DnaJ Recruits DnaK to Protein Aggregates*

Sergio P. Acebrón1,2, Vanesa Fernández-Sáiz1,2,3, Stefka G. Taneva4, Fernando Moro5, and Arturo Muga6

From the Unidad de Biofísica (Consejo Superior de Investigaciones Científicas/Universidad del País Vasco-Euskal Henrike Unibertsitatea) and Departamento de Bioquímica y Biología Molecular, Universidad del País Vasco, P.O. Box 644, Bilbao 48080, Spain

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Members of the Hsp70 protein family are involved in numerous cellular processes that include protein folding and refolding (1, 2), protein translocation across membranes (3), and protein complex assembly and disassembly (4). To fulfill its functional role in protein (re)folding, the Hsp70 protein has to collaborate with co-chaperones that modulate its ATPase cycle and might also carry protein substrates (5). DnaJ is the bacterial representative of the Hsp70 family and, in collaboration with GrpE (nucleotide exchange factor) and DnaJ (Hsp40), prevents protein aggregation and productively solubilizes protein aggregates either alone or together with ClpB (the Hsp100 representative in Escherichia coli) (6). DnaJ is involved in the transfer of substrates to DnaK (7–11) and, together with GrpE, in controlling the time the chaperone spends in the low affinity and high affinity states for substrate proteins (5). These DnaJ roles have been found essential for its efficient reactivation. Our results provide the first experimental evidence of DnaJ-mediated recruiting of ATP-DnaK molecules to the aggregate surface.

The mechanism of Hsp100/Hsp70-dependent disaggregation of aggregated substrates is as yet poorly understood. The chaperone network that leads to productive aggregate reactivation depends on aggregate properties, which would include size, conformational properties of the denatured protein molecules, and number and nature of the intermolecular interactions between unfolded polypeptides. Several findings suggest that the activity of DnaK is required in the early stages of protein disaggregation to extract polypeptides from aggregates (14). Kinetic studies have identified the interaction of DnaK with aggregated proteins as the rate-limiting step of the disaggregation process (15). Analysis of the ClpB variant BAP has also revealed that DnaK is required for the initial substrate unfolding event that leads to aggregate processing (16). The DnaK system might also be involved downstream of ClpB action, taking over the protein once it has been processed by ClpB, preventing its reassociation with the aggregate and therefore promoting substrate reactivation (16).

To understand the role of the DnaK system (K/J/E) at the initial stage of reactivation, it is necessary to study how the components of this system interact with client proteins and particularly with protein aggregates. It seems clear that the chaperone action is driven by ATP hydrolysis, which converts the ATP-bound, low affinity state into the ADP-bound high affinity state for substrate proteins (9, 17–19). This conversion is stimulated by DnaJ, which itself is also able to bind (partially) denatured proteins, avoiding their aggregation (10, 20, 21). Refolding of denatured proteins required both domains of DnaJ: the J domain, which stimulates the ATPase activity of DnaK (17, 22, 23), and the binding substrate domain (23, 24). Based on these observations, it was proposed that after the initial binding of the substrates to DnaJ, they were transferred to the peptide-binding site of DnaK (9–11), which was also proposed to be activated by DnaJ for binding client proteins (22, 25). More recently, a model has been put forward in which binding of DnaJ and ATP-DnaK to the same polypeptide chain leads to formation of ternary complexes. In these complexes, DnaJ, through a proximity effect, efficiently triggers the conversion of ATP-bound DnaK into the high affinity state that locks onto the substrate (7, 8). However, the question of how the components of the DnaK system interact with and assist in the reactivation of protein aggregates is not yet fully answered.

To address this question, we explore in this work the association of DnaK and DnaJ with aggregates of different client proteins and find that the co-chaperone mediates DnaK binding to the aggregate. DnaK is unable by its own to significantly bind to the aggregate surface, a “catalytically” productive chaperone concentration on the aggregate surface being only achieved in
DnaK-Protein Aggregate Interaction

the presence of DnaJ. In contrast, the co-chaperone binds the aggregate in a concentration-dependent manner, the association not requiring additional protein factors to occur. The use of a DnaK mutant in which specific ionic contacts at the substrate binding domain (SBD)\(^7\) (26) were disrupted reinforces the above interpretation. This mutant holds two substitutions (D450A/K548A) at the “latch” and shows a reduced affinity for DnaJ. As a consequence, it is recruited less efficiently by the aggregate-DnaJ complex, which in turn compromises its aggregate reactivation ability. Taken together, our data indicate that DnaK binding to protein aggregates, and therefore their reactivation, depends on DnaJ.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Mutant DnaK2A was generated as previously described (26). Wild type DnaK and the mutant were produced, purified, and extensively dialyzed to obtain nucleotide-free samples (27).

