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The DnaJ chaperone catalytically activates the DnaK chaperone to preferentially bind the σ^{32} heat shock transcriptional regulator

(DnaK chaperone machine/*Escherichia coli* heat shock regulation/protein–protein interaction)

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ABSTRACT **In *Escherichia coli* the heat shock response is under the positive control of the σ^{32} transcription factor. Three of the heat shock proteins, DnaK, DnaI, and GrpE, play a central role in the negative autoregulation of this response at the transcriptional level. Recently, we have shown that the DnaK and DnaJ proteins can compete with RNA polymerase for binding to the σ^{32} transcription factor in the presence of ATP, by forming a stable DnaJ–σ^{32}–DnaK protein complex. Here, we report that DnaJ protein can catalytically activate DnaK's ATPase activity. In addition, DnaJ can activate DnaK to bind to σ^{32} in an ATP-dependent reaction, forming a stable σ^{32}–DnaK complex. Results obtained with two DnaJ mutants, a missense and a truncated version, suggest that the N-terminal portion of DnaJ, which is conserved in all family members, is essential for this activation reaction. The activated form of DnaK binds preferentially to σ^{32} versus the bacteriophage λP protein substrate.**

All organisms respond to a variety of stress conditions through the transient acceleration in the rate of synthesis of a group of proteins, collectively referred to as stress or "heat shock" proteins. During the heat shock response in *Escherichia coli* a group of about 20 proteins is transiently overexpressed. One of them, the DnaK protein, the Hsp70 homolog, was previously shown to belong to the "molecular chaperone" class of proteins (reviewed in refs. 1–4). Molecular chaperones mediate the correct assembly/folding of other polypeptides or macromolecular structures but themselves are not part of the final product. The DnaK protein is capable of binding to various unfolded polypeptides, allowing them to (*i*) maintain their unfolded form, thus preventing premature misfolding, (*ii*) traverse biological membranes, (*iii*) be protected against protein aggregation/denaturation under stress conditions, and (*iv*) disaggregate/disassemble certain protein aggregates (reviewed in refs. 1–4). Pelham (5) proposed that proteins belonging to the Hsp70 family are involved in binding to the hydrophobic domains of other proteins, which are exposed either naturally or as a consequence of stressful conditions. Such binding and release of substrate proteins, which is dependent on ATP binding and hydrolysis, lead to the disassembly of specific protein structures. Recently, in agreement with this, Banecki *et al.* (6) showed that following ATP binding and hydrolysis, progressively fewer hydrophobic structures are exposed on the surface of the DnaK protein.

Phenotypes resulting from mutations in the *dnaK* gene are similar to those resulting from mutations in two other heat shock genes, *dnaJ* and *grpE*. It has been shown that DnaJ and GrpE act together with DnaK in many biological processes, such as the negative autoregulation of the heat shock response (7, 8) and the replication of bacteriophage λ (9, 10) and P1 plasmid (11). In addition, the weak ATPase of DnaK is greatly stimulated in the presence of GrpE and DnaJ (12). The DnaJ protein helps DnaK to hydrolyze ATP, while GrpE accelerates the release of the bound nucleotide (12). Additionally, DnaK, DnaJ, and GrpE function coordinately, in an ATP-dependent manner, to reactivate heat-inactivated RNA polymerase (13) and firefly luciferase (14). Recently, several laboratories reported that DnaJ protein helps DnaK to bind to several substrates, such as the bacteriophage λP protein (15, 16), the *E. coli* transcription σ factor σ^{32} (17, 18), rhodanese (19), and firefly luciferase (14), in an ATP-dependent reaction. It was proposed that DnaJ may act by "tagging" the substrate for subsequent binding by DnaK (11, 16, 18).

We have investigated the details of the mechanism of this DnaK/substrate/DnaJ interaction through the use of DnaJ wild-type and two DnaJ mutant proteins with complementary defects, DnaJ259 and DnaJ12 (20). Our results not only support the above-mentioned tagging hypothesis but also further refine it by demonstrating that DnaJ protein can act catalytically to "activate" DnaK to bind preferentially to some of its polypeptide substrates.

