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DnaK-mediated association of ClpB to protein aggregates. A bichaperone network at the aggregate surface

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ABSTRACT

Intracellular protein aggregates formed under severe thermal stress can be reactivated by the concerted action of the Hsp70 system and Hsp100 chaperones. We analyzed here the interaction of DnaJ/DnaK and ClpB with protein aggregates. We show that aggregate properties modulate chaperone binding, which in turn determines aggregate reactivation efficiency. ClpB binding strictly depends on previous DnaK association with the aggregate. The affinity of ClpB for the aggregate-DnaK complex is low (K_d = 5–10 μM), indicating a weak interaction. Therefore, formation of the DnaK–ClpB bichaperone network is a three step process. After initial DnaJ binding, the cochaperone drives association of DnaK to aggregates, and in the third step, as shown here, DnaK mediates ClpB interaction with the aggregate surface.

Structured summary:
MINT-7258957: *G6PDH* (uniprotkb:P0AC53) and *G6PDH* (uniprotkb:P0AC53) *bind* (MI:0407) by *dynamic light scattering* (MI:0038)MINT-7258951: *alpha glucosidase* (uniprotkb:P21517) and *alpha glucosidase* (uniprotkb:P21517) *bind* (MI:0407) by *dynamic light scattering* (MI:0038)
MINT-7258903: *AdhE* (uniprotkb:P0A9Q7) and *AdhE* (uniprotkb:P0A9Q7) *bind* (MI:0407) by *dynamic light scattering* (MI:0038)
MINT-7258900: *G6PDH* (uniprotkb:P0AC53) and *G6PDH* (uniprotkb:P0AC53) *bind* (MI:0407) by *biophysical* (MI:0013)
MINT-7258974: *DnaK* (uniprotkb:P0A6Y8), *ClpB* (uniprotkb:P63284), *DnaJ* (uniprotkb:P08622) and *G6PDH* (uniprotkb:P0AC53) *physically interact* (MI:0914) by *cosedimentation* (MI:0027)

1. Introduction

After severe heat stress cell survival depends on the ability of a chaperone network to reactivate intracellular protein aggregates. This ability is enhanced by preconditioning cells at sub-lethal temperatures, a phenomenon known as acquired thermotolerance that results in an increased synthesis of chaperones able to solubilize and reactivate the aggregates when physiological conditions are restored. Elimination of protein aggregates can occur through two alternative pathways, namely disaggregation and refolding mediated by the Hsp70–Hsp100 bichaperone system, or proteolysis [1]. Disaggregation of preformed protein aggregates has been shown to be the most important process to acquire thermotolerance [2–4], indicating that cell survival following thermal stress requires the reactivation of thermally inactivated proteins.

Reactivation of protein aggregates requires one or two chaperone systems acting in a concerted manner. In prokaryotes, these are the DnaK system, formed by the chaperone DnaK and the cochaperones DnaJ and GrpE, and the Hsp100 chaperone ClpB. Although the involvement of these chaperones in the disaggregation process is well established, the way they associate with protein aggregates remains as yet poorly understood. Several findings indicate that the DnaK system is required in the early stages of protein disaggregation to extract polypeptides from aggregates [5] or to remodel the aggregate surface [6]. Kinetic studies have identified the interaction of DnaK with aggregated proteins as the rate-limiting step of the disaggregation process [7]. Analysis of a ClpB variant has also revealed that DnaK is engaged in the initial substrate unfolding event that leads to aggregate processing [8]. Following KJE action the translocation of the polypeptide chain through the central channel of ClpB is an important part of the disaggregation process [8], although recent studies suggest that polypeptides can also be processed into the pore without a threading activity [9]. The DnaK system might also be

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involved downstream of ClpB action, interacting with the substrate protein being processed by ClpB, and therefore preventing its reassociation with the aggregate [8].