DnaJ and GrpE were expressed in BL21 cells and purified as described earlier (28, 29). ClpB was obtained as previously reported (30). Protein concentration was determined by the colorimetric Bradford assay (Bio-Rad).

Surface Plasmon Resonance Spectroscopy (SPR) Binding Assays—SPR measurements were performed on a BIACORE 3000 system (BIACORE, Uppsala, Sweden). Neutravidin was coupled to Research grade CM5 sensor chips using the EDC/NHS cross-linking reagent (Amersham Biosciences). DnaJ was biotinylated (NHS-LC-Biotin; Pierce) and coupled to the chips to ∼2200 resonance units. Excess neutravidin was blocked with ethanolamine. For binding studies, 40 μl of DnaK or the mutant (0.75–6 μM) was incubated with a 10-fold higher ATP concentration in 25 mM Hepes, 50 mM KCl, 10 mM MgCl\(_2\), 0.005% (v/v) Tween20, pH 7.6. Protein solution was injected at 30 °C in the above buffer over the chips at a flow rate of 20 μl min\(^{-1}\). Complete regeneration of the chip surfaces was achieved by two 10-μl injections of 1 M urea at a flow rate of 5 μl min\(^{-1}\) as described (31). This treatment did not affect the interaction between immobilized DnaJ and DnaK or DnaK2A, as verified for each experimental series where a DnaK wild-type control was run at the beginning and the end of the series to rule out any possible inactivation of immobilized DnaJ. In all cases studied here, these controls were virtually identical. Background binding to neutravidin was subtracted from each signal to account for nonspecific binding.

ATPase Activity—Steady-state ATPase activity was performed in 40 mM Hepes, pH 7.5, 50 mM KCl, 11 mM magnesium acetate buffer at 30 °C, as described previously (32). DnaK or DnaK2A and ATP concentrations were 3 μM and 1 mM, respectively, and DnaJ was added at varying concentrations. Reactions were followed measuring the absorbance decay at 340 nm for 30 min in a Cary spectrophotometer (Varian).

Refolding of Client Proteins—Luciferase (2.5 μM; Promega) was denatured for 45 min at 25 °C, in 6 M guanidinium hydrochloride, 100 mM Tris·HCl, pH 7.7, 10 mM DTT. Luciferase was diluted to 80 nM in 50 mM Tris·HCl, 55 mM KCl, 15 mM MgCl\(_2\), 5.5 mM DTT, 0.5 mg ml\(^{-1}\) bovine serum albumin, pH 7.7, containing an ATP-regenerating system (4 mM phosphoenolpyruvate and 20 ng ml\(^{-1}\) pyruvate kinase). The diluted sample was incubated 10 min, and afterward 1 μM DnaK or DnaK2A, 0.5 μM GrpE, and different DnaJ concentrations (0.1–10 μM) were added. Reactivation was initiated by the addition of 5 mM ATP. Luciferase activity was measured after a 90-min reactivation period at 25 °C, using the luciferase assay system (Promega E1500) in a Synergy HT (Biotek) luminometer.

Malate Dehydrogenase (MDH)—MDH (2 μM monomer; Sigma) was denatured and aggregated by incubating the protein for 30 min at 47 °C in 50 mM Tris·HCl, 150 mM KCl, 20 mM MgCl\(_2\), 2 mM DTT, pH 7.5. The sample was diluted (final MDH concentration 1 μM) in the presence of 1 μM DnaK or DnaK2A, 1.5 μM ClpB, 0.25 μM GrpE, and 0.05–10 μM DnaJ in the above buffer. Reactivation was started by the addition of 2 mM ATP and was measured at 30 °C in a medium containing the above ATP-regenerating system. MDH activity was recorded after 2 h of reactivation as previously described (33). Turbidity of luciferase and MDH aggregates was recorded under the same experimental conditions used in the refolding assays, on a SLM8100 spectrofluorimeter (Aminco) with both excitation and emission wavelengths set at 550 nm.