MATERIALS AND METHODS

Buffers. Buffer A is 40 mM Hepes·KOH, pH 7.6/100 mM NaCl/5 mM $MgCl_2$/0.1 mM EDTA/1 mM dithiothreitol (DTT). Buffer B is 20 mM Tris·HCl, pH 7.3/100 mM NaCl/5 mM $MgCl_2$. Buffer C is 50 mM Tris·HCl, pH 7.2/10% glycerol/5 mM $MgCl_2$/5 mM 2-mercaptoethanol. Buffer D is 40 mM Hepes·KOH, pH 7.6/100 mM KCl/5 mM $MgCl_2$/1 mM DTT/0.01% Triton X-100.

Protein Purifications. DnaK protein was purified according to Zylicz *et al.* (21), DnaJ and DnaJ259 according to Zylicz *et al.* (22), σ^{32} according to Liberek *et al.* (23), DnaJ12 according to Wall *et al.* (20), and λP according to Tsurimoto *et al.* (24). The purity of all proteins was >90% (except for λP, ≈75%). The concentration of the proteins was estimated by the dye-based Bio-Rad assay using bovine serum albumin as a standard. The molar amounts of proteins were calculated on the basis that all proteins except DnaJ (22), DnaJ259, and DnaJ12 (dimers) are monomers. [^{35}S]Methionine-labeled σ^{32} was purified from an *in vitro* Zubay transcription/translation system (25) according to Liberek *et al.* (23). The resulting radioactive σ^{32} protein was estimated to be about 50% pure at the protein level and >90% radioactively pure.

Velocity Sedimentation Experiments. These were carried out essentially as described by Liberek *et al.* (23).

Half-Life Determination of the DnaK–ATP Complex. The preparation of DnaK complexed with adenine nucleotide and

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the determination of its half-life were done as described before by Liberek *et al.* (12).

HPLC Sizing Chromatography. Protein mixtures were preincubated in 70-μl samples in buffer B for 10 min at 37°C. After incubation, samples were injected into a Progel-TSK G3000SW HPLC column (0.75 cm × 60 cm) at a flow rate of 1 ml/min. Protein detection was performed at 214 nm. In some experiments, various column fractions were precipitated with trichloroacetic acid and proteins were analyzed directly by SDS/PAGE.

P-150 (Bio-Rad) Sizing Chromatography. Protein mixtures were preincubated in 50-μl samples in buffer D for 10 min at 37°C. After incubation, sizing chromatography on a P-150 (Bio-Rad) column (0.5 cm × 7 cm) was performed, and 100-μl fractions were collected and analyzed directly in a scintillation counter.

RESULTS

Recently, several groups reported that DnaJ can interact with DnaK, thus assisting it in its interactions with various substrates (14–19). We investigated this DnaJ/DnaK interaction in more detail by using two complementary DnaJ mutants, DnaJ259 (26) and DnaJ12 (20). In DnaJ259 the histidine at the highly conserved amino acid position 33 is changed to a glutamine (20). This mutation is located within the most highly conserved domain of DnaJ, the so-called "J domain," used to define the various DnaJ family members (27). Mutant DnaJ12 is a truncated protein carrying only the N-terminal 108 amino acids of DnaJ, which includes the J domain, as well as the adjacent (glycine + phenylalanine)-rich motif (20).

As a model substrate, we chose the σ^{32} protein because we recently reported the formation of a DnaK–σ^{32}–DnaJ complex in an ATP-dependent manner (ref. 17 and Fig. 1*A*). When the mutant DnaJ259 protein was substituted for wild-type DnaJ, a DnaJ259–σ^{32} complex readily formed, but a DnaJ259–σ^{32}–DnaK complex was not observed (Fig. 1*B*). This result shows that the DnaJ259 mutant protein can bind σ^{32}, as well as wild-type DnaJ does (17), but it cannot activate DnaK to bind to σ^{32} in the presence of ATP.

