assemblies was investigated by Western blot analysis. DnaK cosedimented with luciferase following KJE-mediated dissociation of IbpAB from assemblies and was present in analogous fractions to IbpAB-luciferase assemblies (Fig 6A). This indicates that DnaK replaces surface-located dynamic IbpAB, thereby preserving assembly size and preventing its aggregation.

In an alternative approach to support a competitive dissociation mechanism of IbpAB by DnaK, we took advantage of the ability of DnaK to bind substrates independent of DnaJ if present at high concentration in the presence of ADP (Liberek *et al*, 1991). Under such conditions, DnaK displays no refolding activity. We performed sedimentation experiments in which DnaK, at 12-fold higher concentration compared with the standard KJE-ATP reaction, and ADP were added to the isolated IbpAB-luciferase assemblies. Indeed, high concentrations of DnaK caused release of IbpAB (Fig 6B), suggesting that DnaK binding is sufficient to displace IbpAB from the assemblies. As control, we incubated the IbpAB-luciferase assemblies with the DnaK-V436F variant, which possess 20-fold lower association rate for binding substrate proteins (Laufen *et al*, 1999). The amount of IbpAB released upon incubation with DnaK-V436F and ADP was substantially lower (Fig 6C). This supports the model that the mere binding of DnaK is sufficient to efficiently outcompete dynamic, surface-located IbpAB from the assemblies.

Hsp70 has unique ability to displace sHsps

We next analysed whether chaperones other than DnaK can also displace sHsps from sHsp-substrate assemblies. We tested for activity of DnaJ, as it binds misfolded proteins and delivers them to DnaK (Hartl *et al*, 2011). We first analysed if DnaJ alone is able to release the inhibitory influence of sHsps on disaggregation by performing order of addition experiments. When IbpAB-luciferase assemblies were initially incubated with DnaJ and the missing components of the KJE-ClpB machinery were added at a later step, no change in the start of refolding reaction was observed (Fig EV3B). DnaJ is therefore not sufficient to shorten the delay

Figure 6. Hsp70 remains associated with the assembly following sHsp displacement.

- A** Purified IbpAB-luciferase assemblies were incubated with buffer or KJE and 2 mM ATP at 25°C for 60 min and resubjected to glycerol gradient sedimentation. Fractions were collected from the top of the gradient, and luciferase and DnaK were visualized by Western blot analysis. To avoid visualizing dominant, unbound DnaK, only the lower, IbpAB-luciferase assembly-containing fractions of the gradient are shown. Sedimentation analysis of KJE only is provided as a control. IbpAB-luciferase assemblies (approximate stoichiometry 1:0.8:1), DnaK, DnaJ and GrpE were present at 100 nM (calculated for luciferase monomer), 1 μM, 0.3 μM and 0.3 μM concentrations, respectively.
- B** DnaK binding to IbpAB-luciferase assemblies is sufficient to release IbpAB. Purified IbpAB-luciferase assemblies (100 nM) were incubated with the indicated components for 60 min and subjected to glycerol gradient sedimentation. Fractions were collected from the top of the gradient and IbpA was visualized by Western blot following SDS-PAGE.
- C** Purified IbpAB-luciferase assemblies (100 nM) were incubated for 60 min with buffer or DnaK (10 μM) or DnaK V436F (10 μM) and subjected to glycerol gradient sedimentation as in Fig 4B, but instead of showing the entire gradients, the fractions corresponding to the released material (1–3) were pooled together and IbpA was visualized by Western blot following SDS-PAGE.

Source data are available online for this figure.

time. Next, a series of sedimentation experiments was performed to directly probe for the ability of DnaJ to dissociate IbpAB from IbpAB-luciferase assemblies (Fig EV3C). High DnaJ concentrations

released only a small amount of IbpAB that was marginal compared with equivalent DnaK concentrations (Fig EV3C). These results confirm DnaK as the crucial component of the KJE chaperone system that is displacing IbpAB from IbpAB–luciferase assemblies.

Next we investigated whether other chaperones that act on protein aggregates are able to release IbpAB from assemblies. We focussed on AAA+ subunits of ATP-dependent proteases, ClpA and ClpC (with its adaptor MecA), which possess disaggregation activity *in vitro* (Dogan et al, 2002; Schlothauer et al, 2003). None of the AAA+ chaperones caused substantial dissociation of IbpAB from the IbpAB–luciferase assemblies as shown by sedimentation experiments (Fig EV3D). These findings further demonstrate the unique ability of KJE to act on sHsp–substrate assemblies.

