The Escherichia coli DjlA and CbpA Proteins Can Substitute for DnaJ in DnaK-Mediated Protein Disaggregation

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The DnaJ (Hsp40) protein of Escherichia coli serves as a cochaperone of DnaK (Hsp70), whose activity is involved in protein folding, protein targeting for degradation, and rescue of proteins from aggregates. Two other E. coli proteins, CbpA and DjlA, which exhibit homology with DnaJ, are known to interact with DnaK and to stimulate its chaperone activity. Although it has been shown that in dnaJ mutants both CbpA and DjlA are essential for growth at temperatures above 37°C, their in vivo role is poorly understood. Here we show that in a dnaJ mutant both CbpA and DjlA are required for efficient protein dissaggregation at 42°C.

Protein aggregation, which has been shown to occur in all cells, may be lethal, as exemplified by the various amyloid diseases, such as type II diabetes, neurodegenerative diseases, and prion diseases (11, 17, 20). As a consequence, mechanisms have evolved to prevent protein aggregation and to solubilize aggregates once they are formed. These mechanisms rely on the activity of various proteases and chaperones.

Members of the Hsp70 (DnaK) protein family are chaperones which assist the correct folding of newly synthesized proteins, refolding of misfolded proteins, protein translocation, disaggregation, and targeting of proteins for degradation (reviewed in reference 2). The folding activity of Hsp70 proteins is energy dependent and is carried out through repeated cycles of ATP hydrolysis. As the intrinsic ATPase activity of Hsp70 proteins is too slow to support in vivo folding of proteins, the aid of cochaperones which stimulate both ATP hydrolysis and ADP-ATP exchange is required (10, 19, 27, 44). The stimulation of ATP hydrolysis is carried out mainly by members of the Hsp40 (DnaJ) family that share a universally conserved domain (J-domain) which is essential for the interaction with the corresponding Hsp70 partner (24, 28, 36, 37). Apart from the conserved J-domain, the rest of the protein primary sequence shows a high degree of diversity among members of the Hsp40 family, which may account for the differences in substrate binding (33) and cellular localization in eukaryotic and prokaryotic cells.

The DnaK protein of Escherichia coli is the most extensively characterized Hsp70 family member, and its activity is important for bacterial viability (2, 31). Null mutations in the dnaK gene result in temperature sensitivity at temperatures below 16°C and above 37°C and in defects in protein folding and disaggregation following heat shock (15, 23, 29, 39). DnaJ, CbpA, and DjlA are three E. coli Hsp40 family members with homologous J-domains which function as cochaperones of DnaK (13, 28, 42). Of these three proteins, DnaJ has been characterized best and has been shown to be the key regulator of the various DnaK activities (21, 24). DnaJ contains four domains: an N-terminal J-domain, a glycine-phenylalanine domain whose function is largely unknown, a zinc finger domain, and a C-terminal domain, which is presumably important for substrate specificity (1, 33, 38). E. coli dnaJ null mutants show temperature sensitivity at temperatures above 42°C (34), loss of motility (35), and a failure to replicate the genomes of bacteriophages λ and P1 and the mini-F plasmid (22, 45). The DnaJ homologues CbpA and DjlA have not been extensively characterized.

CbpA (curved DNA binding protein A) is a 33-kDa protein that was first discovered based on its ability to bind a synthetic curved DNA molecule (40). This protein was subsequently shown to act as a multicopy suppressor for dnaJ mutations (40). Deletion of CbpA results in no detectable phenotype, but it does result in synthetic lethality in a dnaJ mutant background at temperatures above 37°C and below 16°C (12, 41). DjlA is a 30-kDa type III membrane protein. Its N-terminal domain is anchored to the inner membrane, while the rest of the molecule, including its J-domain, faces the cytoplasm (5). DjlA was shown to be required, together with DnaK, for induction of the colonic acid capsule and to be able to replace DnaJ in vitro as a cochaperone for DnaK in refolding of heat-denatured luciferase (13). Although a djlA mutant and a djlA-cbpA double mutant exhibit no characteristic growth phenotypes, a dnaJ-djlA double mutant is temperature sensitive and grows poorly at temperatures above 39°C (12). The growth phenotype of a dnaJ-cbpA-djlA triple mutant is the most acute and resembles that of a dnaJ mutant. These growth phenotypes suggest that CbpA and DjlA may replace DnaJ under certain conditions.