When DnaJ12 was used in similar experiments, no stable complex between DnaJ12 and σ^{32} was detected (result not shown; see Fig. 6). However, despite the absence of a DnaJ12–σ^{32} complex, the DnaJ12 mutant protein was surprisingly capable of activating DnaK to bind to σ^{32} in the presence of ATP, judging by the fact that a DnaK–σ^{32} complex was readily detected in a glycerol gradient (result not shown; see Fig. 6). Furthermore, DnaJ12 was not associated with this DnaK–σ^{32} complex. These preliminary studies were further substantiated by sizing chromatography on an HPLC column. Preincubation of σ^{32} with DnaK and DnaJ12 in the presence of ATP resulted in the formation of an additional peak, corresponding to a DnaK–σ^{32} complex. Additionally, the peaks that correspond to free DnaK and σ^{32} were much smaller in the presence of DnaJ12 than in its absence (Fig. 2*A*). To confirm the identity of the DnaK–σ^{32} complex, proteins in the collected fractions were precipitated with trichloroacetic acid, separated by SDS/PAGE, and stained with Coomassie blue (Fig. 2 *B* and *C*).

DnaJ Acts Catalytically To Help DnaK Bind to σ^{32}. The observation that the DnaJ12 protein can activate DnaK protein for binding to σ^{32}, although itself not part of the final DnaK–σ^{32} complex, suggested that the wild-type DnaJ protein may also activate DnaK for binding to some of its protein substrates. To test this hypothesis, HPLC sizing chromatography of DnaK, σ^{32}, and DnaJ proteins was performed. When

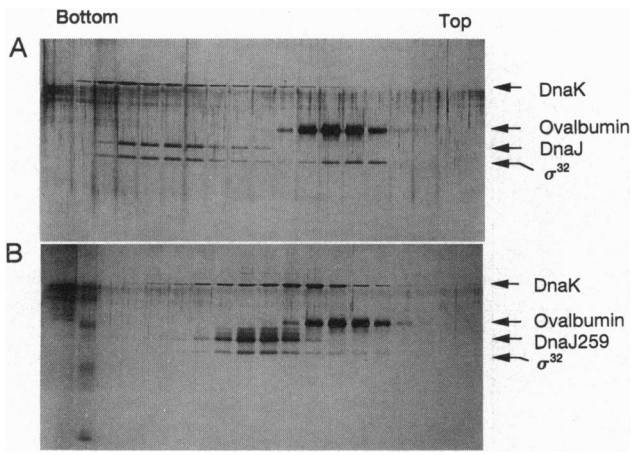

FIG. 1. DnaJ259 binds σ^{32} stably but does not activate DnaK for binding to σ^{32}. The sedimentation of DnaK and σ^{32} in the presence of ATP and DnaJ (*A*) and ATP and DnaJ259 protein (*B*) in 15–35% (vol/vol) glycerol gradients in buffer A is shown. The concentrations of the proteins in the reaction mixtures were 2.7 μM for DnaK, 3 μM for σ^{32}, 2.2 μM for DnaJ, and 2.8 μM for DnaJ259. ATP was present in the reaction mixture at a concentration of 1 mM. Ovalbumin was used as a sedimentation standard. Fractions were collected from the bottom of the gradient and their protein contents were analyzed by SDS/PAGE followed by silver staining.