Hsp70 releases sHsps from aggregates in bacterial cells

All our *in vitro* experimental approaches indicate that IbpAB chaperones, in an Hsp70-dependent process, must dissociate from the misfolded substrate prior to substrate disaggregation and reactivation. To test whether our *in vitro* observations are relevant in the cell and whether dissociation of sHsps from aggregates takes place prior to disaggregation *in vivo*, we made use of *E. coli* $\Delta clpB$ cells, in which aggregates are stably maintained (Mogk et al, 1999). Additionally, the native heat shock promoter of the *dnaK dnaJ* operon was replaced by an IPTG-inducible Lac promoter, which allows manipulating DnaK and DnaJ levels on demand. This experimental set-up enabled us to uncouple Hsp100-dependent and Hsp100-independent Hsp70 activities and to test whether Hsp70-mediated release of sHsp is observed *in vivo* in the absence of ongoing disaggregation. $\Delta clpB$ P_{IPTG} *dnaKJ* cells were first grown in the absence of IPTG, and formation of protein aggregates was induced by a 30-min heat shock at 46°C (Fig 7A). Next, the bacterial culture was transferred to 30°C and divided into two flasks. IPTG was added to one of them to induce the synthesis of DnaK and DnaJ. Following 2 h from induction, the level of DnaK was similar to the level of heat-shocked wild-type cells (Fig 7E). Protein aggregates were isolated from both cultures during the recovery phase, and the levels of IbpAB associated with aggregates were analysed. As expected, the fraction of aggregated proteins remained stable, regardless of DnaK and DnaJ induction, due to the absence of ClpB (Fig 7B and D). However, induction of DnaK and DnaJ led to a substantial decrease in the amount of IbpAB associated with aggregates (Fig 7C and D). The Hsp70 system is therefore able to dissociate IbpAB from protein aggregates *in vivo*, in agreement with the *in vitro* results. IbpAB dissociation proceeded with slow kinetics under the given experimental conditions. However, one should take into account that DnaK, a major and highly abundant bacterial chaperone, was depleted to ~5% of its physiological level before and during heat shock. In combination with the *clpB* deletion, this resulted in massive protein aggregation. Moreover, DnaK levels corresponding to the heat shock-induced levels were only achieved 2 h after addition of IPTG, further rationalizing the delayed release of IbpAB.

Yeast Hsp70 releases Hsp26 from substrate assemblies

We finally tested whether the determined novel Hsp70 activity is evolutionary conserved by repeating key experiments with the respective *S. cerevisiae* chaperones (sHsp: Hsp26; Hsp70 system:

Figure 7. Hsp70 releases sHsps from the fraction of aggregated proteins *in vivo*.

- Experimental scheme. BB6410 (PA1lacO-1) *dnaK, dnaJ, lacIq, ΔclpB* cells were grown at 30°C in LB medium until late log phase, heat-shocked at 46°C for 30 min, then the culture was separated into two flasks and IPTG was added at 1 mM. At indicated time points, aliquots were taken, cells were lysed and protein aggregates were isolated.
- The isolated fraction of aggregates was analysed by SDS-PAGE followed by staining with Coomassie brilliant blue at the indicated time points.
- Levels of IbpA present in aggregates at the indicated time points were determined by quantitative immunoblot analysis using IbpA-specific antibodies.
- Quantification of (B) and (C).
- DnaK cellular levels were determined by quantitative immunoblot analysis using DnaK-specific antibodies.

Source data are available online for this figure.

Ssa1, Ydj1, Sse1 and Hsp104 disaggregase) and MDH as substrate. Order of addition experiments showed that initial incubation of Hsp26–MDH assemblies with the yeast Hsp70 system (SYS: Ssa1,

Ydj1, Sse1) resulted in a faster start of MDH refolding following addition of Hsp104 (Fig 8A), analogous to the results obtained for the bacterial chaperones (Figs 2 and EV2). Assemblies composed of misfolded MDH and Hsp26 were also isolated using sedimentation. Such isolated assemblies were incubated for 180 min in the presence of SYS, SYS and Hsp104 or with buffer only, and subjected to a second round of sedimentation (Fig 8B and C). Assemblies

composed of Hsp26 and misfolded MDH were less stable than those comprising bacterial IbpAB and denatured substrates. Incubation of Hsp26–MDH assemblies with buffer only followed by recentrifugation caused dissociation of almost 40% of Hsp26 molecules, suggesting increased dynamics of Hsp26 binding to Hsp26/MDH core assemblies. Incubation of Hsp26–MDH assemblies with the yeast Hsp70 chaperone system, however, increased the fraction of

Figure 8. Eukaryotic Hsp70 dissociates eukaryotic sHsps from sHsp-substrate assemblies *in vitro*.