Following heat shock, protein aggregates transiently accumulate in E. coli and then disappear due to the activity of various quality control systems, which consist mainly of chaperones and proteases (16, 18, 29, 39, 43). It is known that the DnaK chaperone machinery plays a major role in preventing the formation of protein aggregates and, once they are formed, accumulation of these aggregates. Thus, following heat shock,
aggregation of proteins in dnaK mutants is more pronounced than it is in the wild type (23, 29, 39). Furthermore, in a dnaK mutant the aggregates are not dissolved over time. We used an in vivo protein aggregation system to study the specific involvement of the various DnaJ proteins in this process. This system makes it possible to simultaneously examine the aggregated state of a large number of cellular proteins. The results presented here indicate that CbpA and DjlA can replace DnaJ in solubilization of aggregates. Efficient disaggregation in the absence of DnaJ requires both proteins.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are shown in Table 1.

Growth conditions. Bacterial cultures were grown with aeration in salt-glucose minimal medium as described by Davis and Mingioli (7) at 32°C until the mid-log phase. Protein aggregates were purified following transfer to 42°C. For radioactive labeling the cultures were grown in the same medium but in the presence of [35S]methionine (10 μCi/ml; 1,000 Ci/mmol; Amersham Pharmacia Biotech).

Purification of aggregated proteins. Aggregated proteins were purified basically as described previously (32, 39). Briefly, 20-ml samples were collected from the cultures at different times and immediately centrifuged at 7,600 × g and 4°C. The cell pellets were washed twice with cold TE-PMSF (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 243 mg of phenylmethylsulfonyl fluoride [PMSF] per liter) and frozen at −70°C in 2-ml Microfuge tubes. After addition of 0.5 ml of cold TE-PMSF, cells were lysed by sonication and centrifuged for 30 min at 23,000 × g and 4°C. Then 0.5 ml of cold TE-PMSF was added to the pellets, and the sonication and centrifugation steps were again performed as described above. The pellets were resuspended by sonication in 0.5 ml of cold TE-PMSF and centrifuged for 15 min at 2,000 × g and 4°C in order to pellet intact cells. Then 450-μl portions of the aggregate-containing supernatants were transferred to new 2-ml microcentrifuge tubes and centrifuged for 30 min at 20,000 × g. To lower the concentration of membrane proteins, the pellets were resuspended by sonication in 0.5 ml of cold TE-PMSF containing NP-40 (4:1) and centrifuged for 30 min at 20,000 × g and 4°C. This step was repeated once, and the pelletted aggregates were resuspended in 50 μl of distilled water.

For two-dimensional polyacrylamide gel electrophoresis (PAGE) analysis aggregated proteins were purified from 100-ml cultures.

One-dimensional SDS-PAGE. Gel electrophoresis was carried out by previously described protocols (26) by performing sodium dodecyl sulfate (SDS)—10% PAGE. The gels were silver stained and scanned with an ImageScanner (Amersham Pharmacia Biotech). To quantify DnaK levels, the gels were analyzed by Western blotting by using mouse anti-DnaK monoclonal antibodies (Stressgen) and densitometry.

Two-dimensional SDS-PAGE. For isoelectric focusing, the aggregates were dissolved for 3 h in rehydration solution, which contained 8 M urea, 2 M thiourea, 1% (wt/vol) 3-[3-cholamidopropyl]-dimethylammonio-1-propanesulfonate (CHAP), 20 mM dithiothreitol (DTT), and 0.5% (vol/vol) Pharmalyte 3-10. Fractions corresponding to identical cell numbers were loaded on IPGs (pH 4 to 7; Amersham Pharmacia Biotech) and rehydrated for 24 h.

The isoelectric focusing was performed with a Multiphor II unit (Amersham Pharmacia Biotech) by employing the following voltage profile: linear increase from 0 to 100 V for 100 V-h, constant potential of 100 V for 500 V-h, linear increase from 100 to 500 V for 2,400 V-h, constant potential of 500 V for 2,500 V-h, linear increase from 500 to 3,000 V for 10,000 V-h, and finally constant potential of 3,500 V for 35,000 V-h.

