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Autoregulation of the *Escherichia coli* heat shock response by the DnaK and DnaJ heat shock proteins

(negative autoregulation/protein–protein interactions)

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ABSTRACT **All organisms respond to various forms of stress, including heat shock. The heat shock response has been universally conserved from bacteria to humans. In *Escherichia coli* the heat shock response is under the positive transcriptional control of the σ^{32} polypeptide and involves transient acceleration in the rate of synthesis of a few dozen genes. Three of the heat shock genes—*dnaK*, *dnaJ*, and *grpE*—are special because mutations in any one of these lead to constitutive levels of heat shock gene expression, implying that their products negatively autoregulate their own synthesis. The DnaK, DnaJ, and GrpE proteins have been known to function in various biological situations, including bacteriophage λ replication. Here, we report the formation of an ATP hydrolysis-dependent complex of DnaJ, σ^{32}, and DnaK proteins *in vitro*. This DnaJ–σ^{32}–DnaK complex has been seen under different conditions, including glycerol gradient sedimentation and co-immunoprecipitation. The DnaK and DnaJ proteins in the presence of ATP can interfere with the efficient binding of σ^{32} to the RNA polymerase core, and are capable of disrupting a preexisting σ^{32}–RNA polymerase complex. Our results suggest a possible mechanism for the autoregulation of the heat shock response.**

All organisms respond to environmental stresses, such as heat and ethanol, by the rapid and transient acceleration in the rate of synthesis of a group of proteins collectively called the heat shock proteins (for reviews see refs. 1–4). *Escherichia coli* possesses approximately 20 known heat shock genes whose expression is positively regulated by the σ^{32} polypeptide (the *rpoH* gene product) at the transcriptional level. The σ^{32} polypeptide is extremely unstable *in vivo*, with a half-life of 1 min at normal temperatures, its intracellular levels usually correlating with the magnitude of the heat shock response (refs. 5–7, reviewed in refs. 2 and 8). The heat shock response is negatively autoregulated, since mutations in the *dnaK*, *dnaJ*, or *grpE* heat shock genes lead to an overproduction of the heat shock proteins. Such an overexpression of the heat shock response is due in part to a stabilization of σ^{32} polypeptide in these genetic backgrounds (6, 7, 9).

The major heat shock protein DnaK has been extremely conserved during evolution. It is 50% identical at the amino acid sequence level to the eukaryotic Hsp70 protein. DnaK can bind to various unfolded polypeptides, such as denaturated bovine pancreatic trypsin inhibitor, as well as seemingly folded polypeptides such as *E. coli* GrpE, σ^{32}, phage λ P, and the human antitumor p53 protein (10–16). Such binding is disrupted in the presence of ATP. In addition, overproduction of DnaK aids the translocation of an export-deficient MalB-LacZ hybrid protein (17), and DnaK/DnaJ overproduction can substitute for some of the functions for the missing SecB protein (18). DnaK, also referred to as a "chaperone" (19), usually acts together with two other heat shock proteins, DnaJ and GrpE, to constitute a "chaperone machine" (20).

The DnaJ protein is another example of a heat shock protein that has been extensively conserved in evolution (21). Recently, it has been shown that *E. coli* DnaJ protein can suppress the import defect into the endoplasmic reticulum and mitochondria of YDJ1-defective yeast mutants (22), demonstrating a functional conservation throughout evolution. DnaJ and GrpE work synergistically with DnaK in the replication of bacteriophages λ and P1 (23–25). Part of the role of DnaJ protein is to target both the phage λ P and RepA replication proteins for DnaK action (26–28). In the case of P1 replication the formation of the DnaJ–RepA protein complex is necessary for the subsequent action of DnaK, resulting in the monomerization of RepA protein dimers (26). DnaJ and GrpE also interact with DnaK directly. The GrpE protein forms a stable ATP-sensitive complex with DnaK, whereas GrpE and DnaJ together greatly stimulate the ATPase activity of DnaK (29).