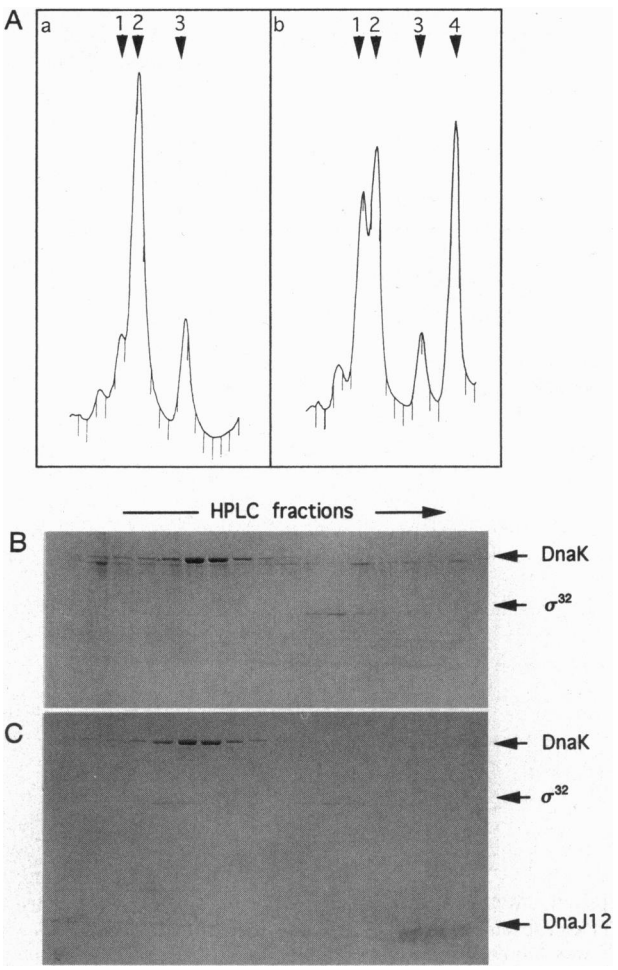

FIG. 2. DnaJ12 activates DnaK for binding to σ^{32} protein. The interaction of DnaK with σ^{32} was observed by sizing chromatography on HPLC. (*A*) Traces: a, 3.4 μM DnaK incubated with 3.4 μM σ^{32} and 1 mM ATP; b, 3.4 μM DnaK incubated with 3.4 μM σ^{32} and 6.5 μM DnaJ12 and 1 mM ATP. Arrowheads mark the peaks corresponding to the σ^{32}–DnaK complex (1), DnaK protein (2), σ^{32} (3), or DnaJ12 (4). (*B* and *C*) The appropriate fractions around the peaks were collected after sizing chromatography on an HPLC column and analyzed by SDS/PAGE to confirm the identity of proteins.

DnaK was preincubated with σ^{32} in the presence of ADP or ATP, only a very small peak corresponding to the σ^{32}–DnaK complex was detected (somewhat larger in the case when ADP was used) (Fig. 3*A*, traces a and b). The same experiment was repeated in the presence of wild-type DnaJ protein, at an 18-fold less molar ratio than DnaK. In this case, when ADP was used, the result obtained was identical to that seen in the absence of DnaJ. However, when ATP was present together with DnaJ in the preincubation mixture, the peak corresponding to the DnaK–σ^{32} complex increased significantly (Fig. 3*A*, traces e and f). The presence of σ^{32} and DnaK in this peak was verified by SDS/PAGE (Fig. 3*B*). Both the position of the peak and its protein composition clearly showed that DnaJ was not part of this complex.