After consecutive equilibration of the gels in solutions containing DTT and iodoacetamide as previously described (14), separation in the second dimension was done in polyacrylamide gels of 12.5% T and 2.6% C with the Investigator 2-D electrophoresis system (Genomic Solutions) at approximately 4 W per gel. The gels were stained with Coomassie blue reagent and scanned with an ImageScanner (Amersham Pharmacia Biotech).

Disaggregation in cell extracts. The disaggregation procedure was carried out as described by Mogk et al. (29). Purification of proteins for in vitro studies. (i) DnaJ, CbpA, and DjlA ΔTMR The genes encoding DnaJ, CbpA, and DjlA ΔTMR were cloned into plasmid pET11a (the N-terminal transmembrane domain of DjlA was deleted) and transformed into E. coli K-12 strain ER2566 from which these three genes had been deleted (ER2566-1) (Table 1). A fresh colony was used to inoculate 1.6 liters of 2× YT medium containing ampicillin (100 mg/liter) at 30°C, and the culture was grown with aeration to a concentration of about 3 × 108 cells ml−1 before induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 5 h. The cells were pelleted by centrifugation, frozen, and resuspended in 15 ml of 25 mM triethanolamine (TEA) (pH 8.3) containing lysosyme (0.2 mg/ml) and a protease inhibitor cocktail (Sigma). All subsequent steps were performed at 4°C. After the solution was stirred for 30 min, the cells were lysed by passage through a French press, and cell debris were removed by centrifugation at 23,000 × g for 30 min. Streptomycin sulfate (final concentration, 1% [wt/vol]) was added to the supernatant, and the solution was stirred for 1 h. After centrifugation at 23,000 × g for 30 min, the proteins (DnaJ ΔTMR, CbpA, or DjlA) were found exclusively in the pellet. The pellet was resuspended by stirring it in a solution containing 50 mM Tris-HCl (pH 8.0), 2 M urea (4 M urea for DjlA ΔTMR), 5 mM DTT, and 0.1% Triton X-100. After centrifugation (23,000 × g, 30 min) the supernatant, which contained the proteins, was concentrated to a volume of approximately 1 ml either by addition of ammonium sulfate (0.28 g/ml) for DnaJ or by the use of an Amicon membrane (YM-10) for DjlA and CbpA. The concentrated solution was filtered and loaded onto a HiLoad 16/60 Superdex 200 column (Amersham Pharmacia Biotech). The protein was eluted at a rate of 1 ml/min with a solution containing 40 mM potassium phosphate (pH 7.0), 200 mM KCl, 0.1 mM EDTA, and 5 mM β-mercaptoethanol (for CbpA and DjlA ΔTMR) and 2 M urea (only for DnaJ). The fractions which yielded a single band on an SDS-PAGE gel with Coomassie blue staining were concentrated and frozen at −70°C after addition of glycerol to a final concentration of 20% (vol/vol). For DnaJ, dialysis against a solution containing 40 mM potassium phosphate (pH 7.0), 200 mM KCl, 0.1 mM EDTA, and 5 mM β-mercaptoethanol was performed before the addition of glycerol.

(ii) DnaK. The dnaK gene was cloned into plasmid pET11a and transformed into the ER2566 strain. A fresh colony was used to inoculate 2.4 liters of 2× YT medium containing ampicillin (100 mg/liter) at 30°C, and the culture was grown with aeration to a concentration of about 3 × 1010 cells ml−1 before induction with 0.5 mM IPTG for 3 h. The cells were pelleted by centrifugation, frozen, and resuspended in 35 ml of 25 mM TEA (pH 7.6) containing lysosyme (0.2 mg/ml), a protease inhibitor cocktail (Sigma), and 5 mM β-mercaptoethanol. All subsequent steps were performed at 4°C. After the solution was stirred for 30 min, the cells were lysed by passage through a French press, and cell debris was removed by centrifugation at 23,000 × g for 30 min. Nucleic acids were precipitated by

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### Table 1. Strains used

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<td>ER2566</td>
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RESULTS

Aggregated proteins are solubilized in a dnaJ mutant. It has been shown previously that the activity of DnaK is crucial for the prevention of intracellular protein aggregation and resolubilization of formed aggregates (23, 29, 39). We examined whether this is also the case for DnaJ, since various studies have shown that this protein is the major cochaperone of DnaK. Protein aggregates were purified from bacterial cultures of a dnaJ mutant, a dnaK mutant, and a wild-type strain at various times following a shift from 32 to 42°C.