We have previously shown that purified DnaK but not DnaK756 mutant protein interacts with σ^{32} and that this interaction is sensitive to ATP hydrolysis (11). Recently, Gamer *et al.* (14) showed, using crude extracts and affinity chromatography, that DnaK, DnaJ, and GrpE proteins also interact with σ^{32} transcription factor. In this work we show that purified DnaK and DnaJ proteins form a stable complex with σ^{32} in the presence of ATP. Such an association prevents σ^{32} from binding to RNA polymerase. In addition, DnaK and DnaJ can also actively "strip" σ^{32} from its complex with RNA polymerase.

MATERIALS AND METHODS

Protein Purification. DnaK, DnaJ, GrpE, σ^{32}, and RNA polymerase were purified according to refs. 11, 30, and 31. The purity of all enzymes was >90% except σ^{70} (≈75%). Protein concentrations were estimated by the Bio-Rad assay and the molar ratio was determined on the basis that all proteins are monomers except DnaJ (dimer).

Glycerol Gradient Centrifugation. Reaction mixtures (60 μl) in buffer A (40 mM Hepes/KOH, pH 7.6/100 mM NaCl/1 mM dithiothreitol/5 mM $MgCl_2$/0.1 mM EDTA) were assembled on ice. The mixtures were incubated for 10 min at 37°C and then loaded directly onto a 3.2-ml linear 15–35% (vol/vol) glycerol gradient in buffer A and centrifuged at 48,000 rpm for 24 hr at 2°C in a Beckman SW60 rotor. Fractions were collected from the bottom of the tube and analyzed by SDS/PAGE, followed by Coomassie blue staining, silver staining, and/or Western blot analysis with antibodies to σ^{32} and ^{125}I-labeled staphylococcal protein A or a chemiluminescent detection system using Lumigen PPD as a substrate (Boehringer Mannheim). In the experiments with

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RNA polymerase, the glycerol gradient was increased to 20–40% (vol/vol) and the centrifugation time was lowered to 20 hr. Where indicated, ATP was used at 1 mM in the reaction mixtures and at 100 μM throughout the gradient.

Coimmunoprecipitation Experiments. The reaction mixtures in buffer A were assembled on ice and then incubated for 15 min at room temperature. Ten microliters of polyclonal anti-DnaK rabbit serum was added and the mixtures were incubated for 1 hr on ice. Forty microliters of a 1:1 (vol/vol) mixture of protein A-agarose in buffer B (10 mM Tris·HCl, pH 7.2/150 mM NaCl/5 mM EDTA/1% Triton X-100) was added. After an additional 30-min incubation on ice the protein A beads were washed five times with 500 μl of buffer B. The σ^{32} protein that associated with protein A-agarose was detected by SDS/PAGE, followed by Western blot analysis with polyclonal antibodies to σ^{32} as described above.

RESULTS

The σ^{32} Transcription Factor Interacts with DnaJ Protein. Because we had previously demonstrated that the DnaK chaperone protein can form a weak, ATP hydrolysis-sensitive complex with σ^{32} (11), we investigated whether the DnaJ chaperone can also interact with σ^{32}. Such a possible interaction between the DnaJ and σ^{32} protein was investigated by glycerol gradient centrifugation. Under such conditions, DnaJ is known to sediment as a dimer (30), while σ^{32} sediments as a monomer (11). When σ^{32} was preincubated with DnaJ (approximate molar ratio 1:1) for 10 min at 37°C and the mixture was sedimented in a glycerol gradient, approximately 75% of the σ^{32} protein was found in a complex with DnaJ (Fig. 1). The DnaJ–σ^{32} complex, judging from its sedimentation behavior, as well as the separation of the proteins by SDS/PAGE followed by Coomassie blue staining, most likely consists of a monomer of σ^{32} and a dimer of DnaJ. This complex formed equally well in the presence and absence of ATP (results not shown).

The interaction between σ^{32} and DnaJ has been additionally demonstrated by the following observations: (*i*) σ^{32} protein binds to a DnaJ affinity column, and (*ii*) part of DnaJ protein copurifies with σ^{32} during Q-Sepharose chromatography (results not shown). Our results with purified proteins confirm and extend those of Gamer *et al.* (14), who demonstrated a σ^{32}–DnaJ interaction by using histidine-tagged σ^{32} bound to a nickel affinity column and passing crude extracts over it.

