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A cycle of binding and release of the DnaK, DnaJ and GrpE chaperones regulates activity of the *Escherichia coli* heat shock transcription factor σ^{32}

Jürgen Gamer, Gerd Multhaup, Toshifumi Tomoyasu, John S.McCarty, Stefan Rüdiger, Hans-Joachim Schönfeld[1], Christiane Schirra, Hermann Bujard and Bernd Bukau[2]

Zentrum für Molekulare Biologie, Universität Heidelberg, Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany and [1]Hoffmann-La Roche AG, CH-4002 Basel, Switzerland

[2]Corresponding author

The chaperone system formed by DnaK, DnaJ and GrpE mediates stress-dependent negative modulation of the *Escherichia coli* heat shock response, probably through association with the heat shock promoter-specific σ^{32} subunit of RNA polymerase. Interactions of the DnaK system with σ^{32} were analysed. DnaJ and DnaK bind free, but not RNA polymerase-bound, σ^{32} with dissociation constants of 20 nM and 5 μM respectively. Association and dissociation rates of DnaJ–σ^{32} complexes are 5900- and 20-fold higher respectively than those of DnaK–σ^{32} complexes in the absence of ATP. ATP destabilizes DnaK–σ^{32} interactions. DnaJ, through rapid association with σ^{32} and stimulation of hydrolysis of DnaK-bound ATP, mediates efficient binding of DnaK to σ^{32} in the presence of ATP, resulting in DnaK–DnaJ–σ^{32} complexes containing ADP. GrpE binding to these complexes stimulates nucleotide release and subsequent complex dissociation by ATP. We propose that the principles of this cycle also operate in other chaperone activities of the DnaK system. DnaK and DnaJ cooperatively inhibit σ^{32} activity in heat shock gene transcription and GrpE partially reverses this inhibition. These data indicate that reversible inhibition of σ^{32} activity through transient association of DnaK and DnaJ is a central regulatory element of the heat shock response.

Keywords: heat shock protein/heat shock response/Hsp70/protein folding/sigma factor

Introduction

Heat shock proteins (HSPs) constitute a cellular system for folding, repair and degradation of proteins (Hendrick and Hartl, 1993; Georgopoulos *et al.*, 1994). Their levels are tightly adjusted to the metabolic and environmental status of the cell by regulation at the transcriptional level (Morimoto *et al.*, 1994). In *Escherichia coli* the genes encoding cytosolic HSPs form a regulon that is positively controlled by the *rpoH* gene product, the heat shock promoter-specific σ^{32} subunit of RNA polymerase (Gross *et al.*, 1990; Bukau, 1993; Yura *et al.*, 1993; Georgopoulos *et al.*, 1994). The level and activity of σ^{32} are limiting for heat shock gene transcription (Grossman *et al.*, 1987; Straus *et al.*, 1989; Tilly *et al.*, 1989). Induction of heat shock gene transcription by stress is achieved through an increase in the level of σ^{32}, due to increased σ^{32} synthesis and stability (Straus *et al.*, 1987) and, possibly, through activation of σ^{32}. Down-regulation of transcription during the shut-off phase of the stress response is achieved through a decrease in the levels and inactivation of σ^{32} (Straus *et al.*, 1989).

The DnaK chaperone system formed by the HSPs DnaK, DnaJ and GrpE negatively modulates transcription of heat shock genes. It mediates inactivation and degradation of σ^{32} (Straus *et al.*, 1989, 1990; Tilly *et al.*, 1989; C.Gross, personal communication) and repression of *rpoH* translation during the shut-off phase of the heat shock response (Grossman *et al.*, 1987; Straus *et al.*, 1990). Components of the DnaK system have been shown to interact physically with σ^{32} in crude cell extracts and in a purified system (Gamer *et al.*, 1992; Liberek and Georgopoulos, 1993). DnaJ is capable of binding σ^{32} independently of DnaK and the nucleotide exchange factor GrpE. DnaK was reported to have a rather low affinity for σ^{32} and to bind σ^{32} with higher efficiency in the presence of DnaJ and ATP (Liberek and Georgopoulos, 1993; Liberek *et al.*, 1995). Based on glycerol gradient centrifugation experiments it has been claimed that DnaK and DnaJ, through interaction with RNA polymerase (RNAP)-bound σ^{32}, strip σ^{32} from RNAP (Liberek and Georgopoulos, 1993). Together these findings raise the possibility that inactivation and degradation of σ^{32} are mediated through direct association of these chaperones with the σ^{32}-dependent transcription machinery. Homeostatic regulation models (Georgopoulos *et al.*, 1990; Craig and Gross, 1991; Bukau, 1993) propose that induction of a heat shock response after stress treatment relies on sequestering of the DnaK system through binding to damaged proteins accumulating during stress. Chaperone sequestering results in activation and stabilization of σ^{32} and, consequently, induction of heat shock gene transcription. HSP-mediated repair or degradation of damaged proteins ameliorates the inducing signal and frees the DnaK system to shut off the heat shock response.