Glucose-6-phosphate Dehydrogenase (G6PDH)—G6PDH (2.5 μM; Sigma) was incubated for 15 min at 52 °C in 100 mM Tris·HCl, 150 mM KCl, 20 mM MgCl\(_2\), 10 mM DTT, pH 7.5, to denature and aggregate the protein. The sample was then diluted (final protein concentration 1 μM) in the same buffer containing 6.4 μM DnaK, 0.25 μM GrpE, 0.05–10 μM DnaJ. Reactivation was measured at 25 °C in the presence of the ATP-regenerating system, as previously described (34).

Chaperone Binding Assays—Association of chaperones with protein aggregates was characterized by SDS-PAGE analysis of the pellets obtained after centrifugation of the protein complexes (33). Luciferase (80 μM) in 6 M guanidinium hydrochloride was diluted 50 times the absence of chaperones and incubated for 10 min at 25 °C to allow protein aggregation. After the addition of 1 μM DnaK or DnaK2A and DnaJ (0.1–1 μM), the mixture containing 1 μM luciferase and 3 mM ATP was incubated for 10 min at 25 °C in refolding buffer and centrifuged in a Beckman Optima ultracentrifuge at 95,600 × g for 30 min at 4 °C. MDH was denatured as described above and mixed with 1 μM DnaK or DnaK2A, 1.5 μM ClpB, and 0.05–5 μM DnaJ. The sample was incubated for 10 min at 30 °C in refolding buffer containing 3 mM ATP and centrifuged (76,000 × g, 30 min, 4 °C) (33). The procedure used with G6PDH was the same as described above for its refolding but in the absence of GrpE. Aggregated G6PDH (1 μM), DnaK (6.4 μM), and DnaJ (0.05–10 μM) were incubated for 10 min at 25 °C in refolding buffer containing 3 mM ATP. Protein complexes were centrifuged at 76,000 × g to separate aggregate-bound from free chaperones. The resulting pellets and controls, containing known amounts of native proteins, were analyzed by SDS-PAGE (12.5%). Commercial ADP was further purified by ion exchange chromatography to remove contaminating ATP (35).
DnaK-Protein Aggregate Interaction

The amount of aggregate-bound proteins was quantitated by densitometry of the gel bands with a gel scanner G-800 and the Quantity One software from Bio-Rad. Each data point is an average of at least two experiments and was estimated by subtracting the amount of the corresponding protein in pellets of control experiments.

RESULTS

DnaJ Mediates DnaK Interaction with Protein Aggregates—It has been suggested that the DnaK system functions upstream of ClpB to reactivate protein aggregates. However, how the components of this system interact with protein aggregates remains as yet unknown. To address this question, the interaction of DnaK and DnaJ (the only components of the system that can bind client proteins) with aggregates of luciferase (Fig. 1A) and of MDH (Fig. 1B) has been analyzed. Experiments show that DnaK is unable on its own to significantly interact with aggregates of either protein substrates, but it binds in the presence of DnaJ and ATP. In contrast, the co-chaperone binds to the aggregates in the absence of DnaK, suggesting that the interaction of the chaperone with aggregate-bound DnaJ drives its association with protein aggregates. Since reactivation of MDH aggregates requires the combined action of the DnaK system and ClpB, we also tested whether ClpB had any influence on the way the DnaK system binds to aggregates. A similar analysis to that shown in Fig. 1B but carried out in the presence of ClpB indicates that the Hsp100 bacterial homolog does not modify the DnaJ-dependence of DnaK binding to protein aggregates (Fig. 1B). As expected for an interaction that depends on ATP to occur, DnaJ-mediated association of DnaK to protein aggregates is not observed for the ADP-bound or apo-forms of the chaperone (Fig. 1, C and D).

Two factors might affect the association of chaperones with protein aggregates, namely the time the protein substrate spends under denaturing and reactivation conditions. The first one (i.e. the time at denaturing temperatures) determines both the size of the aggregate and the conformational properties of the denatured protein molecules within the aggregate. These properties have been related to the ability of different combinations of chaperones to reactivate protein aggregates (36, 37) and might influence chaperone binding to the aggregate. Special care has been taken to denature and aggregate protein substrates under the same experimental conditions (protein concentration, temperature, and incubation time) to obtain samples that, after cooling or diluting them from denaturant-containing solutions, maintain homogeneous size and secondary structure (data not shown). Therefore, chaperones bind to stable protein aggregates that do not evolve with time. The second one (i.e. the effect of the incubation time on the association reaction) has been analyzed by performing binding experiments at different incubation times. The results (not shown) indicate that the system reaches a binding equilibrium within the first 10 min of incubation in the presence of ATP. It should be noted that less than 10% of the nucleotid is hydrolyzed during the initial incubation at 25 °C and subsequent centrifugation at 4 °C. Long incubation times might change the equilibrium as the amount of ATP hydrolyzed increases.