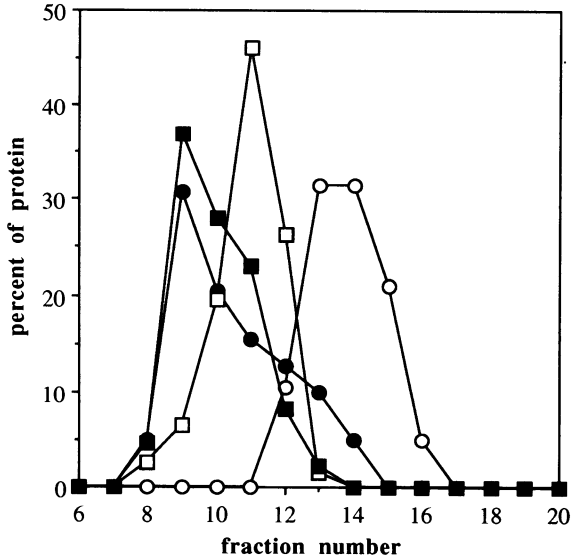

FIG. 1. DnaJ heat shock protein interacts with σ^{32}. Cosedimentation of σ^{32} with DnaJ in a glycerol gradient is shown. ○ and ●, σ^{32} (1.8 μM) preincubated without or with DnaJ (1.9 μM), respectively; □ and ■, DnaJ (1.9 μM) preincubated without or with σ^{32} (1.8 μM), respectively. The amounts of σ^{32} and DnaJ were determined by SDS/PAGE followed by Coomassie blue staining of proteins and densitometry.

Formation of the DnaJ–σ^{32}–DnaK Complex Is ATP Dependent. Next, we determined the relative affinity of σ^{32} for DnaK or DnaJ. In the absence of ATP, when the molar ratio of DnaK, DnaJ, and σ^{32} was approximately 1:1:1 in the incubation mixture, the majority of σ^{32} was found in a complex with DnaJ only (Fig. 2). This result demonstrates that DnaJ binds to σ^{32} much better than DnaK does. This conclusion is consistent with the observation that, when the amount of DnaK in the preincubation mixture was increased to 10-fold above that of σ^{32} and DnaJ, the formation of the DnaJ–σ^{32}–DnaK complex was readily seen. However, as expected, the bulk of DnaK sedimented in a position characteristic of that of a DnaK monomer (results not shown).

A surprising observation made during the course of this work was that the presence of ATP greatly stimulated the quantitative formation of DnaJ–σ^{32}–DnaK complex even when the molar ratio of the three proteins was 1:1:1 (Fig. 2). For the efficient formation of this complex, the presence of ATP is absolutely required; i.e., when ATP was replaced either by its nonhydrolyzable analogs adenosine 5′-[β,γ-imido]triphosphate and adenosine 5′-[γ-thio]triphosphate or by ADP, the DnaJ–σ^{32}–DnaK complex did not form. The DnaJ–σ^{32}–DnaK complex must be fairly stable, since it is seen after a 24-hr centrifugation and persists at any concentration of ATP used in the gradient (ranging from 0 to 1 mM ATP; results not shown). In a control experiment, we were unable to demonstrate a direct physical interaction between DnaK and DnaJ under any of our experimental conditions.

When the third heat shock protein, GrpE, was included in the reaction mixture the extent of formation of the DnaJ–σ^{32}–DnaK complex in the presence of ATP was essentially the same as that seen without GrpE. Furthermore, the GrpE protein was never seen to associate with the DnaJ–σ^{32}–DnaK complex (results not shown).

To further confirm the validity of the results obtained by sedimentation analysis, we performed co-immunoprecipitation experiments as well. Using anti-DnaK antibodies, we were able to efficiently co-immunoprecipitate σ^{32} with DnaK protein only when either DnaJ and ATP was present or DnaJ