A Yeast Hsp70 system acts prior to disaggregation of Hsp26–MDH assemblies. An order of addition experiment. MDH (5 μ M) denatured in the presence of Hsp26 (5 μ M) was diluted 10-fold and first incubated with the components listed in the legend and then complemented with the missing components of the functional Hsp104–SYS (Ssa1, Ydj1, Sse1) bichaperone system present at 1.5 μ M, 4 μ M, 2 μ M and 0.15 μ M concentrations, respectively. Data are the mean $\pm$ SD of three independent experiments.

B Experimental scheme. Purified Hsp26–MDH assemblies were incubated with indicated components and subjected to glycerol gradient sedimentation.

C SYS (Ssa1, Ydj1, Sse1) dissociates Hsp26 from Hsp26–MDH assemblies. Fractions were collected from the top of the gradient, and MDH and Hsp26 were visualized by Western blot following SDS–PAGE. The band intensities of MDH and Hsp26 retained in the core assembly (fractions 5–12) or released from the assembly (fractions 1–3) were quantified and presented on the graphs on the left. Hsp26–MDH assemblies (approximate stoichiometry 1.25:1), Ssa1, Ydj1, Sse1 and Hsp104 were present at 400 nM (calculated for MDH monomers), 4 μ M, 2 μ M, 0.15 μ M and 1.5 μ M concentrations, respectively.

Source data are available online for this figure.

released Hsp26 to 80%, indicating a conserved Hsp70 function in sHsp displacement (Fig 8C); 20% of Hsp26 remained associated with MDH assemblies suggesting that this fraction of Hsp26 is not surface-exposed but is an integral part of the assembly. This indicates that Hsp26–MDH assemblies are also composed of a stable Hsp26–MDH core structure and a dynamic Hsp26 outer shell. Hsp26 displacement by SYS did not result in release of free MDH, which still sedimented at almost the same position in the gradient as observed before for bacterial chaperones. Substrate solubilization and reactivation required addition of Hsp104, as seen by quantitative movement of MDH to the top fractions of the gradient indicative for formation of active MDH (Fig 8C). Accordingly, under these experimental conditions, all Hsp26 was found in the top fractions of the gradient. We finally showed that the bacterial KJE system also efficiently releases Hsp26 from Hsp26–MDH assemblies (Fig EV5A and B), indicating that Hsp70–sHsp interplay does not involve species-specific cooperation consistent with a mechanism based on the direct binding of Hsp70 to sequestered substrates. Together, these experiments show that the Hsp70 system harbours an evolutionary conserved activity to displace sHsps from substrate assemblies, preparing the assemblies for efficient substrate reactivation.

Discussion

sHsps act as the first line of defence during protein misfolding stress and are key factors in modifying protein aggregation. Their presence results in the formation of small-sized sHsp–substrate assemblies as compared to protein aggregates formed in sHsp absence. This assembly formation has beneficial effects on rates and yields of Hsp70–Hsp100-mediated protein disaggregation and reactivation. This positive impact can be explained by an altered conformational state of the bound substrate and an increased surface offering more

substrate binding sites for chaperone association. However, here we demonstrate that sHsps have ambivalent effects as their presence also causes a delay in substrate recovery and even inhibits the disaggregation and refolding processes at limited Hsp70 concentrations. Thus, while increasing the binding-competent surface of the sHsp–substrate assemblies, sHsps also seem to reduce chaperone binding, presumably through direct competition. Additionally, binding of sHsps to substrates is likely also confining the fate of the bound substrate to the refolding pathway.

In this work, we unravel a novel, evolutionary conserved function of Hsp70 chaperones, which directly affects the ambivalent influence of sHsps on the disaggregation process. We show that Hsp70 binding allows for efficient, permanent dissociation of surface-located sHsps from sHsp–substrate assemblies at the initial phase of disaggregation (Fig 9). This Hsp70 activity is unique and is not executed by other chaperones that either act on protein aggregates or can bind misfolded proteins (Fig EV3). It also does not involve cooperation with the Hsp100 partner protein. This has important evolutionary implications as the Hsp100 disaggregase is absent in metazoa, which encode for more potent Hsp70 chaperone systems that exhibit robust disaggregation activity (Nillekoda *et al*, 2015). The conserved key role of Hsp70 in initiating protein disaggregation strongly suggests that the activity of Hsp70 towards sHsp–substrate assemblies established here for bacterial and yeast chaperones is conserved in all kingdoms of life.