We focus here in the association of the chaperones involved in aggregate reactivation in *Escherichia coli*, DnaJ, DnaK and ClpB, with protein aggregates. We find that the amount of aggregate-bound chaperones depends on aggregate properties (size and conformation), which in turn are modulated by the particular substrate protein considered and the denaturation method used to obtain aggregates. Furthermore, our data extend previous observations showing that DnaJ drives DnaK binding to the aggregate [10], since they demonstrate that ClpB association to the aggregate surface is a low affinity (K_d = 5–10 μM), DnaK-mediated process.

2. Materials and methods

2.1. Protein expression and purification

DnaK was expressed, purified, and extensively dialyzed to obtain nucleotide-free samples [11]. DnaJ, GrpE and ClpB were obtained as previously reported [12–14]. G6PDH and α-glucosidase were purchased from Sigma, AdhE was expressed and purified as previously described [15]. Protein concentration was determined by the colorimetric Bradford assay (Bio-Rad), except for DnaK, that was determined spectrophotometrically using $\varepsilon_{280} = 15.8 \times 10^3\ M^{-1}\ cm^{-1}$ [16]. ClpB concentration refers to its monomeric form.

2.2. Refolding of client proteins

G6PDH (10 μM) was denatured at 50 or 70 °C during 30 min in 50 mM Tris–HCl, pH 7.5, 150 mM KCl , 20 mM $MgCl_2$ and 10 mM DTT. Denatured and aggregated G6PDH was diluted to 0.4 μM in the same buffer containing an ATP-regenerating system (3 mM ATP, 6 mM phosphoenolpyruvate and 20 ng/mL pyruvate kinase) and chaperones: 0.7 μM DnaJ, 0.35 μM GrpE and different DnaK concentrations (3.5–15 μM), in the presence or absence of increasing ClpB concentrations (0.5–20 μM monomer). G6PDH activity was measured after a 60-min reactivation period at 30 °C as described previously [17]. AdhE (10 μM) and α-glucosidase (10 μM) were denatured at 62 °C for 30 min and 42 °C for 20 min, respectively, diluted (0.4 μM) and incubated as described above for G6PDH. AdhE and α-glucosidase activity was measured as reported [15,18]. One hundred percent refolding was assume to be the activity corresponding to the same concentration of native protein before denaturation. Turbidity of G6PDH aggregates was measured under the same experimental conditions used in the refolding assays on a Fluorolog-3 (Jobin Yvon) spectrofluorimeter with both excitation and emission wavelengths set at 500 nm.

2.3. Dynamic light scattering (DLS)

Substrate proteins (10 μM) were denatured in filtrated refolding buffer under the same experimental conditions described in the refolding assays. Average size of the aggregates was monitored in a Zetasizer nanoseries DLS (Malvern), which can accurately analyze particles with hydrodynamic diameter between 0.6 and 6000 nm. Each experimentally determined size value is the average of 12 measurements of the same sample. A peak which contains more than 95% of the total input was always found.

2.4. Fourier transformed infrared spectroscopy (FT-IR)

G6PDH, AdhE and α-glucosidase were dialyzed against deuterated buffer containing 50 mM Tris–HCl, pD 7.5, 150 mM KCl, 20 mM $MgCl_2$, 10 mM DTT and concentrated to ~10 μM. Samples were placed in a thermostated IR cell between two CaF_2 windows separated by a 50 μM Teflon spacer. They were denatured at the temperatures indicated above, and during the denaturation process spectra were recorded in a Nexus 870 FT-IR spectrometer (Thermo) equipped with a MCT detector. Each spectrum consists of 200 independent scans measured at a spectral resolution of 2 cm^{-1}. Protein spectra were obtained by subtracting the corresponding buffer spectra. Data treatment and amide I band decomposition was performed as described [19].

2.5. Chaperone binding assays

Association of chaperones with protein aggregates was characterized by electrophoresis of the pellets obtained after centrifugation of the protein mixtures, as described [10]. Aggregates (1 μM) were incubated 10 min at 30 °C in refolding buffer with the desired combination of chaperones: 0.7 μM DnaJ and different DnaK (3.5–15 μM) or/and ClpB concentrations (0.5–20 μM). GrpE was omitted to avoid aggregate reactivation. Samples were centrifuged in a Beckman Optima ultracentrifuge at 95 600×*g* for 30 min at 4 °C, to separate free and aggregate-bound chaperones. The resulting pellets were analyzed by SDS–PAGE (7.5% or 12.5%). The amount of aggregate-bound proteins was estimated by densitometry of the gel bands with a gel scanner G-800 and the Quantity One software (Bio-Rad). Each data point is the average of at least three independent experiments and was estimated by subtracting the amount of protein found in pellets of control experiments, containing native G6PDH.