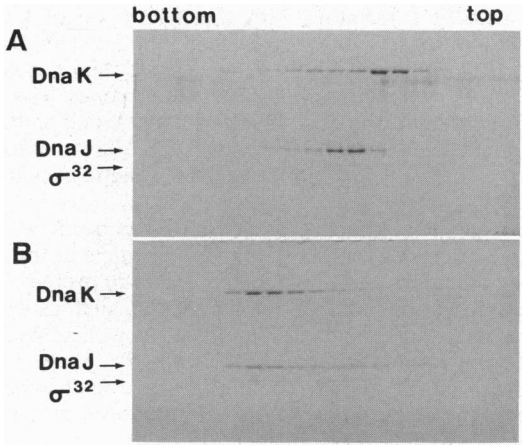

FIG. 2. Efficient formation of the DnaJ–σ^{32}–DnaK complex is ATP dependent. Sedimentation of DnaK, DnaJ, and σ^{32} in a glycerol gradient in the absence (*A*) or presence (*B*) of ATP is shown. The concentrations of the proteins in the reaction mixture were as follows: σ^{32}, 1.8 μM; DnaJ, 1.8 μM; DnaK, 2 μM. Fractions collected from the gradient were analyzed by SDS/PAGE followed by Coomassie blue staining.

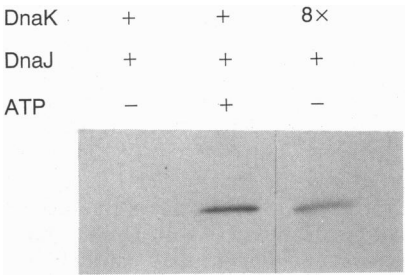

FIG. 3. Co-immunoprecipitation of σ^{32} protein by anti-DnaK polyclonal antibodies in the presence of DnaK, DnaJ, and ATP. σ^{32} at 150 nM was preincubated with 240 nM (except 1.92 μM in the case indicated 8×) and 180 nM DnaJ in 80 μl of buffer A for 15 min at 22°C, in the presence of 1 mM ATP when indicated.

and a 13-fold molar excess of DnaK were present in the preincubation reaction mixture (Fig. 3). The presence of DnaK and DnaJ proteins in equimolar amounts with σ^{32}, in the absence of ATP, did not result in the efficient co-immunoprecipitation of σ^{32} (Fig. 3). The presence of the DnaJ–σ^{32}–DnaK complex was demonstrated by nondenaturing gel electrophoresis, as well as sizing column chromatography (results not shown).

σ^{70} Does Not Form a Stable Complex with DnaJ and DnaK. To determine whether the DnaJ–σ^{32}–DnaK complex is specific for σ^{32} we performed a series of experiments with the canonical *E. coli* σ factor, σ^{70}. No evidence for a DnaJ–σ^{70}, DnaK–σ^{70}, or DnaJ–σ^{70}–DnaK complex could be seen after a 10-min preincubation at 37°C and glycerol gradient centrifugation (Fig. 4). Furthermore, in a competition experiment we established that the presence of σ^{70} did not appreciably interfere with the formation of the DnaJ–σ^{32}–DnaK complex (Fig. 4).

DnaK and DnaJ Compete with RNA Polymerase for Binding to σ^{32}. To better understand the mechanism of heat shock autoregulation, we then asked whether the DnaJ and DnaK proteins can compete with the RNA polymerase core for σ^{32} binding. In all cases examined (DnaK, DnaJ, and ATP or DnaK, DnaJ, GrpE, and ATP), σ^{32} was preferentially associated with the RNA polymerase core rather than with DnaK or DnaJ—i.e., after glycerol gradient centrifugation, less than 20% of σ^{32} was found to be in the DnaJ–σ^{32}–DnaK complex, whereas the rest cosedimented with the RNA polymerase core (results not shown).

In vivo, other σ factors may compete with σ^{32} for binding to the RNA polymerase core. Therefore, we included σ^{70} in our experiments to determine whether its presence can influence the fraction of σ^{32} that partitions with either RNA polymerase core or DnaK/DnaJ. In a control experiment when only σ^{70}, σ^{32}, and RNA polymerase core were present, approximately 90% of σ^{32} was associated with RNA polymerase (Fig. 5). The remaining σ^{32} sedimented as a free monomer near the top of the gradient. However, when DnaK, DnaJ, GrpE, and ATP were also included in the preincubation mixture, the majority of σ^{32} was detected in the DnaJ–σ^{32}–DnaK complex (Fig. 5). The formation of this complex did not depend on the order of protein addition—i.e., when RNA polymerase core was preincubated with σ^{32} and σ^{70} and then DnaK, DnaJ, and GrpE were added, the DnaJ–σ^{32}–DnaK complex formed equally efficiently.