If the above finding holds for DnaK and DnaJ binding to different protein aggregates, the amount of aggregate-bound chaperone should be sensitive to co-chaperone concentration. To test this hypothesis, binding experiments at constant DnaK and protein aggregate concentrations and increasing DnaJ amounts were carried out. When this type of experiments is performed with aggregates of MDH (Fig. 2) and of G6PDH (Fig. 3), the amount of aggregate-bound DnaK increases with DnaJ concentration, indicating that the co-chaperone binds to the aggregate surface in a concentration-dependent manner and that association of DnaK depends on previous DnaJ binding. If DnaK could bind aggregates by itself, a constant aggregate-bound chaperone concentration should be observed, and this is clearly not the case. Data shown in Figs. 2 and 3 also reveal that: (i) the concentration of aggregate-associated DnaK increases...
This suggests that during the functional cycle, DnaK molecules higher than 1, in contrast to what is experimentally observed. only with DnaJ at the aggregate surface, this value could not be exponentially with co-chaperone concentration up to 1 μM DnaJ and slightly decreases at higher co-chaperone concentrations (Figs. 2B and 3B); (ii) in contrast to DnaK and DnaJ, the interaction of ClpB with MDH aggregates is not sensitive to co-chaperone concentration (Fig. 2B); and (iii) the reactivation yield of both client proteins shows the same DnaJ concentration dependence observed for DnaK binding to their aggregates (Figs. 2C and 3C). These data indicate that DnaK binds to the client protein aggregate once the co-chaperone is bound at the aggregate surface. The estimated aggregate-bound DnaK/DnaJ molar ratio increases at DnaJ concentrations below 0.05 μM and exponentially decreases above this co-chaperone concentration for both substrate proteins (Figs. 2C and 3C). If DnaK would interact only with DnaJ at the aggregate surface, this value could not be higher than 1, in contrast to what is experimentally observed. This suggests that during the functional cycle, DnaK molecules bound to the DnaJ-aggregate complex are transferred to the aggregate. The comparison of the reactivation yields and the estimated aggregate-bound DnaK/DnaJ molar ratios also indicates that the values that provide the highest reactivation yields are between 3 and 1 for MDH and between 4 and 2 for G6PDH. It should be noted that the percentage of aggregate-bound DnaK is at most 24 and 16% of the initially added protein for MDH and G6PDH, respectively.

A DnaK Mutant with Impaired Affinity for DnaJ Shows Defective Binding to Protein Aggregates and Reactivation Yields—To further demonstrate that the ability of DnaK to interact with and reanimate protein aggregates depends on DnaJ, the association of WT DnaK and DnaK2A (Fig. 4A) with DnaJ and protein aggregates has been characterized. This DnaK mutant has two amino acid replacements (D540A and K548A) located in the “latch” region that have a small effect on (i) protein conformation, (ii) peptide association kinetics, (iii) protein and peptide-protein thermal stability, and (iv) interdomain communication. However, DnaK2A was unable to refold luciferase (26).
Interaction with DnaJ—DnaJ stimulates DnaK ATPase activity in a concentration-dependent manner (9). Therefore, to estimate the affinity of WT DnaK and DnaK2A for DnaJ, we have followed the stimulation of their ATPase activity at increasing co-chaperone concentrations (Fig. 4B). Maximum ATPase activity enhancement was achieved at a DnaJ/Hsp70 molar ratio of 0.5 for WT DnaK and 1 for DnaK2A. These data suggest a decrease in the affinity of DnaK2A for DnaJ.