3. Results

3.1. Aggregate properties determine the amount of bound chaperones

Protein aggregate properties such as conformation and size modulate the refolding efficiency of different chaperone combinations [20]. To test if these properties also control chaperone binding to the aggregate surface, the conformation and size of thermally denatured and aggregated G6PDH, α-glucosidase and AdhE were analyzed (Fig. 1). We have used two types of aggregates, small and large, from three different substrate proteins: G6PDH, α-glucosidase and AdhE. Small and large aggregates of G6PDH are obtained after denaturing the protein at low (50 °C) or high (70 °C) temperatures. Denaturation of α-glucosidase (47 °C) and AdhE (62 °C) results in aggregates of similar size to large aggregates of G6PDH (Fig. 1A). Their conformational properties were characterized by IR, a specially well suited spectroscopic technique to study protein aggregation [21] (Fig. 1B). Among the infrared absorption bands, the so called Amide I band (1700–1600 cm^{-1}) is the most commonly used in conformational studies of proteins, and contains several components whose assignment to specific types of secondary structures has been described [19]. Protein aggregation induces the appearance of a characteristic low frequency IR band (1617 cm^{-1}) whose intensity, relative to the overall Amide I band area, gives an estimation of the number of residues involved in the inter-molecular β-structure characteristic of protein aggregation and depends on the denaturation method and the particular sequence under study (Fig. 1C). However, there is a significant difference in the level of β-structure between small and large aggregates.

The efficiency of the bichaperone system to reactivate both types of aggregates differs: while small aggregates are quantitatively refolded, the ability to reactivate large aggregates is remarkably low and similar for the three proteins (Fig. 1D), in good agreement with previous studies using G6PDH [17] and