We provide evidence that Hsp70 displaces sHsps from the assemblies by a competitive dissociation mechanism as mere binding of Hsp70 to the assemblies is sufficient to cause sHsp release. The competition model that we propose predicts that direct interactions between Hsp70 and sHsps are not necessary for sHsp displacement by Hsp70. Indeed, species-specific cooperation between Hsp70 and sHsps in disaggregation reactions was neither observed here nor in former studies and direct interactions between both chaperones

Figure 9. Schematic model of the Hsp100–Hsp70 bichaperone system action on sHsp–substrate assemblies.

sHsp–substrate assemblies consist of a stable sHsp–substrate core and an dynamic outer shell, comprising the majority of sHsps. Surface-exposed sHsps are dynamically binding and dissociating from the sHsp–substrate assemblies, with the equilibrium shifted towards the bound state. Hsp70 outcompetes sHsps in binding to the assembly surface, protecting the sHsp–substrate core and rendering sHsps in the unbound state. Once the sHsps are displaced from the surface, the misfolded polypeptides are extracted from the assemblies and refolded by a concerted action of the Hsp100–Hsp70 bichaperone system.

have not been reported (Giese & Vierling, 2002; Mogk *et al*, 2003b; Friedrich *et al*, 2004; Bepperling *et al*, 2012). The efficient dissociation of sHsps from the assemblies is dependent on a functional Hsp70 chaperone cycle. Hsp70 has to be activated for binding to the substrate by J-proteins (e.g. DnaJ) to compete with sHsps for binding to the assemblies. DnaJ itself, however, is not able to dissociate sHsps from assemblies. We speculate that the interaction between DnaJ and substrate is too transient to efficiently compete with dynamic IbpAB. Alternatively DnaJ may recognize distinct substrate segments compared with IbpAB allowing for their simultaneous binding at the complex surface.

The proposed competition model demands for a dynamic behaviour of sHsps. Our work defines two distinct sHsp populations within sHsp–substrate assemblies forming specific substructures with diverse dynamics. The first and less abundant population of sHsps forms an integral, immobile part of sHsp–substrate assemblies. This population of sHsps is only liberated during Hsp100 disaggregase activity, which results in the complete disintegration of the assemblies. The second and more abundant sHsp fraction is surface-exposed and dynamically interacts with the assembly. The presence of two sHsp populations within sHsp–substrate assemblies is supported by kinetic studies revealing biphasic kinetics of sHsp–substrate interactions: substrates are rapidly bound by a small number of sHsps, and extra sHsps are subsequently recruited to the assembly (Stengel *et al*, 2010). We suggest that the first of these two populations represents the core assembly population defined in this work and the latter represents the surface-exposed dynamic fraction that can be displaced by Hsp70. We consider two scenarios for the displacement of surface-exposed sHsps by Hsp70, given that sHsps associate with substrates via multiple binding sites (Jaya *et al*, 2009; Fu *et al*, 2013). First, sHsps completely dissociate from the assemblies and are prevented from re-association by Hsp70 binding. Second, sHsps only partially dissociate from the assembly by losing some substrate interactions while retaining others. Binding of Hsp70 to the core assembly at a site that becomes vacant upon partial sHsp release might then ultimately cause sHsp dissociation.

We also predict that the outer shell of dynamic sHsps has various important physiological functions. First, it maintains size and solubility of the core assembly by shielding hydrophobic sites within the substrate that are exposed at the surface of the assemblies. Second, it forms a selective barrier that is only penetrated by the Hsp70 chaperone system but not by, for example, AAA+ proteases, which have disaggregation potential and are linked to proteolysis. The sHsp shell thereby indirectly targets sequestered substrates of the core structure to refolding pathways. Third, it increases the demand for Hsp70 chaperone activity, as sHsp–substrate assemblies require higher Hsp70 concentrations for disaggregation compared with aggregated substrates. We suggest that such mechanism allows to protect sequestered substrates under unfavourable folding conditions such as chronic stress, which reduces the availability of free Hsp70 chaperones.

Taken together, our work unravels a novel function of Hsp70 in protein disaggregation, namely the displacement of surface-located sHsps from sHsp–substrate assemblies. This Hsp70 activity directs sequestered substrates to refolding pathways. The Hsp70 function is independent from the cooperating Hsp100 disaggregase and is therefore expected to also play crucial roles in metazoan protein quality control systems.