As an additional method to estimate the affinity of WT DnaK and the mutant for DnaJ, we have used SPR. WT DnaK is able to bind immobilized DnaJ in the presence of ATP, an interaction that can be followed as an increase in resonance units by SPR (Fig. 4C) (34, 38). This signal has been shown to represent a functionally relevant interaction between both proteins involving the HPD motif. As previously described (38), the association and dissociation phases were fit independently to a first order rate kinetics and a single exponential decay, respectively. This fitting procedure explains reasonably well the complex interaction between DnaK and immobilized DnaJ. The same experiment was carried out with DnaK2A to confirm its lower affinity for DnaJ suggested by ATPase activity measurements. As seen in Fig. 4C, under the same experimental conditions, the increase in resonance units for the mutant is around 30% of that obtained for WT DnaK. This might be caused by a decreased affinity for immobilized DnaJ. The equilibrium dissociation constants, as well as the apparent association and dissociation rate constants, were derived from kinetic measurements at different chaperone concentrations (Fig. 4D and Table 1). As compared with WT DnaK that shows a $K_d$ similar to previously reported values (9, 38), the affinity of DnaK2A for DnaJ is decreased as revealed by a 3-fold increase of its $K_d$ values. The lower affinity of the mutant for DnaJ is mainly due to a decrease (3.3-fold) of the association rate constant for DnaK2A (Table 1). Therefore, our data indicate that the interactions that helix B establishes with the $\beta$ subdomain at the latch modulate the affinity of DnaK for immobilized DnaJ. It should also be mentioned that the good agreement between ATPase activity and SPR measurements indicates that the effect of DnaJ immobilization on the interaction is, if at all real, a subtle one.

Reactivation of Protein Aggregates—As DnaJ mediates the interaction of DnaK with protein aggregates, the lower affinity of DnaK2A for DnaJ should recruit fewer chaperone molecules

![FIGURE 4. A, schematic diagram of the SBD of DnaK. The ionic contacts disrupted by the mutations and the bound substrate are indicated. B, stimulation of the ATPase activity of WT DnaK and DnaK2A by DnaJ. The steady-state ATPase activity of WT DnaK (filled circles) and DnaK2A (squares) was measured at 30 °C at increasing DnaJ concentrations (0.05–4 μM), as described under “Experimental Procedures.” C, interaction of DnaK and DnaK2A with DnaJ monitored by SPR. Experimental interaction curve of WT DnaK (solid line) and DnaK2A (broken line) with DnaJ. The interaction between Hsp70 (4 μM) and the co-chaperone was analyzed in the presence of 40 μM ATP in the running buffer. D, analysis of the interaction with DnaJ. Apparent association constants ($K_{a\text{obs}}$) for WT DnaK (filled circles) and DnaK2A (squares) as a function of Hsp70 concentration. Kinetic constants $k_{+1}$ and $k_{-1}$, shown in Table 1).]

### TABLE 1

|          | $k_{+1}$ $\times 10^6$ M$^{-1}$s$^{-1}$ | $k_{-1}$ s$^{-1}$ | $K_d$ μM |
|----------|---------------------------------------|-------------------|-----------|
| DnaK     | 1.95 ± 0.2                            | 0.032 ± 0.004     | 1.52 ± 0.09 |
| DnaK2A   | 0.619 ± 0.025                         | 0.029 ± 0.001     | 4.6 ± 0.33  |
at the aggregate interface, which in turn would cause a decrease in the reactivation yield. To explore this possibility, we have analyzed the ability of WT DnaK and DnaK2A to interact with and reanimate protein aggregates of luciferase (Fig. 5) and MDH (Fig. 6). Using luciferase as denatured substrate, the electrophoretic analysis indicates that the amount of chaperone bound to the aggregate increases in parallel with the concentration of aggregate-bound DnaJ and with the affinity of the co-chaperone for DnaK or DnaK2A (Fig. 5A). As expected from the 3-fold reduction of the affinity of DnaK2A for DnaJ, as compared with WT DnaK, the densitometric estimation shows that less mutant protein binds to the aggregate-DnaJ complex at substoichiometric co-chaperone concentrations (K/J molar ratio 1:0.1; Fig. 5B). Binding data are perfectly paralleled by aggregate reactivation yields (Fig. 5C). In contrast to WT DnaK, DnaK2A was not able to significantly reanimate luciferase aggregates when mixed with substoichiometric amounts of DnaJ, as we found previously (26), due to the low number of DnaK2A molecules bound to the aggregate. However, when the co-chaperone is added at stoichiometric amounts, the lower affinity of the mutant for DnaJ is compensated, and the amount of aggregate-bound DnaK2A increases 4 times (Fig. 5B), thus becoming as active as WT DnaK in protein reactivation (Fig. 5C).