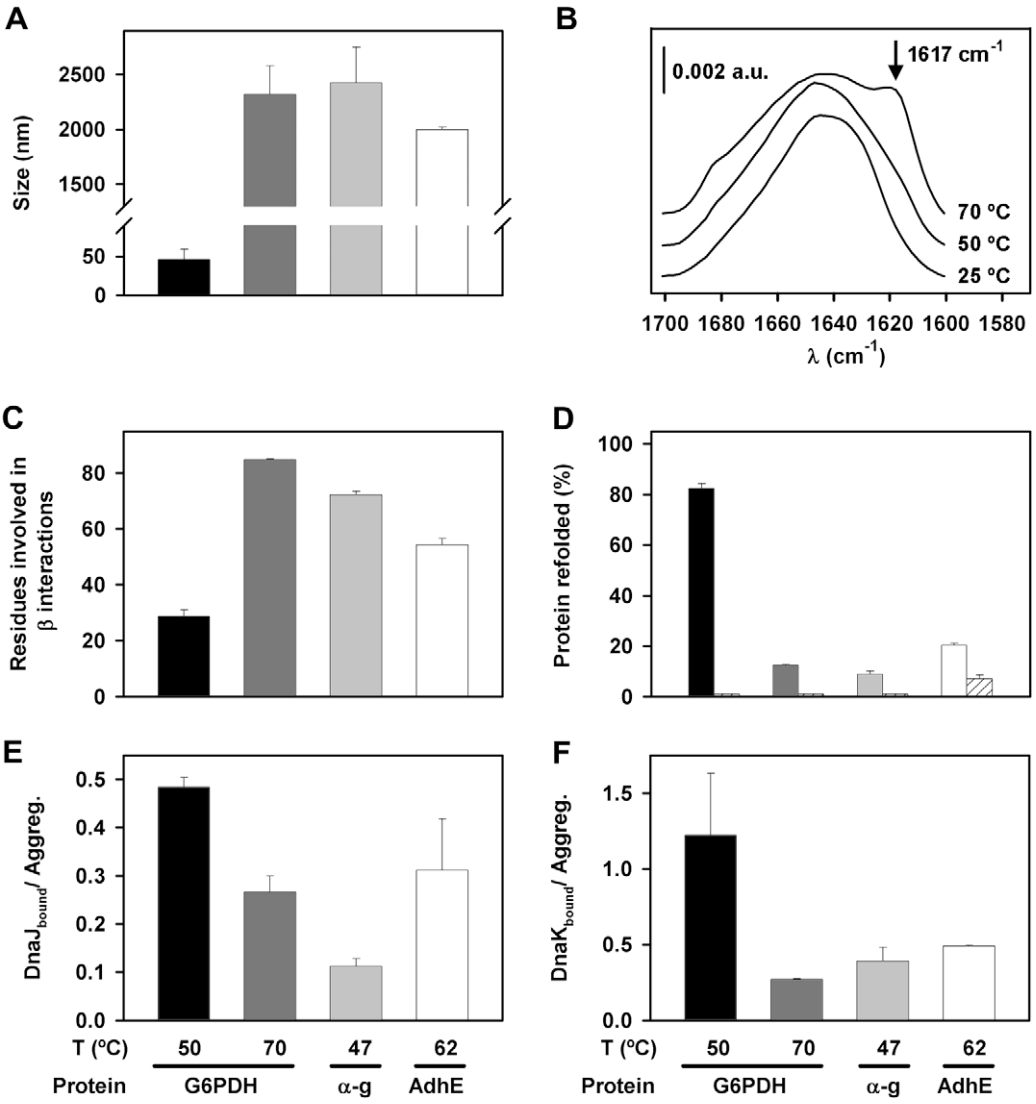

Fig. 1. Aggregate properties regulate chaperone binding and reactivation efficiency. (A) Average size of the aggregates obtained after denaturing 10 μM G6PDH at 50 °C (15 min) and 70 °C (30 min), α-glucosidase at 42 °C (20 min) and AdhE at 62 °C (30 min), as determined by dynamic light scattering (see Section 2). (B) Amide I region (1600–1700 cm^{-1}) of the IR spectra of native and aggregated G6PDH denatured at different temperatures. (C) Estimation of the number of residues involved in inter-molecular interactions for the three substrates, made considering the relative intensity of the 1617 cm^{-1} band component and the number of residues of each protein (486, 574 and 891 amino acids for G6PDH, α-glucosidase and AdhE, respectively). (D) Aggregate reactivation. Aggregates formed as in A were diluted to 0.4 μM and refolded in the presence of 0.5 μM ClpB, 3.5 μM DnaK, 0.7 μM DnaJ and 0.35 μM GrpE for 1 h at 30 °C. The activity of samples treated identically in the absence of chaperones is also shown (stripped columns). Interaction of DnaJ (E) and DnaK (F) with protein aggregates formed as in A, and diluted (0.4 μM) in the presence of 3 mM ATP, 0.7 μM DnaJ, 3.5 μM DnaK and 0.5 μM ClpB.

α-glucosidase [22]. We asked whether this could be a consequence of a defective interaction of any of the chaperones with large aggregates. To test if this was the case for the DnaK system, binding of DnaJ (Fig. 1E) and DnaK (Fig. 1F) to these protein aggregates was analyzed. DnaJ (Fig. 1E) and DnaK (Fig. 1F) bind about 2.2 and 5 times less to large aggregates of G6PDH, which contain an increased amount of inter-molecular β-structure, than to small ones. Interestingly, the refolding efficiency of large aggregates drops (6.2-fold) similarly to DnaK binding to these aggregates. The correlation between chaperone binding and aggregate properties is not so clear for DnaJ binding to large (2000–2400 nm) aggregates of the three substrate proteins analyzed (Fig. 1E). However, a comparison of Fig. 1C and F suggests that as the number of inter-molecular interactions among denatured protein molecules increases, the amount of aggregate-bound DnaK decreases.