Materials and Methods

Proteins

Purifications of the proteins were performed as described previously: DnaK, DnaJ, GrpE, ClpB, IbpA, His-IbpB (N-terminal His-tag) and GFP (Ratajczak *et al*, 2009), and Ssa1, Sse1, Ydj1, Hsp104 wt, Hsp104 D484K and Hsp26 (Seyffer *et al*, 2012; Lipinska *et al*, 2013). Pyruvate kinase and creatine kinase were purchased from Sigma, pig heart muscle MDH from Roche and firefly luciferase from Promega. Protein concentrations were determined with the Bio-Rad Bradford assay using BSA as standard. Protein concentrations refer to monomers.

Dynamic light scattering

Particle sizing was performed using Malvern Instruments ZetaSizer Nano S dynamic light scattering instrument. Measurements were taken in buffer F (50 mM Tris, pH 7.4, 150 mM KCl, 20 mM Mg acetate, 2 mM DTT). Luciferase was present at 1.5 μ M, IbpA at 3 μ M, IbpB at 7 μ M concentration. Conditions were as follows: measurement volume—40 μ L, scattering angle—173°, wavelength—633 nm, temperature—25°C. For every measurement, minimum five 10-s runs were averaged and particle size distribution was calculated by fitting to 70 size bins between 0.4 and 10,000 nm. Results are shown as particle distribution by volume distribution of analysed species.

Luciferase denaturation and refolding experiments

Luciferase (1.5 μ M) was denatured at 48°C for 10 min in buffer A (50 mM Tris, pH 7.5, 150 mM KCl, 20 mM MgCl₂, 2 mM DTT) either with or without IbpA (3 μ M) and His-IbpB (7 μ M). Protein refolding was started by 40-fold dilution of denatured luciferase in the chaperone cocktail. Unless noted otherwise, the chaperone concentrations used were as follows: DnaK 1 μ M, DnaJ 0.3 μ M, GrpE 0.3 μ M, ClpB 1.5 μ M. All assays were performed in the presence of an ATP-regenerating system (3 mM phosphoenol pyruvate, 20 μ g/ml pyruvate kinase, 2 mM ATP). The disaggregation reaction was carried out at 25°C. Luciferase activities were measured using a Sirius Luminometer (Berthold). Refolding rates were calculated from the linear range of increase in luciferase activity.

GFP denaturation and refolding experiments

The experiments were performed essentially as described previously (Ratajczak *et al*, 2009). GFP (150 μ M) was thermally inactivated in buffer B (40 mM Tris (pH 7.5), 10% (v/v) glycerol, 150 mM NaCl and 100 mM 2-mercaptoethanol) by incubation for 10 min at 85°C. After denaturation, GFP aggregates were diluted to a final concentration of 1.5 μ M in buffer A supplemented with sHsps (3 μ M IbpA and 7 μ M IbpB) as indicated, and incubation proceeded for 10 min at 48°C. Reactivation was initiated by threefold dilution of GFP in buffer C (40 mM Tris (pH 7.8), 85 mM potassium glutamate, 20 mM magnesium acetate, 10% (v/v) glycerol and 5 mM 2-mercaptoethanol) supplemented with 10 mM ATP and an ATP regeneration system comprising creatine phosphate (120 mM) and creatine kinase (0.2 mg/ml) and chaperone proteins (DnaK, DnaJ,

GrpE, and Hsp104 D484K at final concentrations of 1, 0.3, 0.3, and 1 μM , respectively). The disaggregation reaction was carried out at 25°C in 400 μl final volume in a spectrofluorometric cuvette. The fluorescence of refolded GFP was measured in a PerkinElmer LS50B spectrofluorimeter with excitation at 395 nm and emission at 510 nm.

MDH denaturation and refolding experiments

MDH (5 μM) was denatured in the presence of Hsp26 (5 μM) in buffer D (50 mM HEPES, pH 7.5, 50 mM KCl, 5 mM MgCl_2 , 2 mM DTT). Protein refolding was started by diluting aggregated MDH to 500 nM in the chaperone cocktail: Ssa1 4 μM , Ydj1 2 μM , Sse1 0.15 μM , Hsp104 1.5 μM . All assays were performed in the presence of an ATP-regenerating system (3 mM phosphoenol pyruvate; 20 $\mu\text{g}/\text{ml}$ pyruvate kinase; 2 mM ATP). The disaggregation reaction was carried out at 30°C. Determination of enzymatic activities followed published protocols (Mogk et al, 2003a).