In all such experiments the combined presence of DnaK, DnaJ, GrpE, and ATP resulted in the removal of more than 50% of σ^{32} from the RNA polymerase core. Although the precise role, if any, that GrpE plays in this σ^{32} release/sequestration reaction is not known, it was nevertheless included, because its presence resulted in a more quantitative

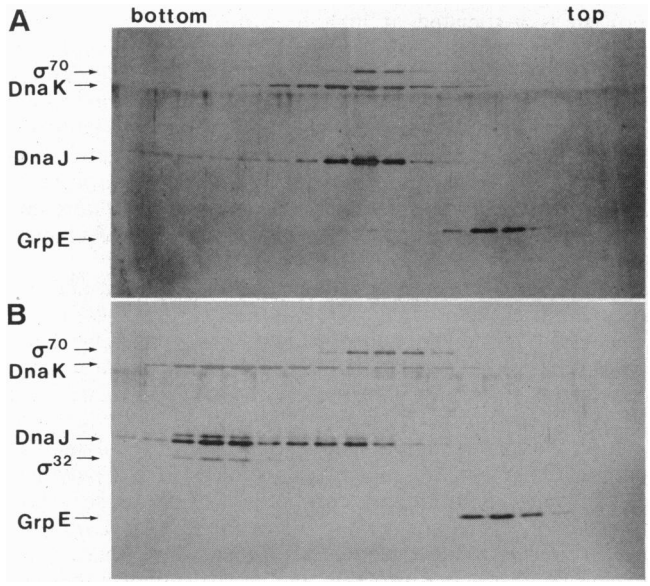

FIG. 4. DnaK and DnaJ preferentially bind to σ^{32} but not to σ^{70}. (*A*) σ^{70} does not form a complex with DnaK and DnaJ. (*B*) σ^{70} does not interfere with formation of the DnaJ–σ^{32}–DnaK complex. The concentrations of the various proteins used were as follows: σ^{70}, 1.5 μM; DnaK, 2 μM; DnaJ, 2 μM; σ^{32}, 1 μM; and GrpE, 1.7 μM. ATP was present in both *A* and *B*. Fractions were analyzed by SDS/PAGE followed by silver staining.