Following 15 min of incubation at 42°C, protein aggregates were detected in the wild-type strain (Fig. 1A). The presence of aggregates was only transient as their level dramatically decreased following further incubation at 42°C. In a dnaK mutant, the aggregates accumulated to a higher level than they accumulated in the wild type, and they were not resolved with time. These results are consistent with previously described data (29, 39). Following a 15-min incubation at the higher temperature, the level of aggregated proteins in the dnaJ mutant was significantly higher than the level in the wild type and comparable to the level in the dnaK mutant. However, in contrast to the dnaK mutant, the level of aggregates was significantly reduced after 45 min of incubation at 42°C (Fig. 1A). This result was unexpected, as DnaJ is thought to modulate most DnaK activities in vivo.

In order to quantitatively compare the disaggregation process in the wild type and mutants, the experiment described above was performed in a culture medium containing [35S]methionine. Aggregates were purified, and the radioactivity was determined for each time. The results indicate that there was no significant disaggregation of proteins in the dnaK mutant at 45 min (Fig. 1B). In contrast, the protein disaggregation in the dnaJ mutant resembled that in the wild-type strain. Thus, the efficient solubilization of aggregates in the absence of DnaJ suggests that the function of DnaJ may be assumed by other intracellular factors.

Protein disaggregation is not perturbed in cbpA or djlA single mutants. To determine if the DnaJ homologues CbpA and DjlA are essential for protein disaggregation in vivo, we studied protein aggregation in mutants lacking one of these proteins. Protein aggregates were purified from dnaJ, dnaK, or djlA single mutants at 0, 15, and 45 min following a shift to 42°C. No effect on protein disaggregation could be detected in a cbpA mutant or in a djlA mutant (Fig. 2). Furthermore, the level of protein aggregation 15 min following the shift to 42°C in either of these mutants was much lower than that in the dnaJ mutant, indicating that prevention of protein aggregation in these mutants was also unaffected.

Both CbpA and DjlA are required for disaggregation in a dnaJ mutant. Since neither a cbpA mutation nor a djlA mutation could abolish in vivo disaggregation, we examined the possibility that a lack of one of the DnaJ homologues is compensated for by the presence of the others. To do this, disaggregation results were compared for all three double mutants (dnaJ-cbpA, dnaJ-djlA, and cbpA-djlA) and for the dnaJ-cbpA-djlA triple mutant. These results (Fig. 3) clearly indicated that when the dnaJ mutation is coupled to a mutation in one of the homologues (dnaJ-djlA and dnaJ-cbpA), the cells are unable to resolve aggregated proteins at 42°C, and the phenotype closely

addition of 1% (wt/vol) streptomycin sulfate to the supernatant, and the solution was stirred for 1 h before the nucleic acids were pelleted by centrifugation at 23,000 × g for 30 min. The nucleic-acid-free supernatant was dialyzed twice against 8 liters of 25 mM TEA (pH 7.6) for 1 h. The precipitate which formed during dialysis was removed by centrifugation at 23,000 × g for 30 min, and the clear supernatant was loaded onto a 20-ml HiPrep 16/10 Q Sepharose FF column (Amersham Pharmacia Biotech) that was previously equilibrated with 25 mM TEA (pH 7.8). Proteins were eluted at a rate of 5 ml/min with a 200-ml linear 0 to 1 M NaCl gradient. Fractions rich in DnaK (as determined by SDS-PAGE) were pooled, concentrated (YM-30 membrane; Amicon), and loaded onto a HiLoad 16/60 Superdex 200 column (Amersham Pharmacia Biotech). The protein was eluted at a rate of 1 ml/min in 40 mM potassium phosphate (pH 7.0)–200 mM KC1–0.1 mM EDTA–5 mM β-mercaptoethanol. Fractions which yielded a single band on an SDS-PAGE gel following Coomassie blue staining were concentrated and stored at −80°C after addition of glycerol to a final concentration of 20% (v/v).