SDS–PAGE, immunoblotting and protein quantifications

Gel electrophoresis was performed using 4–20% ExpressPlus PAGE Gels (Genescript) and stained with Coomassie brilliant blue. Immunoblotting was performed according to the standard procedures, using rabbit anti-sera specific for IbpA, DnaK, Hsp26, MDH or luciferase as primary antibody, and developed either with a Vistra ECF fluorescence immunoblotting kit (Amersham) using alkaline phosphatase-conjugated anti-rabbit IgG as secondary antibodies (Vector Laboratories) or, alternatively SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), using (H+L) HRP-conjugated anti-rabbit IgG (Bio-Rad) as secondary antibodies. Developed immunoblots were scanned using a fluorimager Image-Quant LAS 4000 or ChemiDoc MP Imaging System (Bio-Rad) and quantified using ImageJ software.

sHsp–substrate assembly isolation and sedimentation analysis

Luciferase (1.5 μM) was denatured at 48°C for 10 min in buffer A in the presence of IbpA (3 μM) and His-IbpB (7 μM). To isolate the IbpAB–luciferase assemblies from unbound IbpAB, 600 μl of preformed assemblies was applied on a 3.2 ml 10–60% glycerol gradient in buffer A. Samples were centrifuged at 4°C in a Beckman SW 60 rotor at 160,000 g for 1.5 h; fractions were collected from the top. Protein distribution in each fraction was verified by SDS–PAGE followed by immunoblotting. Fractions containing IbpA–IbpB–luciferase assemblies (approximate stoichiometry 1:0.8:1) were pooled together, and the substrate concentration was determined by optical densitometry following SDS–PAGE as 1 μM (luciferase monomer). To analyse the effect of chaperones on isolated assemblies, the assemblies were diluted 1:10 in chaperone cocktail (DnaK 1 μM , DnaJ 0.3 μM , GrpE 0.3 μM , ClpB 1.5 μM) and subjected to a second round of sedimentation under conditions listed above.

MDH (5 μM) was denatured at 47°C for 30 min in buffer D in the presence of Hsp26 (15 μM). To isolate the Hsp26–MDH assemblies from unbound sHsps, 600 μl of the assemblies was applied on a 3.2 ml 10–60% glycerol gradient in buffer D. Samples were centrifuged at 4°C in a Beckman SW 60 rotor at 210,000 g for 80 min;

fractions were collected from the top. Protein distribution in each fraction was verified by SDS–PAGE followed by immunoblotting. Fractions containing Hsp26–MDH assembly (approximate stoichiometry 1.25:1) were pooled together, and substrate concentration was determined by optical densitometry following SDS–PAGE as 1.6 μM (MDH monomer). To analyse the effect of chaperones on isolated assemblies, the assemblies were diluted 1:4 in chaperone cocktail (Ssa1 4 μM , Ydj1 2 μM , Sse1 0.15 μM , Hsp104 1.5 μM or, alternatively, DnaK 1 μM , DnaJ 0.3 μM , GrpE 0.3 μM , ClpB 1.5 μM) and subjected to a second round of sedimentation under conditions listed above.

Isolation of aggregated proteins *in vivo*

The *E. coli* strain used was a derivative of MC4100 and is described in Mogk et al (2003a). The strain was cultured at 30°C in Luria broth (LB). Chloramphenicol and kanamycin were used at final concentrations of 10 and 20 $\mu\text{g}/\text{ml}$, respectively. Temperature-shift experiments were performed in orbital shaking water baths. The cells were grown overnight to OD = 0.7, heat-shocked at 46°C for 30 min and shifted back to 30°C. To induce synthesis of DnaK and DnaJ, IPTG was added to 1 mM. Four hours after the heat shock spectinomycin was added to 50 $\mu\text{g}/\text{ml}$ to the cultures to prevent the residual accumulation of DnaK and DnaJ synthesized due to PA1/lacO-1 promoter leakage in IPTG non-induced culture. At indicated time points, 10-ml aliquots were collected and the aggregated protein fraction was isolated as described in Tomoyasu et al (2001).

Expanded View for this article is available online.

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Author contributions

SZw, AK, NBN and IO performed the experiments. SZw, AK, NBN, SZi, BB, AM and KL designed the experiments. AP performed and analysed the DLS experiments. SZw, AM and KL wrote the manuscript. SZw, AM, BB and KL conceived the project.

Conflict of interest

The authors declare that they have no conflict of interest.

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