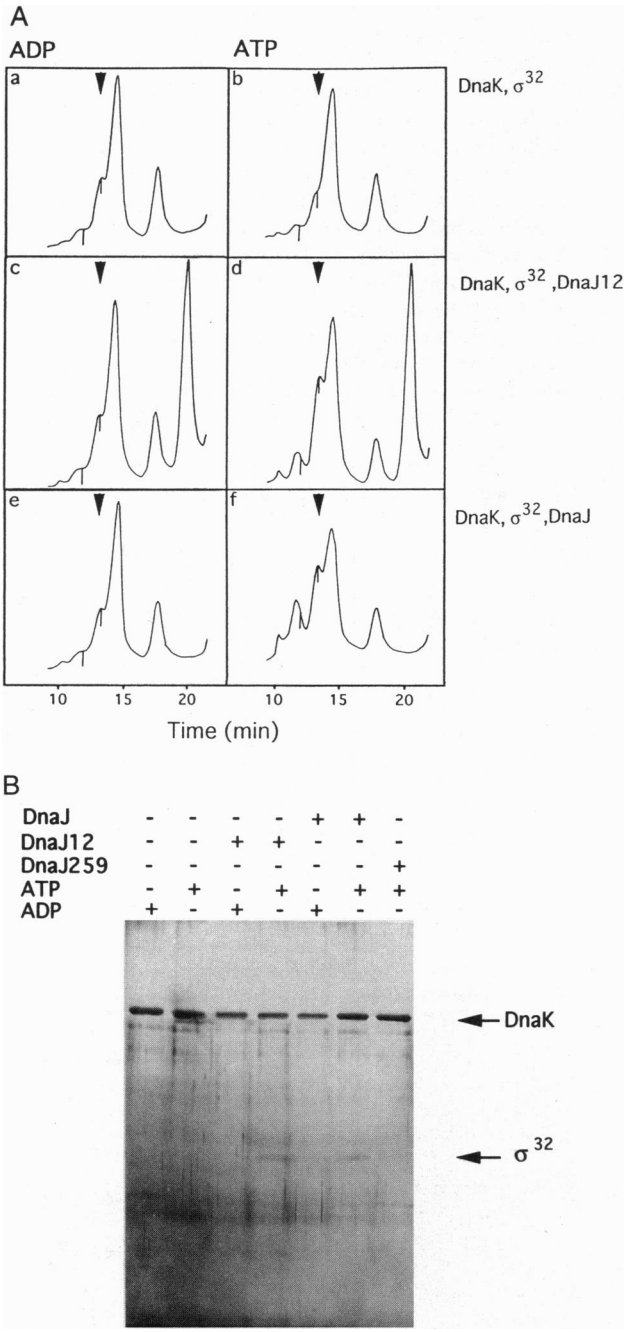

FIG. 3. Wild-type DnaJ and DnaJ12 protein in the presence of ATP can activate DnaK to bind σ^{32} protein. Interaction of DnaK with σ^{32} was followed by sizing chromatography on an HPLC column. Traces: a, 1.6 μM DnaK incubated with 1.7 μM σ^{32} and 100 μM ADP; b, 1.6 μM DnaK incubated with 1.7 μM σ^{32} and 100 μM ATP; c, 1.6 μM DnaK incubated with 1.7 μM σ^{32}, 4.7 μM DnaJ12, and 100 μM ADP; d, 1.6 μM DnaK incubated with 1.7 μM σ^{32}, 4.7 μM DnaJ12, and 100 μM ATP; e, 1.6 μM DnaK incubated with 1.7 μM σ^{32}, 90 nM DnaJ, and 100 μM ADP; f, 1.6 μM DnaK incubated with 1.7 μM σ^{32}, 90 nM DnaJ, and 100 μM ATP. Arrowheads indicate the peak corresponding to the σ^{32}–DnaK complex. (*B*) The peak fractions, identified by arrowheads in *A*, were analyzed by SDS/PAGE to confirm the identity of the proteins.

The other peaks in Fig. 3*A* correspond to free DnaK (14.5 min), free σ^{32} (18 min), free DnaJ12 (20.6 min; traces c and d), as well as other uncharacterized protein complexes (aggregates?) (11.6 min; trace f). The exact protein composition of the fast eluting protein complexes was not determined primarily due to poor recovery of the material. When DnaJ12 was used, the results were similar to those obtained with wild-type DnaJ protein. The peak corresponding to the σ^{32}–DnaK complex was larger when ATP, as opposed to ADP, was used (Fig. 3*A*, traces c and d, and *B*). The concentration of DnaJ12 used in this activation reaction was about 50-fold higher than that of wild-type DnaJ. Therefore, we decided to titrate the amount of DnaJ12 needed for activation of DnaK in a separate experiment. For this purpose, ^{35}S-labeled σ^{32} was purified from an *in vitro* transcription/translation system and shown to be capable of associating with the core RNA polymerase and promoting transcription (results not shown). The amount of σ^{32} in complex with DnaK was determined following sizing chromatography on a small P-150 (Bio-Rad) column and scintillation counting. The results confirmed that an $\approx$3-fold excess of DnaJ12 to DnaK and σ^{32} is needed for efficient σ^{32}–DnaK complex formation (Fig. 4). The ability of another DnaJ mutant, DnaJ259, to activate DnaK for binding to σ^{32} was also tested. Contrary to wild-type DnaJ or DnaJ12, the DnaJ259 mutant was not capable of activating DnaK for binding to σ^{32} in the concentration tested in the HPLC sizing chromatography experiments (result not shown) and in the experiments with sizing of ^{35}S-labeled σ^{32} on a P-150 column (Fig. 4).