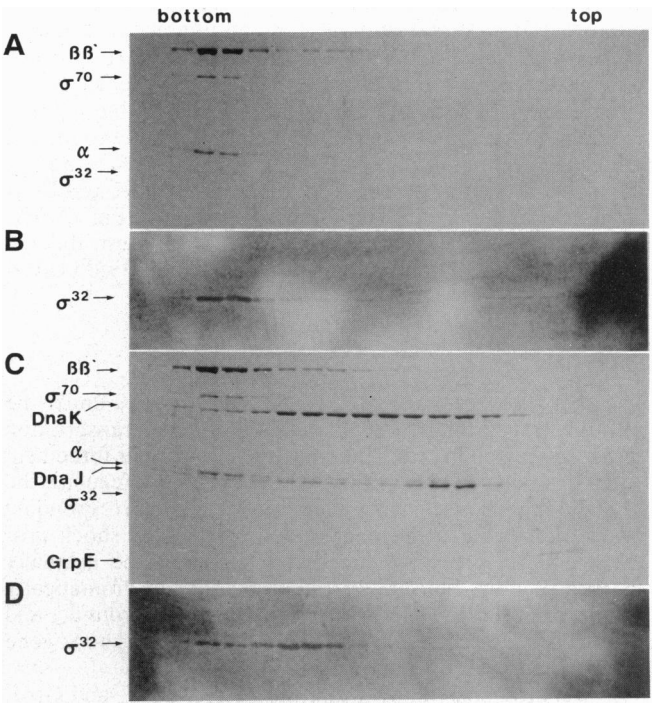

FIG. 5. DnaK, DnaJ, and GrpE heat shock proteins efficiently prevent σ^{32} from binding to RNA polymerase core. (*A* and *B*) Sedimentation of σ^{32} protein after incubation with RNA polymerase core. (*C* and *D*) Sedimentation of σ^{32} after its sequestration by DnaK, DnaJ, GrpE, and ATP. RNA polymerase core at 1.3 μM, 1.5 μM σ^{70}, and 0.7 μM σ^{32} were preincubated with 1 mM ATP without (*A* and *B*) or with (*C* and *D*) 6.4 μM DnaK, 2.8 μM DnaJ, and 1.5 μM GrpE and sedimented in a linear 20–40% glycerol gradient. The presence of the individual proteins in the various fractions after centrifugation was established by SDS/PAGE followed by Coomassie blue staining (*A* and *C*). Since σ^{32} does not stain well with Coomassie blue, its presence was verified by Western blot analysis, using anti-σ^{32} polyclonal antibodies and the Lumigen PPD chemiluminescent detection system (*B* and *D*). α, β, and β' mark the positions of RNA polymerase subunits on the SDS/polyacrylamide gel.

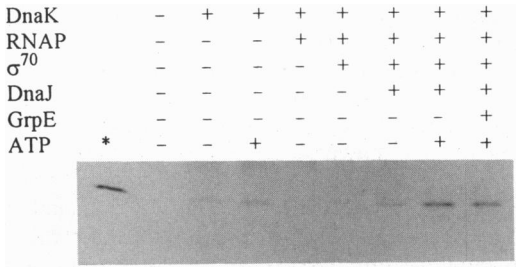

FIG. 6. DnaK, DnaJ, and GrpE heat shock proteins efficiently prevent σ^{32} from binding to the RNA polymerase core. Co-immunoprecipitations of σ^{32} with DnaK in the additional presence of RNA polymerase core, σ^{70}, DnaJ, GrpE, and ATP are shown. σ^{32} at 150 nM was preincubated with 1.6 μM DnaK, 310 nM core RNA polymerase, 350 nM σ^{70}, 0.7 μM DnaJ, 0.8 μM GrpE, and 1 mM ATP (as indicated) for 15 min in 80 μl of buffer A. Immunoprecipitation with anti-DnaK polyclonal antibodies and detection of σ^{32} were performed as described in the text.
*Lane where purified σ^{32} protein was used as a marker.

release of σ^{32} (in two out of three experiments). As a control, when either DnaK or DnaJ was incubated alone with the RNA polymerase core, σ^{32} and σ^{70}, and ATP, no appreciable release of σ^{32} from RNA polymerase was observed (results not shown).

To confirm the results obtained by the glycerol gradient centrifugation method, we performed a series of co-immunoprecipitation experiments using anti-DnaK antibodies. When DnaK and σ^{32} were the only proteins present in the reaction mixture only a limited amount of σ^{32} co-immunoprecipitated with DnaK (Fig. 6). Furthermore, the addition of RNA polymerase core with or without σ^{70} abolished the DnaK–σ^{32} interaction as judged by the fact that no detectable σ^{32} was co-immunoprecipitated. However, it was possible to restore efficient co-immunoprecipitation of σ^{32} with DnaK by adding either DnaJ (partial restoration; notice that DnaK and DnaJ are used in molar excess) or both DnaJ and ATP (Fig. 6). The presence of GrpE protein in the reaction mixture did not affect the extent of co-immunoprecipitation of σ^{32} with DnaK (Fig. 6).

DISCUSSION

The heat shock response in *E. coli* is known to be under the positive transcriptional regulation of the σ^{32} transcription factor (reviewed in refs. 2 and 8). It is known that three heat shock proteins—DnaK, DnaJ, and GrpE—autoregulate the heat shock responses, since mutations in their corresponding genes often lead to the overproduction of heat shock proteins. This is probably due to the fact that the normally extremely short half-life of σ^{32} ($\approx$60 sec) is dramatically stabilized in *dnaK*, *dnaJ*, or *grpE* mutant backgrounds, and the levels of σ^{32} correlate with the extent of heat shock gene expression, at least at 42°C (2, 6–9).