Interestingly, neither the amount of aggregate-bound DnaK (data not shown) nor the reactivation yield (Fig. 2) increased with DnaK concentration, indicating that, under these experimental conditions the lowest DnaK concentration used was enough to saturate the aggregate surface. It should be noted that the DnaJ concentration used here also saturates the aggregate surface, as previously reported [10], since higher cochaperone concentrations do not significantly change DnaJ binding and refolding yield. Interestingly, when the same experiments were carried out at increasing ClpB concentration, the reactivation efficiency of the bichaperone system is significantly enhanced for all protein substrates (Fig. 2A). The time dependence of G6PDH aggregates reactivation at different ClpB concentrations also reveals that the reactivation rate increases up to 10 μM ClpB (Fig. 2B). A similar time evolution is observed for enzyme activity recovery and the decrease in aggregate turbidity (Fig. 2C), indicating that, as described for other substrates [5,10], disaggregation and refolding are coupled reactions. Therefore, the limiting step in the reactivation reaction would be the extraction of unfolded monomers from the aggregate. These data demonstrate that increasing ClpB

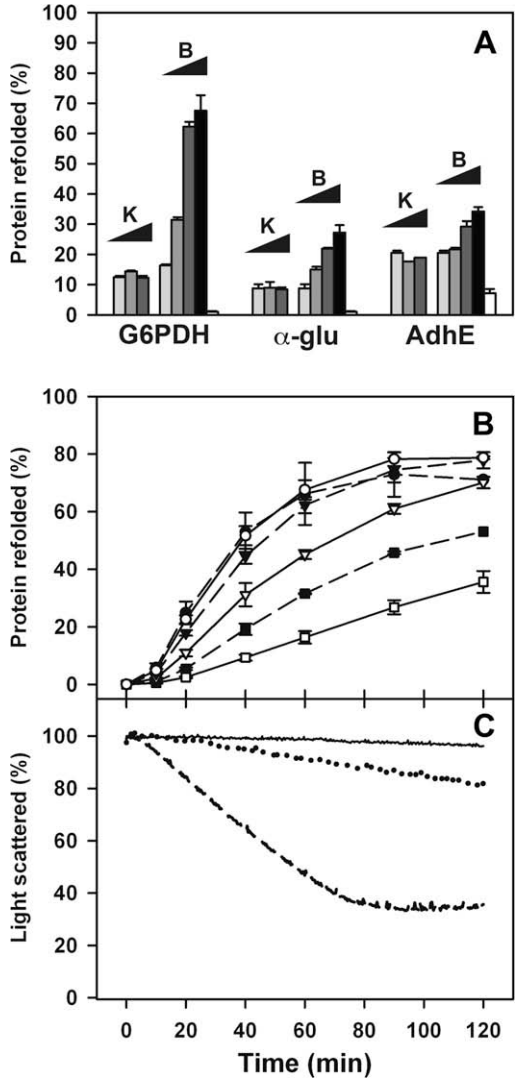

Fig. 2. Effect of increasing chaperone concentration on aggregate reactivation. (A) Protein aggregates, obtained as in Fig. 1, were diluted (0.4 μM) in the presence of 0.7 μM DnaJ, 0.35 μM GrpE, 0.5 μM ClpB and different concentrations of DnaK (3.5, 7, 15 μM); or 0.7 μM DnaJ and 0.35 μM GrpE, 3.5 μM DnaK and increasing ClpB concentrations (0.5, 1, 2 5, 10 μM). After incubating the samples for 1 h at 30 °C in the presence of an ATP-regenerating system, the activity of substrate proteins was measured. White bars represent the residual activity of each protein under the same experimental conditions but in the absence of chaperones. (B) Time dependence of the reactivation of G6PDH aggregates (0.4 μM) that were diluted in the presence of 0.7 μM DnaJ and 0.35 μM GrpE, 3.5 μM DnaK and increasing ClpB concentrations: 0.5 μM (empty squares), 1 μM (filled squares), 2 μM (empty triangles), 5 μM (filled triangles), 10 μM (empty circles), and 20 μM (filled circles). (C) Changes in the turbidity of G6PDH aggregates (0.4 μM) in the absence of chaperones (continuous line) and in the presence of 0.7 μM DnaJ and 0.35 μM GrpE, 3.5 μM DnaK, and 0.5 μM (dotted line) or 10 μM (dashed line) ClpB.