We previously showed that the DnaJ protein specifically stimulates the hydrolysis of DnaK-bound ATP (12). Next, we compared the ability of DnaJ and the various mutant proteins to activate DnaK for binding to σ^{32} as well as their influence on the hydrolysis of the DnaK-bound ATP. To do this, the complex between DnaK and [α-^{32}P]ATP was isolated as pub-

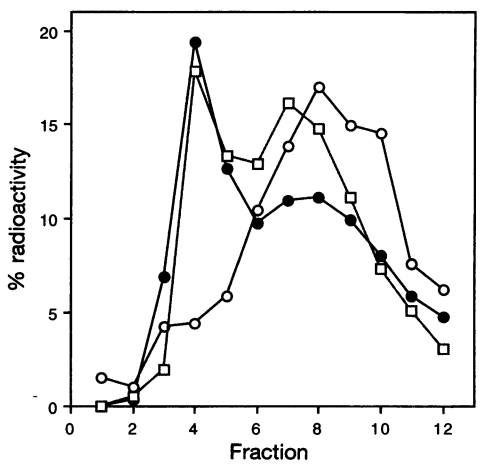

FIG. 4. DnaJ259 protein does not activate DnaK for binding to σ^{32}. Interaction of ^{35}S-labeled σ^{32} with DnaK was followed by sizing chromatography on a P-150 (Bio-Rad) column. ^{35}S-labeled σ^{32} (0.095 μM) was preincubated with 0.11 μM DnaK and 0.34 μM DnaJ (●), 0.34 μM DnaJ12 (□), or 0.34 μM DnaJ259 (○) for 10 min at 37°C in 50 μl of buffer D and loaded onto a P-150 column. Following sizing chromatography, the radioactivity in various fractions was counted in a scintillation counter.

lished (12). The presence of a small amount of DnaJ protein (50-fold less molar ratio than DnaK) shortened the half-life of ATP in the DnaK–ATP complex from 9.1 to 1.8 min (Fig. 5). When the DnaJ12 truncated protein was used in the same assay, albeit at a somewhat higher concentration than DnaJ (8-fold less molar ratio than DnaK), the half-life of ATP decreased to nearly the same extent as in the presence of the wild-type DnaJ protein (2.3 min). However, when the DnaJ259 mutant protein was used under the same conditions, no significant change in the half-life of DnaK-bound ATP was detected (7.6 min), as reported (20). These results demonstrate that wild-type DnaJ and DnaJ12 help DnaK to hydrolyze bound nucleotide in a catalytic manner.

DnaJ-Activated DnaK Has a Higher Affinity Toward σ^{32} than λP. The next question addressed was whether DnaJ confers a specificity to DnaK in terms of substrate binding. The bacteriophage λP protein was chosen as an alternative substrate because we have previously extensively characterized its interaction with DnaK (16). As shown earlier, in a glycerol gradient, in the absence of ATP, DnaK bound to σ^{32} and λP (in fact, DnaK bound better to λP than σ^{32}; Fig. 6*A*). As expected (16, 23), in the presence of ATP, no stable interaction between DnaK and λP or σ^{32} was detected (Fig. 6*B*). However, when ATP and DnaJ protein were present, the DnaK–σ^{32}–DnaJ complex formed efficiently (Fig. 6*C*). Under these specific conditions, no stable interaction was seen between λP protein and DnaK or DnaK/DnaJ in the presence of ATP, as judged by its sedimentation position. In agreement with our previous results, in the presence of DnaJ12 and ATP, DnaK formed a complex with σ^{32} but not λP protein (Fig. 6*D*). DnaJ 12 was not associated with the DnaK–σ^{32} complex (Fig. 6*D*). Similar results showing that DnaK protein, activated by either wild-type DnaJ or DnaJ12, exhibits a higher affinity toward σ^{32} than λP were obtained by sizing chromatography on an HPLC column (results not shown).

DISCUSSION

DnaK protein, the Hsp70 homologue of *E. coli*, plays a central role in cell physiology, especially under stress conditions, by acting synergistically with two other heat shock proteins, DnaJ and GrpE (1–4). Recently, it was proposed (11, 16, 18) that DnaJ protein binds to various substrate proteins and thus "tags" them for further action by DnaK. Here, we refine this suggestion by showing that the DnaJ protein (*i*) can specifically activate DnaK to bind preferentially to some of its protein substrates, (*ii*) does not necessarily have to be a part of the DnaK–substrate complex, and (*iii*) acts catalytically, since substoichiometric amounts are sufficient to activate DnaK for binding to σ^{32} and to accelerate DnaK's ATPase activity.