We analysed association of the DnaK chaperone system with σ^{32} in detail. We identified a σ^{32} binding/release cycle which controls σ^{32} activity and assigned functions for DnaJ, DnaK, GrpE and ATP in this cycle.

Results

ATP-dependent binding of DnaK to σ^{32}

The parameters of the interaction of DnaK with σ^{32} were determined by gel filtration. Some experiments were performed using C-terminal histidine-tagged σ^{32} (σ^{32}-C-his). Tagging allows convenient purification of σ^{32} by

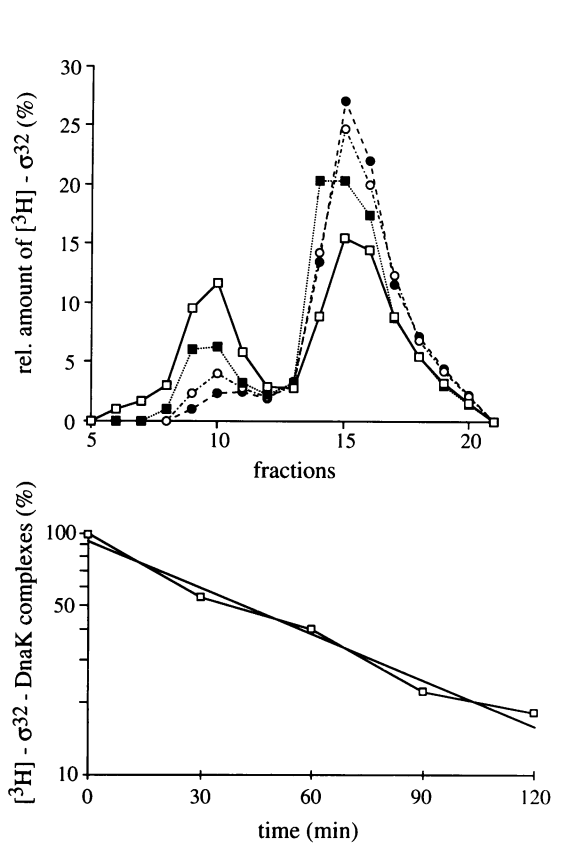

Fig. 1. Stability of DnaK–σ^{32} complexes in the absence of ATP. DnaK (4 μM) and [^{3}H]σ^{32} (5 μM) were incubated in 20 μl buffer T for 30 min at 30°C, followed by addition of 180 μl unlabeled σ^{32}-C-his (20 μM final concentration in a 200 μl volume). At 1, 30, 60, 90 and 120 min after addition of σ^{32}-C-his (chase), samples were immediately subjected to Superdex 200 gel filtration followed by scintillation counting of the eluted fractions. (**A**) Elution profiles of [^{3}H]σ^{32} at 1 (□), 30 (■), 60 (○) and 120 (●) min after σ^{32}-C-his addition (90 min value not shown). The amount of [^{3}H]σ^{32} in each fraction is represented as a percentage of the total [^{3}H]σ^{32} of each run. (**B**) Chase time-dependent decrease in the amount of [^{3}H]σ^{32}–DnaK complex. The amount of complex (fractions 8–11) found after 1 min of σ^{32}-C-his addition was set as 100%.

metal affinity chromatography (Gamer *et al.*, 1992) without affecting σ^{32} function in heat shock gene transcription *in vivo* (Gamer *et al.*, 1992) or *in vitro* (see Figure 8A) and chaperone-dependent modulation of the stress response *in vivo* (Gamer *et al.*, 1992). To verify that σ^{32}-C-his has the same properties as authentic σ^{32}, key experiments were repeated with σ^{32} and identical results were obtained.