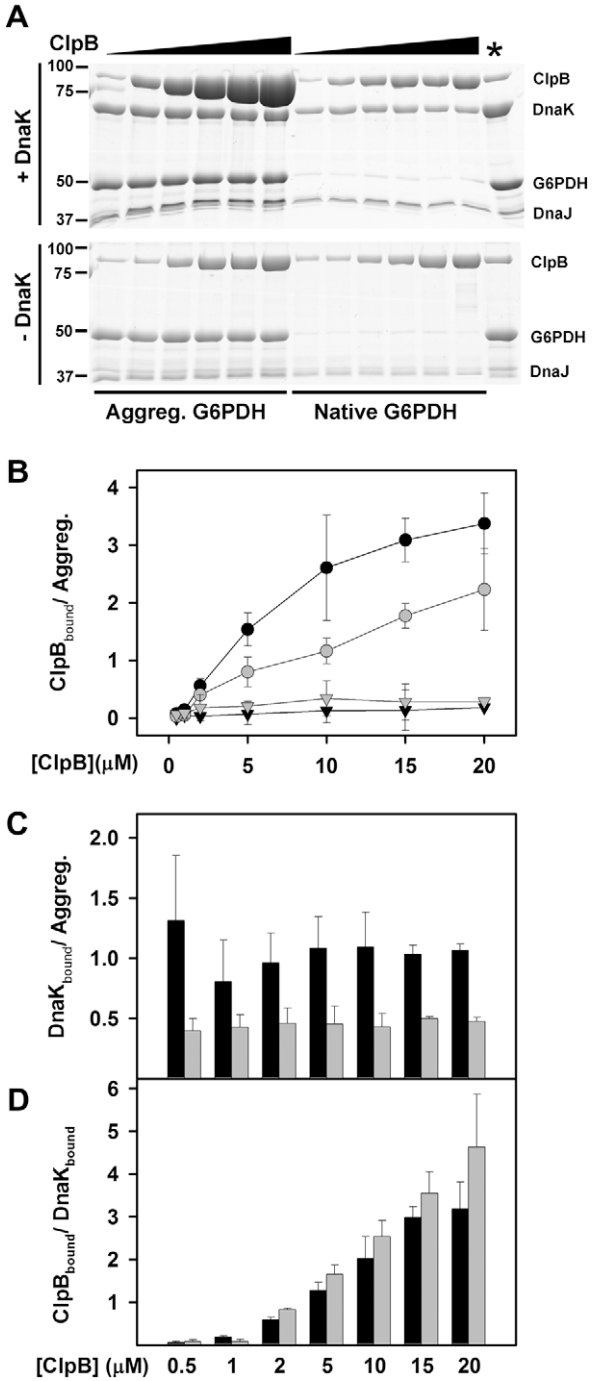

Fig. 3. DnaK-mediated association of ClpB to protein aggregates. (A) Interaction of ClpB with G6PDH aggregates. G6PDH (10 μM) was denatured at 70 °C/30 min. Aggregated or native G6PDH was diluted to 1 μM in the presence or absence of DnaK (3.5 μM) and different ClpB concentrations (1, 2, 5, 10, 15, 20 μM). The sample also contained 0.7 μM DnaJ and 3 mM ATP, but not GrpE to avoid aggregate reactivation. Samples were incubated 10 min and centrifuged to separate free and aggregate-bound chaperones. The resulting pellets were analyzed by electrophoresis to estimate the amount of aggregate-bound chaperones. (B) Estimation of the amount of aggregate-bound ClpB. Aggregates (10 μM) were formed at 50 °C/15 min (black symbols) or 70 °C/30 min (gray symbols) and were diluted to 1 μM in buffer containing 0.7 μM DnaJ, 3 mM ATP, increasing ClpB concentrations, and in the presence (circles) or absence (triangles) of 3.5 μM DnaK. (C) Effect of aggregate properties and ClpB concentration on DnaK binding. Binding assays with aggregates formed at 50 °C/15 min (black) or 70 °C/30 min (gray) were performed as indicated in B. (D) Aggregate-bound ClpB (monomer)/DnaK molar ratio. Binding assays with aggregates formed at 50 °C/15 min (black) or 70 °C/30 min (gray) were performed as in B.

concentrations can rescue reactivation of large aggregates with an efficiency that depends on the particular substrate protein and most likely the denaturation method.