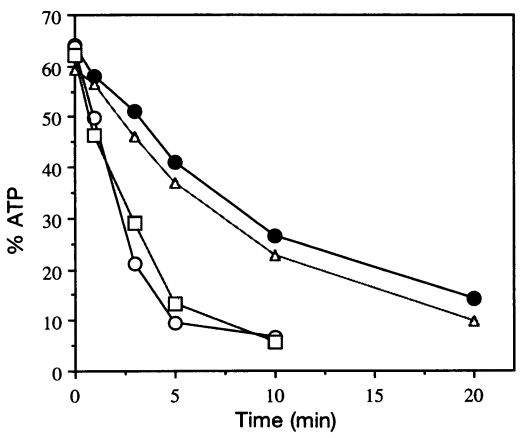

FIG. 5. DnaJ catalytically stimulates the hydrolysis of DnaK-bound ATP. A complex between DnaK and [α-^{32}P]ATP was isolated as described (see text). DnaK (1.2 μM) complexed with ATP was incubated without (●) or with 22 nM DnaJ (□), 22 nM DnaJ259 (△), or 150 nM DnaJ12 (○). At the indicated times, samples were withdrawn and spotted on a PEI-cellulose plate. Following one-dimensional chromatography and autoradiography, the spots corresponding to ATP and ADP were cut out and assayed in a scintillation counter, and the percent ATP was calculated.

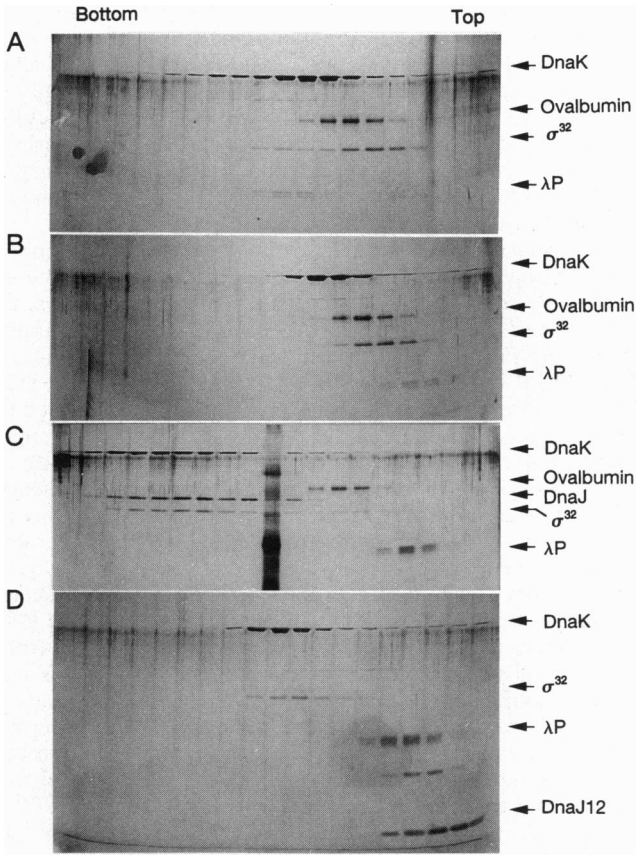

FIG. 6. DnaJ or DnaJ12 can activate DnaK for preferential binding to σ^{32}, as opposed to the λP substrate protein. Sedimentation occurred in a 15–35% (vol/vol) glycerol gradient in buffer A of a protein mixture containing 8.4 μM DnaK, 2.6 μM σ^{32}, and 3.5 μM λP (*A*), 8.4 μM DnaK, 2.6 μM σ^{32}, 3.5 μM λP, and 1 mM ATP (*B*), 2.7 μM DnaK, 2.6 μM σ^{32}, 3.5 μM λP, 2.5 μM DnaJ, and 1 mM ATP and 2.7 μM DnaK, 2.6 μM σ^{32}, 3.5 μM λP, 5.3 μM DnaJ12, and 1 mM ATP (*D*). Protein mixtures were incubated for 10 min at 37°C followed by sedimentation. In *A–C* ovalbumin was used as a standard for sedimentation position. Fractions were collected from the bottom of the gradient and their proteins were analyzed by SDS/PAGE followed by silver staining.