(ii) ClpB and GrpE. ClpB was purified as described previously (9). GrpE was purchased from Stressgen (catalog no. SPP-650).

FIG. 1. Protein aggregation in a dnaJ mutant. (A) Cultures of E. coli MC4100 and various dnaJ and dnaK mutant derivatives were shifted to 42°C, and aggregated proteins were purified at 0, 15, and 45 min. Absorbance (optical density at 600 nm) was measured for every sample, and fractions corresponding to identical numbers of cells were separated by SDS—10% PAGE, followed by silver staining. (B) Cultures of wild-type strain MC4100 (W.T.) and dnaJ and dnaK mutants were radiolabeled with [35S]methionine. Samples were removed at 0, 15, and 45 min after a shift to 42°C, and aggregated proteins were purified. The radioactive counts at zero time were subtracted, and the samples were normalized so that the 15-min level was defined as 1.0.
suggest that in *E. coli* nor CbpA alone is sufficient for efficient resolubilization of protein aggregates.

**DnaK levels in the dnaJ, djlA, and cbpA mutants.** DnaJ enhances the ATPase activity of DnaK, the major factor in the disaggregation process. It was therefore important to determine the level of DnaK in the various mutants to ensure that the observed effects of the *dnaJ* mutations on disaggregation were not simply due to changes in the intracellular DnaK levels. Moreover, as the *dnaK* gene is located upstream of the *dnaJ* gene, it was important to determine that its expression was not affected by the downstream *dnaJ* disruption mutation. The results presented in Fig. 4 indicate that the levels of DnaK in the mutants defective in disaggregation were higher than the levels in the wild type. The highest DnaK levels observed were those in the triple mutant; these levels were three- and twofold higher than the levels in the wild type after 15 and 45 min of incubation at 42°C, respectively. Therefore, the defective disaggregation process observed in the double and triple mutants was not due to reduced DnaK levels.

**Protein disaggregation in the dnaJ-djlA-cbpA triple mutant.** The high levels of DnaK in the triple mutant lacking all the DnaJ homologues (*dnaJ-djlA-cbpA*) may have contributed to the relatively low level of aggregates following 15 min of incubation at 42°C (Fig. 3), since DnaK is the major factor in preventing aggregation. However, the accumulation of aggregates after 45 min of incubation at 42°C was greatest in the triple mutant. Thus, it is conceivable that the various members of the DnaJ family have different substrate specificities (33) and therefore more species of proteins were aggregated in the triple mutant. It is also possible that accumulation of extragenic suppressors in the ΔdnaK strain, but not in the triple J mutant strain, modulated the level of aggregates. In order to characterize individual proteins found in the aggregates of the various mutants, we separated the aggregates by two-dimensional gel electrophoresis (Fig. 5). We could not detect the presence of proteins that were specifically resolubilized from aggregates in bacteria with only CbpA and in bacteria with only DjlA (*dnaJ-djlA* and *dnaJ-cbpA* mutants, respectively). There were also no significant differences between the aggregated proteomes of the two double mutants (Fig. 5). Therefore, the greater accumulation of aggregates in the triple mutant lacking all the DnaJ homologues likely reflected a generalized block in protein disaggregation.

**CbpA and DjlA promote disaggregation in vitro.** DnaJ is the major and most-studied Hsp40 and was shown to interact with DnaK in protein refolding, as well as in protein disaggregation.
extracts were obtained from an E. coli (20 ng/ml), DNAK (2 μM), ATP (5 mM), phosphoenolpyruvate (3 mM), pyruvate kinase (20 ng/ml), DNAK (2 μM), ClpB (2 μM), GrpE (0.2 μM), and 0.4 μM DNAJ, 0.4 μM DNAJ, 0.4 μM CbpA, or none of the J-proteins. After 4 h of incubation in preheated extracts deprived of DNAJ, to stimulate ATPase activity and refold proteins (4, 13). To determine whether CbpA and DJLA can also substitute for DNAJ in this DNAK-mediated protein disaggregation process, we examined their ability to assist DNAK in resolving aggregated proteins in preheated extracts deprived of DNAJ proteins (obtained from a dnaJ-cbpA-djLA triple mutant). The results summarized in Fig. 6 indicate that either CbpA or DJLA could partially substitute for DNAJ in this DNAK-mediated protein disaggregation process. These results are compatible with the in vivo results, indicating that DNAJ is the major protein in this group and cannot be completely replaced by any one of the J-proteins.