The dissociation constant (K_d) of DnaK–σ^{32} complexes in the absence of ATP was determined by two experiments. First, ^{3}H-labeled σ^{32} (Figure 1A) or unlabeled σ^{32}-C-his (not shown) was incubated with DnaK at 30°C to allow complex formation, followed by gel filtration separation of complexes from free σ^{32} at 4°C. This approach allows determination of the K_d of DnaK–σ^{32} complexes, since these complexes are highly stable at 4°C in the absence of ATP. For ^{3}H-labeled σ^{32}, based on the relative amounts of free [^{3}H]σ^{32} and [^{3}H]σ^{32}–DnaK complexes (open squares), we calculated the K_d of the complex to be ~5 μM. For σ^{32}-C-his, based on data obtained by incubation with different concentrations of DnaK and subsequent complex isolation, the K_d of DnaK–σ^{32}-C-his complexes was calculated to be ~5 μM as well (data not shown). A similar K_d of DnaK–σ^{32} complexes was obtained in a second experiment, where the ability of a radiolabeled peptide with known affinity for DnaK (K_d 3 μM), peptide C of vesicular stomatitis virus glycoprotein (Landry *et al.*, 1992), to compete with σ^{32} for DnaK binding was determined (not shown).

The dissociation rate of DnaK–σ^{32} complexes in the absence of ATP was determined by a chase of preformed [^{3}H]σ^{32}–DnaK complexes with an excess of unlabeled σ^{32}. [^{3}H]σ^{32}–DnaK complexes were highly stable ($t_{1/2}$ 40 min), yielding a dissociation rate constant (k_d) of 2.8×10^{-4}/s (Figure 1A and B). On the basis of the determined K_d and k_d values, the association rate constant (k_a) of the DnaK–σ^{32} complex was calculated to be very low (56/s/M). These experiments show that although association of DnaK with σ^{32} in the absence of ATP is very slow, it has significant affinity for σ^{32} due to its low k_d.

The effect of ATP on the formation and stability of DnaK–σ^{32}-C-his complexes was examined by gel filtration. DnaK and σ^{32}-C-his were incubated at 30°C under conditions allowing efficient σ^{32}-C-his binding to DnaK in the absence of ATP (Figure 2, upper left panel). When ATP was present during this incubation σ^{32}-C-his exhibited a broad elution with significant amounts eluting in molecular weight fractions between free and DnaK-bound σ^{32}-C-his, indicating repeated dissociation and association of complexes during isolation. To monitor complex stability an excess of peptide C as chase was added to the incubation mixture at two time points (Figure 2, upper right panel). The majority of σ^{32}-C-his was in the free form after 2 min of peptide C addition and almost all σ^{32}-C-his was free after 20 min. We conclude that DnaK cannot stably bind to σ^{32}-C-his in the presence of ATP and, furthermore, DnaK binds σ^{32}-C-his via its regular substrate binding site.

Binding of DnaJ to σ^{32}

Complexes between DnaJ and σ^{32} were detected by gel filtration isolation (data not shown). However, σ^{32} exhibited a broad elution behavior consistent with instability of the complexes during filtration and/or interaction of DnaJ with the gel matrix. This behavior prevented determination of binding parameters of DnaJ–σ^{32} complexes by gel filtration. As an alternative approach we used the BIAcore system, which allows detection of protein interactions at the surface of a sensor chip. Selective binding of the analyte to the immobilized ligand on the sensor chip is monitored in real time using the optical phenomenon surface plasmon resonance (SPR; Malmquist, 1993). For DnaK this technique could not detect association with σ^{32} because of the very low k_a of DnaK–σ^{32} complexes, well outside the dynamic range of BIAcore. For DnaJ, SPR spectra of the interaction with σ^{32} coupled to the chip were detected at various DnaJ concentrations (Figure 3). Upon injection DnaJ specifically associated with immobilized σ^{32} yielding 297 (3.7×10^{-8} M DnaJ) to 484 (1.1×10^{-7} M DnaJ) response units (Figure 3A). Computerized evaluation of these data revealed that the association reaction is monophasic, indicating that binding occurs between single DnaJ and σ^{32} molecules and is fast (k_a 3.3×10^5/M/s). This association was specific, as DnaJ did not bind to a sensor chip