Renaturation of protein aggregates involves two sequential steps, an initial solubilization of the aggregate with extraction of protein monomers or small size aggregates that are refolded in the second step. To take into account the possibility that DnaK2A could solubilize large protein aggregates but not refold the extracted protein molecules at the lowest K/J molar ratio assayed here, the ability of this mutant to solubilize luciferase aggregates was investigated at both K/J molar ratios (1:0.1 and 1:1). Extraction of protein monomers or small size aggregates by the DnaK system results in a continuous decrease of the sample turbidity, as experimentally detected for WT DnaK (Fig. 5D). The same DnaJ concentration dependence is observed for the decrease in aggregate turbidity and the increase in enzyme activity (Fig. 5C), indicating that, as described for other protein substrates (33), disaggregation and refolding reactions are tightly coupled. DnaK2A is unable to solubilize luciferase aggregates at the lowest DnaJ concentration, indicating that a decreased affinity of DnaK2A for DnaJ is involved in the failure of this mutant to productively reactivate large protein aggregates.

When the same experiments are carried out using MDH as denatured and aggregated substrate, similar results are obtained (Fig. 6). The only significant difference is that the amount of mutant bound to the aggregate surface is around 0.1 fold less than that found for the WT protein at a K/J molar ratio 1:0.1 (Fig. 6A and B). An increase in the molar ratio to 1:1 doubles the amount of aggregate-bound mutant without varying much that of WT DnaK. As observed with luciferase, the reactivation yields (Fig. 6C) agree well with binding data, indicating that the affinity of DnaK for DnaJ drives chaperone binding to the protein aggregate, which in turn triggers extraction (Fig. 6D) and refolding of substrate protein molecules.

The functionality of the mutant was tested by complementation experiments in the E. coli temperature-sensitive ΔdnaK strain BB1553 (39). As compared with WT DnaK, at 25 μM...

**FIGURE 5. Interaction of WT DnaK and DnaK2A with luciferase aggregates.** A, SDS-PAGE of aggregate-bound DnaK and DnaJ. DnaK or DnaK2A (1 μM) and aggregated or native luciferase (1 μM) were mixed with different DnaJ concentrations (0.1 and 1 μM) in the presence of 3 mM ATP. After incubating the samples for 10 min at 25 °C, they were centrifuged, and the resulting pellets were analyzed by electrophoresis. The lane marked with an asterisk contains 20% (DnaK), 10% (DnaJ; 1 μM), and 20% (luciferase) of the initially added protein. B, estimation of the relative amount of WT DnaK and DnaK2A bound to luciferase aggregates. C, reactivation of luciferase aggregates (80 nM final concentration) by WT DnaK and DnaK2A (1 μM), GrpE (0.5 μM), and different DnaJ concentrations (0.1 and 1 μM). D, changes in the turbidity of luciferase aggregates (80 nM) in the absence of chaperones (solid line), and after the addition of 1 μM WT DnaK (filled symbols) or DnaK2A (open symbols). Besides Hsp70, the samples also contain GrpE (0.5 μM) and DnaJ (0.1 μM (circles) or 1 μM (triangles)).
isopropyl 1-thio-D-galactopyranoside, mutant DnaK2A shows a severe growth defect at 40 °C (Fig. 7A). However, DnaK2A can stably bind peptide substrates in the 25–42 °C temperature range (26), and its expression level is similar to that of WT DnaK (Fig. 7C). Lack of complementation could be explained by the reduced affinity of DnaK2A for DnaJ that might decrease the efficiency of the (re)folding process, especially the reactivation of protein aggregates formed under prolonged stress conditions. If this were the case, the mutant should be able to complement growth of an E. coli temperature-sensitive dnaK strain that overexpresses DnaJ. DnaK2A supports growth of strain CG800, which produces around 50 times more DnaJ than BB1553 (40), at 43 °C (Fig. 7B), indicating that what we have described above for in vitro conditions might also apply to the in vivo situation.

DISCUSSION

Hsp40 proteins have been involved in (i) the regulation of the Hsp70 folding cycle, (ii) binding of client proteins through specific protein domains, and (iii) formation of unique pairs with Hsp70 proteins that facilitate certain processes at specific locations within the cell (41–43). In the particular case of the bacterial system, it has been proposed that DnaJ, together with GrpE, regulates the timing of the ATPase cycle (i.e. the time DnaK spends in the high affinity ADP state and in the low affin-
ity ATP state) (5). Two other functional properties, based on its ability to bind client proteins, have been assigned to DnaJ: preventing substrate protein aggregation and transferring client polypeptides to DnaK for productive folding (21, 44). Substrate protein-DnaJ interaction, which has been demonstrated in vitro for luciferase (44), has been suggested as an important initial step in the model of the chaperone cycle. Our findings add another important function to those listed above for DnaJ, namely mediating association of DnaK to protein aggregates under conditions when the chaperone itself cannot bind. This observation is the first experimental evidence of an aggregate-bound DnaJ-mediated recruiting of low affinity ATP-DnaK molecules.