3.2. DnaK drives ClpB binding to protein aggregates

To understand the effect of increasing ClpB concentrations on the reactivation yield, binding of DnaK and ClpB to G6PDH aggregates was studied under different experimental conditions (Fig. 3). It was previously shown that in the presence of ATP, wt ClpB does not stably interact with protein aggregates [10,23]. However, we find here that significant amounts of ClpB bind to

the protein aggregate when its concentration is raised, and interestingly, that the association only takes place in the presence of DnaK and protein aggregates, indicating that ClpB binding is a DnaK-mediated process (Fig. 3A). As expected from the ATP dependence of DnaJ–DnaK complex formation [24], association of the bichaperone network with protein aggregates strictly depends on ATP (not shown). The binding reaction shows a concentration-dependence that saturates around 15 μM ClpB, the estimated K_d for the interaction being in the micromolar range (5–10 μM). An important difference regarding the amount of aggregate-bound ClpB is observed when aggregates obtained at 70 and 50 °C are compared. For the same initially added ClpB, the aggregate-bound chaperone is around half for aggregates formed at 70 °C (Fig. 3B). This difference could be due to a less efficient DnaJ/DnaK binding to these aggregates, as shown before in similar experiments (Fig. 1). Data in Fig. 3C demonstrate that this is the case, since the amount of aggregate-bound DnaK is between 3.3- and 2-fold lower for aggregates formed at 70 °C. When the estimated aggregate-bound ClpB monomer/DnaK molar ratio is plotted as a function of ClpB monomer concentration, its value reaches a plateau at around 4–5, indicating the presence of substoichiometric amounts of ClpB hexamers relative to DnaK at the aggregate surface (Fig. 3D). Interestingly, this ratio is the same for both types of aggregates, supporting the hypothesis of an aggregate-bound DnaK-mediated recruitment of ClpB. This interpretation also implies that the number of aggregate-bound ClpB molecules would depend on the ability of DnaJ/DnaK to differently bind distinct protein aggregates.

4. Discussion

The efficient reactivation of protein aggregates requires the collaboration of members of the chaperone families Hsp40, Hsp70 and Hsp100 [3,4]. Despite the importance of this process, information on how these proteins build the functional chaperone network is scarce [25,26]. Our results indicate that their association with the aggregate surface takes place in three steps: (i) DnaJ binding, (ii) DnaJ-mediated recruiting of DnaK [10], and (iii) DnaK-dependent association of ClpB with the aggregate surface. The finding that DnaK mediates the interaction of ClpB with the aggregate would be consistent with the previously observed facilitated access of aggregated proteins to the ClpB pore site by the DnaK system [27].

Previous studies on the interaction of ClpB with protein substrates have demonstrated that the life-time of stable ClpB-substrate complexes decreases as the ClpB ATPase activity increases, suggesting that the protein conformation that stably binds substrates is the ATP-bound state [28–31]. Thus, wt ClpB and a trap mutant of the protein that lacks ATPase activity form stable complexes with substrate proteins in the presence of ATPγS and ATP, respectively [28–31]. Interestingly, complex formation was not previously reported under more physiological conditions, i.e., wt ClpB in the presence of ATP. This apparently contradicts data presented herein, and might be explained as follows. The experimental conditions used in previous studies (0.3–2.5 μM hexameric ClpB in the absence of DnaK) result also in our hands in non-significant complex formation. We show that to achieve aggregate-ClpB complex formation two additional experimental conditions are required: (i) the presence of DnaJ/DnaK, and (ii) a ClpB concentration close to or higher than the K_d (5–10 μM) estimated for complex formation. This later condition explains why this interaction was not detected in the presence of DnaK at low ClpB concentration (0.3 μM hexamer) [10,28–31].