Although the DnaJ12 truncated mutant protein (N-terminal 108 amino acids of DnaJ) does not form a stable complex with σ^{32}, it can (*i*) catalytically stimulate DnaK's ATPase activity and (*ii*) allow DnaK to form a stable complex with σ^{32}. Comparison of the relative ability of DnaJ and DnaJ12 to either stimulate the ATP hydrolysis step by DnaK or allow the σ^{32}–DnaK complex formation showed that wild-type DnaJ has an ≈10-fold higher specific activity than DnaJ12. These results suggest that DnaK's ATPase activation and higher binding for the substrate are related processes. This suggestion is further supported by the observation that the DnaJ259 mutant protein does not stimulate either DnaK's ATPase activity or its ability to bind to σ^{32}. The observation that wild-type DnaJ and DnaJ259 proteins can form a complex with σ^{32}, yet only wild-type DnaJ functions in the formation of a DnaK–σ^{32} complex, suggests that it is the "activation" of DnaK and not that of σ^{32} that is important for efficient DnaK–σ^{32} complex formation. These results also suggest that the C-terminal part of DnaJ most likely contains the protein's substrate binding domain, whereas the N-terminal part, which contains the

highly conserved J domain (20, 27), is the one that interacts with DnaK. DnaJ's interaction with DnaK must be transient, since no stable complex between wild-type DnaK and wild-type DnaJ can be detected under our experimental conditions. Most likely, DnaJ transiently interacts with the DnaK–ATP form, thus accelerating ATP hydrolysis and hence the formation of the DnaK–ADP form, which is coupled to a change in DnaK's conformation, a result previously suggested by limited trypsin digestion experiments (20).

The enhancement of DnaK's binding to σ^{32} by DnaJ requires ATP hydrolysis, since when the nonhydrolyzable ATP analog, ATP[γ-S], or ADP was substituted in the reaction assays, no activation of DnaK for binding to σ^{32} was observed (results not shown and Fig. 3). The observations that (*i*) in the absence of DnaJ, ATP hydrolysis destroys the DnaK–σ^{32} complex (12) and (*ii*) in the presence of DnaJ, ATP hydrolysis is needed to promote the formation of the DnaK–σ^{32} complex strongly suggest a qualitative difference in the conformational state of DnaK following ATP hydrolysis in the absence or the presence of DnaJ. Our results are consistent with the observations of Schmid *et al.* (28)—namely, that ATP[γ-S] binding accelerates to the same extent the rate of substrate binding and substrate release from DnaK, thus exerting no overall effect on DnaK binding to its substrate. The recent studies by Palleros *et al.* (29) and Schmid *et al.* (28) showed that the release of substrate from a DnaK–substrate complex (in the absence of DnaJ) is not directly coupled to ATP hydrolysis. Whereas ATP binding causes a 47-fold increase in the rate of DnaK–peptide complex formation, it causes an even greater, 440-fold, increase in the rate of peptide dissociation (28) and as a net result, the release of substrate from DnaK is favored in the presence of ATP.

The activation model of DnaK by DnaJ suggested by our results in this paper may have physiological relevance, since there is $\approx$10-fold more DnaK than DnaJ in an *E. coli* cell (30). Although our results cannot be fully explained by the tagging model (11, 16, 18), nevertheless the two models are not necessarily mutually exclusive and it is quite possible that both are applicable *in vivo*, depending on either the particular physiological conditions—i.e., DnaJ availability—or the specific polypeptide substrates. This last possibility is supported by our findings, that when DnaJ is present in equimolar amounts to DnaK and σ^{32}, the DnaK–σ^{32}–DnaJ complex forms efficiently. Most likely in this case, two separate reactions are occurring: (*i*) activation of DnaK protein by DnaJ for substrate binding and (*ii*) the formation of a DnaJ–σ^{32} complex. These two reactions result in the formation of the DnaJ–σ^{32}–DnaK protein complex. When submolar concentrations of DnaJ protein are used, the activation of DnaK for binding to σ^{32} occurs, but the substoichiometric presence of DnaJ does not allow the quantitative formation of the DnaK–σ^{32}–DnaJ complex. Consistent with the activation model is our previous observation—namely, that part of DnaK copurifies with σ^{32} (23). However, there were no significant amounts of DnaJ protein associated with either DnaK or σ^{32} during these steps of protein purification (23).

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