The lower affinity of DnaK2A for DnaJ has allowed us to explore in more detail the requirements of the DnaK system to reactivate protein aggregates. Maximum refolding yields parallel the relative affinity of DnaK and DnaK2A for DnaJ, as expected from the known requirement of DnaJ for productive refolding. The lower affinity of DnaK2A for DnaJ has to be compensated for by increasing the co-chaperone concentration. This finding led us to propose a working hypothesis in which (i) DnaK loses the ability to interact with protein aggregates and (ii) chaperone binding, and therefore the number of aggregate-bound DnaK molecules, is controlled by its affinity for DnaJ that can directly interact with the aggregates (Fig. 8). Below a certain aggregate-bound DnaK2A concentration, the chaperone system is not able to reanimate large protein aggregates. The values of DnaK molecules bound to one denatured substrate protein molecule plotted in Figs. 2 and 3, which are $\leq 1$, should be discussed bearing in mind that only a small fraction of denatured protein molecules in the aggregate are accessible to the chaperone, and thus that their number could be significantly larger at the aggregate surface. This interpretation would be in agreement with the early proposal that several chaperone molecules can bind to the same misfolded polypeptide substrate (34).

Our data also show that maximum reactivation yields require a fine balance of aggregate-bound DnaK and DnaJ, most likely reflecting the need to couple both protein activities to increase the efficiency of the reactivation process. The fact that the estimated aggregate-bound DnaK/DnaJ molar ratio is $>1$ (Figs. 2C and 3C) also suggests that one DnaJ molecule can transfer several DnaK molecules to the aggregate surface, where it could interact with denatured substrate. Above 0.05 $\mu M$ co-chaperone, the estimated aggregate-bound DnaK/DnaJ molar ratio exponentially decreases. This dependence suggests that DnaK and DnaJ could compete for common substrate binding sites, so that increasing DnaJ concentrations inhibit DnaK binding to the aggregate surface. Indeed, it has been shown that DnaJ competes with DnaK for a peptide with a single and common binding motif (7). Although DnaJ shares most binding motifs with DnaK (45), they also display some differences in their substrate-binding properties. DnaK interacts with both the backbone and side chains of a peptide substrate (46, 47), thus admitting only 1- and 2-peptides, and DnaJ is believed to interact with only the side chains of the substrate, since it binds both 1- and D-peptides (45, 48). Therefore, it is possible that both DnaK and DnaJ can interact with the same misfolded protein molecules that usually contain several binding sites for both DnaK and DnaJ. Formation of ternary complex (DnaJ-substrate-DnaK) has been previously

![Diagram](attachment:3)
The structure and dynamics of the interdomain interface are modulated by hydrophobic and electrostatic interactions between residues at helices A and B and amino acids at the terminal region, of the J domain of auxilin, and on binding data, a small conformational (56), and biophysical (57) studies that DnaK has a binding site for Hsp70. Based on the x-ray structure of a bovine Hsc70 deletion mutant that lacks the 10-kDa C-terminal region, of the J domain of auxilin, and on binding data, a model for the interaction between these proteins has been put forward (58, 59). This model places the J domain of auxilin near the interdomain interface, where it could simultaneously interact with both the nucleotide binding domain and SBD of Hsp70. The structure and dynamics of the interdomain interface are modulated by hydrophobic and electrostatic interactions between residues at helices A and B and amino acids at the nucleotide binding domain and the β-sandwich domain, some of which form the latch (26, 54). In this context, our data point to the latch as an important structural region responsible for the fine tuning of DnaK affinity for DnaJ. Ionic contacts between helix B and the β-sandwich modulate lid dynamics (26) and, as we demonstrate here, also the interaction of DnaK with DnaJ.

Our findings might have physiological implications, since the concentration of DnaJ has been estimated to be ~30–40 times lower than that of DnaK (60). Under these conditions, DnaK2A would be defective as an aggregate-removing agent, since it would need a higher DnaJ concentration to properly perform this function, as shown in the complementation assays. In the proposed scenario of an unfolded polypeptide entering the chaperone folding cycle through its interaction with either ATP-ligated DnaK or with DnaJ (19), our findings identify DnaJ as the pivotal protagonist in the association of the DnaK system with protein aggregates during their chaperone-mediated reactivation (Fig. 8).