We cannot establish whether ClpB interacts with DnaK at the aggregate surface or with denatured substrate proteins after being remodeled by the DnaK system, that could make accessible binding sites for ClpB. Formation of a transient DnaK–ClpB complex would facilitate direct transfer of intermediate conformations between chaperones, avoiding the otherwise possible reassociation of these partly folded intermediates with the aggregates that would hamper their effective reactivation. The following experimental evidences suggest that the hypothesis of a direct interaction between chaperones at the aggregate surface might occur. First, the highest aggregate-bound ClpB hexamer/DnaK molar ratio observed is close to but lower than one, within the ClpB concentration range used (0.17–3.3 μM hexamer). Second, the low affinity of ClpB for DnaK-bound protein aggregates resembles that described for TClpB binding to TDnaK in solution (K_d = 17 μM) [25], indicating that in both cases the interaction is similarly weak. The previous observations that productive cooperation between Hsp70 and Hsp100 homologs from different organisms to reactivate protein aggregates does not occur [25], and that the rate of ATP hydrolysis by the combination of ClpB and the DnaK system in the presence of substrate is greater than the sum of the separate rates [32] provide additional support for an interaction between the two chaperones. However, in contrast to the *Tantalus thermophilus* proteins, *E. coli* chaperones would not stably interact in solution and would instead associate with the aggregate in a sequential reaction in which the requirement of each component for an efficient reactivation can be finely tuned.

We also find that for the same sequence (G6PDH), the denaturation method determines aggregate properties (size and conformation), which in turn modulate the amount of aggregate-bound chaperone. Chaperones bind better to small size aggregates containing denatured molecules with a low content of inter-molecular β-structure. This finding, proposed in a previous work [17] and demonstrated herein, most likely reflects a higher accessibility at the aggregate surface of binding sites for the cochaperone and chaperones. The ability of the DnaK system to reactivate small protein aggregates could be related to the enhanced chaperone binding and to the few non-native inter-molecular contacts that must be rearranged to process a substrate monomer. The accessibility of potential DnaJ/DnaK binding sites at the surface of large protein aggregates would be reduced due to a decrease in the surface-to-volume ratio, and to a higher extent of unfolding of the polypeptide chains that allows more residues to take part in the stable inter-molecular β-structure. This would also explain the correlation found between DnaK binding to large protein aggregates and the number of residues involved in inter-molecular interactions. DnaK interacts with both the backbone and side chains of a peptide substrate [33,34], and therefore hydrophobic amino acid stretches engaged in inter-molecular contacts will not be available for chaperone binding. In contrast, DnaJ is believed to interact with only the side chains of the substrate protein since it binds both L- and D-peptides [34,35], and thus cochaperone binding to these aggregates would not depend so clearly on the conformation that the unfolded substrates adopt at the aggregate surface, but more on the particular protein sequence under study. An increase in intramolecular β-sheet structure would also result in a greater energy requirement to extract an unfolded monomer from the aggregate, the limiting step in the reactivation reaction [5], since more non-native inter-molecular interactions must be disrupted. The consequence is that the DnaK system is not competent by itself to reactivate these protein aggregates, and requires ClpB to do it efficiently. This interpretation is in agreement with the ClpB requirement to reactivate small and large aggregates of luciferase rich in inter-molecular β-structure found by Liberek and coworkers [20]. In this study, small aggregates were obtained by fragmentation of large ones with K/J/E, and therefore if the conformation of the aggregated substrate is maintained during fragmentation, as it was suggested [20], the number of non-native inter-molecular

interactions and the energy needed to disrupt them will also be maintained during the initial extraction step.

In summary, the main finding of this study is that chaperone association to protein aggregates proceeds sequentially. In the first step, aggregate-bound DnaJ drives DnaK to the aggregate surface [10], and, as shown here, in the second one, DnaK mediates ClpB binding to the aggregate, where the joint effort of the bichaperone network allows extraction of unfolded substrate molecules for subsequent folding.

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