It was previously shown that the DnaK, DnaJ, and GrpE chaperone proteins act together in many biological processes as well (6, 23). In this paper we report on the formation of a complex of DnaJ, σ^{32}, and DnaK, which is capable of the sequestration of the σ^{32} transcription factor from RNA polymerase. Recently, Hoffmann *et al.* (28) reported the formation of an analogous complex of λ P, DnaK, and DnaJ in the presence of ATP. Also, Langer *et al.* (32) reported that the presence of Mg^{2+}ATP and DnaJ alters the manner by which DnaK interacts with unfolded rhodanese protein. However, in contrast to the results of both Hoffmann *et al.* (28) and Langer *et al.* (32), in our system the GrpE protein neither interferes with nor disrupts a preexisting DnaJ–substrate–DnaK complex. If anything, in the presence of GrpE, the sequestration of σ^{32} from RNA polymerase by DnaK and DnaJ is more effective. In agreement with this differential role of GrpE in our system, it has been recently shown that in the presence of GrpE, DnaK exhibits a much higher affinity for the DnaJ–λ P complex than for λ P alone (27). A similar mechanism may be operating in our system as well—i.e., in the presence of GrpE, DnaK may exhibit a much higher affinity for the σ^{32}–DnaJ complex than for any other protein present in the reaction mixture. A likely possibility is that GrpE interacts with DnaK, thus changing its conformation and enabling it to efficiently bind to the DnaJ–σ^{32} complex, as in the case of λ P protein (27). Consistent with such a potential role of GrpE, mutations in the *grpE* gene result in a constitutive induction of the heat shock response in *E. coli* (6).

Normally, DnaK protein binds to one of its substrates, such as λ P, λ O, human p53, or unfolded bovine pancreatic trypsin inhibitor and releases them after ATP hydrolysis. However, in the case of the DnaJ–σ^{32}–DnaK complex, the DnaJ protein, which is part of the complex, must also change the manner by which DnaK interacts with σ^{32}, since in the absence of DnaJ the interaction between σ^{32} and DnaK is disrupted in the presence of ATP (11, 14). The DnaJ–σ^{32}–DnaK complex, once formed in the presence of ATP, is stable and can be isolated after sedimentation in the absence or presence of ATP. When a 10-fold excess of DnaK was used in the absence of ATP, a DnaJ–σ^{32}–DnaK complex was readily seen. This result can be explained in two different ways: (*i*) there is no change in the affinity of DnaK for the DnaJ–σ^{32} complex, and the observed result is simply due to a mass effect, and (*ii*) there exists a minor DnaK form (perhaps due to the hydrolysis of contaminating traces of ATP) with a high affinity for the DnaJ–σ^{32} complex. Our results do not allow us to distinguish between these two possibilities. ATP hydrolysis is not always needed to stabilize a DnaJ–substrate–DnaK complex, as the case of the P1 bacteriophage RepA protein exemplifies. Wickner *et al.* (26) have shown that the DnaJ–RepA complex is acted upon by DnaK in the presence of ATP, resulting not only in its disruption but in the actual monomerization of RepA. However, a stable DnaJ–RepA–DnaK complex is never observed (26).

Recently, Sherman and Goldberg (33) showed that DnaK protein is associated at high temperature with a rapidly degraded mutant alkaline phosphatase protein, PhoA61, and that various mutations in *dnaK*, *dnaJ*, or *grpE* differentially affect its degradation rate. Perhaps the binding of σ^{32} to the DnaJ and DnaK proteins makes it a better substrate for proteases (2, 8). It is possible that in vivo GrpE protein acts at this step, by allowing the transfer of σ^{32} to a protease system(s) analogous to its function in the transfer of unfolded rhodanese from DnaK/DnaJ to the GroEL/GroES chaperonin system (32).

Our work with purified proteins has confirmed and extended our previous findings (11), as well as those of Gamer *et al.* (14). The major conclusion of our work is that DnaK and DnaJ proteins form a very stable complex with σ^{32} transcription factor, especially in the presence of ATP. The DnaK and DnaJ proteins in the presence of ATP have been shown to interfere with the efficient binding of σ^{32} to RNA polymerase core and to actively disrupt a preexisting σ^{32}–RNA polymerase complex. This finding correlates with the observation made *in vivo* by Straus *et al.* (34), namely that the activity of σ^{32} protein is reduced under conditions where there is an excess of heat shock proteins. Our work supports the previously suggested model of regulation of the heat shock response (8, 35) and helps explain why mutations in either *dnaK* or *dnaJ* lead to high constitutive levels of heat shock gene expression (6, 7).

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