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DnaK-Protein Aggregate Interaction

demonstrated for rhodanase and a chimeric protein containing binding sites for both chaperones (7, 8). The requirement of DnaJ and ATP for chaperone binding to protein aggregates could be due to the conformation that the misfolded protein adopts at the aggregate surface (37), where the binding sites might not be accessible to DnaK alone. Formation of ternary complexes would favor the interaction between DnaJ and DnaK by an entropic proximity effect, resulting in the stimulation of the ATP hydrolysis (7, 8). Nucleotide hydrolysis by this complex could also play a role in destabilizing the intermolecular hydrophobic interactions that stabilize the aggregate, thus allowing the substrate molecule to be locally unfolded with the concomitant exposure of chaperone binding sites that will be locked in the DnaK-DnaJ complex for further processing. The good correspondence between the DnaK/DnaJ molar ratios that give maximum reactivation yields, between 4 and 1 (Figs. 2 and 3), and the ATPase activation values, around 2 (Fig. 4), supports the above interpretation. However, we cannot rule out a possible DnaJ-induced substrate rearrangement that could facilitate DnaK binding to the aggregate, although neither the size nor the secondary structure, as seen by CD spectroscopy, of the denatured substrate molecules is significantly modified in the presence of DnaJ (data not shown). In this context, our data lend experimental support to the recently proposed mechanism of chaperone-mediated unfolding of stable protein aggregates, known as entropic pulling (49). Hsp70 molecules would convert the energy of ATP hydrolysis into a force capable of accelerating the local unfolding, solubilization, and assisted native refolding of stable protein aggregates.

A second finding in this work is related to the role of the latch in modulating the affinity of DnaK for DnaJ. We recently reported that mutations affecting the latch in DnaK2A do not modify the peptide binding ability of the protein (accessibility and thermal stability of the complex) within the 25–42 °C temperature range, although they compromise its reactivating activity at any temperature (26). Substrate association and dissociation rate constants for DnaK2A are around 2-fold higher than those of WT DnaK, without changing the affinity for the peptide (26), in agreement with the values reported for similar full-length Hsc70 mutants (50, 51). However, the increase is significantly higher (~100-fold) for lidless variants of DnaK (52, 53) and for the single point mutant DnaK1A (26), suggesting that interactions other than those forming the latch are more effective in controlling the accessibility of the peptide binding site. Data presented herein indicate that the latch seems to be more important in defining the affinity of DnaK for DnaJ than for peptide substrates, as previously suggested. The interpretation of these data is not straightforward, since DnaK-DnaJ complex formation is a highly complex process that requires the ATPase and the substrate binding domain of DnaK, interdomain communication, and the coupled events of ATP hydrolysis and locking in of substrate in the binding cavity of DnaK (31). The observed decrease (3.3-fold) in the association constant might be due to a defective interaction of DnaJ with the SBD of DnaK2A, which holds both amino acid replacements. The alternative of a defective interdomain communication and/or interaction with the ATPase domain is less likely, since allosteric communication between both DnaK2A domains is WT-like, as seen by proteolysis (27) and differential infrared spectroscopy (54) (data not shown), and the ATPase domain remains unaltered in the mutant.

The contact sites between any J-protein and Hsp70 are currently unknown, although a region of Hsp70 has been involved in J-domain interactions in E. coli and Saccharomyces cerevisiae (55). It has been proposed on the basis of genetic (56), biochemical (56), and biophysical (57) studies that DnaK has a binding surface for DnaJ on the underside cleft of its ATPase domain, therefore explaining the DnaJ-mediated stimulation of ATP hydrolysis by DnaK. Alternatively, DnaJ could interact with two different binding sites in DnaK, one in the nucleotide binding domain and the other in the SBD. Based on the x-ray structure of a bovine Hsc70 deletion mutant that lacks the 10-kDa C-terminal region, of the J domain of auxilin, and on binding data, a model for the interaction between these proteins has been put forward (58, 59). This model places the J domain of auxilin near the interdomain interface, where it could simultaneously interact with both the nucleotide binding domain and SBD of Hsp70. To date, it is not known whether the interaction of DnaK and Hsp70 involves the same J-protein (51).
DnaK-Protein Aggregate Interaction

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