Fate of disaggregated proteins. One basic question is whether the proteins disaggregated in vivo are subsequently degraded by the cellular proteolytic machinery. To answer this question, we constructed an experimental in vivo system using two features of the enzyme homoserine transsuccinylase (HTS) of E. coli: the fact that its synthesis is repressed in the presence of methionine and the finding that it is completely aggregated and disappears from the soluble fraction upon a shift to 46°C (16). The fate of HTS aggregates, obtained by a 15-min incubation at 46°C, could be monitored subsequent to the addition of methionine, which prevented synthesis of new HTS molecules. The results presented in Fig. 7 indicate that when the cultures were shifted back to a lower temperature, HTS gradually disappeared from the aggregated fraction and could be detected as soluble protein. Therefore, it appears that most of the aggregated HTS was renatured and not proteolyzed.

DISCUSSION

The results presented in this paper indicate that the DJLA and CbpA proteins of E. coli can partially substitute for DNAJ in the DNAK-mediated protein disaggregation process. These results revealed a hitherto unknown role for the CbpA and DJLA cochaperones, whose exact in vivo function has not been elucidated yet. It should be noted that previous in vitro studies have indicated that DNAJ is essential for DNAK-mediated protein disaggregation (29, 39), while our study showed that DNAJ can be partially replaced by CbpA and DJLA, both in vitro and in vivo. Our results are compatible with previously published data indicating that both dnaJ-cbpA and dnaJ-djLA double mutants are temperature sensitive, while the cbpA-djLA double mutant is not temperature sensitive (12). The temperature sensitivity of these mutants could be due to their inability to solubilize protein aggregates, as shown here.

To determine whether the disaggregated proteins are renatured or proteolyzed, we monitored the fate of aggregated HTS and demonstrated that most of it was renatured. This experiment was made possible by the fact that addition of methionine could specifically block HTS synthesis while not
reducing the levels of chaperones and proteases. This experi-
ment provides further proof for the hypothesis that disaggre-
gated proteins go back into the pool of active cellular proteins.

Our data indicating that DnaJ is not completely essential for in vivo protein disaggregation apparently contradicts the previ-
ously published data of Kedzierska et al. (23), who did not observe protein disaggregation in a dnaJ single mutant. How-
ever, the incubation temperature in their experiment was 45°C, a temperature at which growth of dnaJ mutants is completely abolished. Under these harsh conditions, the ability of CbpA and DjlA to promote disaggregation may be compromised, perhaps by malfunctioning of other cellular proteins which are also important in this process.

One protein that plays a critical role in protein disaggrega-
tion is DnaK, which utilizes DnaJ as its major cochaperone. The results presented in Fig. 5 indicate that the levels of DnaK were higher in mutants defective in disaggregation than in other strains, ruling out the possibility that the observed disaggregation defects were simply due to lower intracellular lev-
els of DnaK. Moreover, the level of DnaK was highest in the triple mutant that lacked all three DnaJ homologues.

Taken together, the data presented here clearly demonstrate that the DnaJ function in disaggregation can be substantially replaced by the homologues of this protein, DjlA and CbpA. A study of the aggregated proteins in double mutants lacking DnaJ and either CbpA or DjlA indicated that the aggregated proteomes are similar and that there is no detectable specific group of proteins that can be resolubilized by one of the homologues. Therefore, as far as our results indicate, the DnaJ homologues appear to be interchangeable.

Here we studied protein aggregation at 42°C, a physiological growth temperature for E. coli. Upon a shift to this tempera-
ture, heat shock proteins, such as DnaK and DnaJ, are not present in high enough concentrations to take care of the higher level of protein misfolding and aggregation. The protein quality control system is then involved in folding of newly synthesized and misfolded proteins, as well as in resolving the aggregated proteins (6, 30, 43). Under these cellular conditions the amount of DnaJ may become limiting for efficiently regu-
ulating the activity of DnaK, making the role of CbpA and DjlA physiologically significant.

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