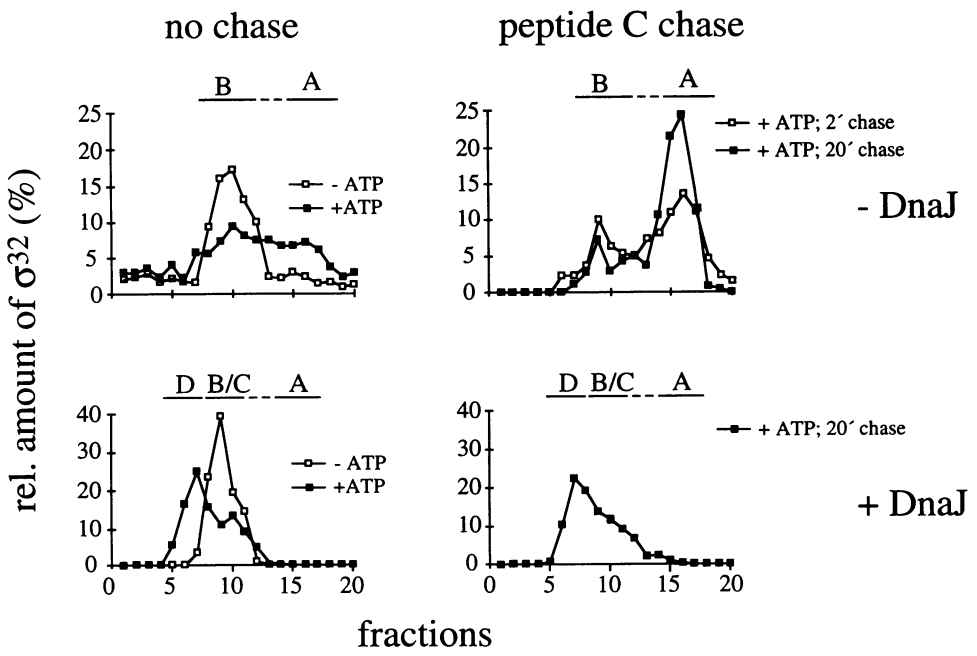

Fig. 2. Stability of chaperone–σ^{32}-C-his complexes in the presence of ATP and peptide C competitor. In experiments without DnaJ (upper panel), DnaK (9 μM) and σ^{32}-C-his (3 μM) were incubated for 20 min at 30°C in buffer T without or with ATP (1 mM) and peptide C (30 μM), followed by Superdex 200 gel filtration chromatography at 4°C. Peptide C was either added 2 min before injection (2 min chase) or added simultaneously with DnaK to σ^{32}-C-his (20 min chase). For samples with ATP the column was pre-equilibrated with buffer T containing 200 μM ATP. In experiments with DnaJ (lower panel), DnaK (4 μM), DnaJ (4 μM) and σ^{32}-C-his (1 μM) were incubated for 20 min at 30°C in buffer T without or with ATP (1 mM) and peptide C (10 μM, 20 min chase) followed by gel filtration. σ^{32}-C-his was visualized by immunoblotting with σ^{32}-specific antiserum after SDS–PAGE and quantified by densitometric scanning. A, B, C and D indicate the peak fractions of free σ^{32}-C-his, DnaK–σ^{32}-C-his, DnaJ–σ^{32}-C-his and DnaK–DnaJ–σ^{32}-C-his complexes respectively.

in the absense of immobilized σ^{32} (data not shown) and bovine serum albumin did not bind to a sensor chip with immobilized σ^{32} (Figure 3B). Dissociation of DnaJ from σ^{32} detected during injection of buffer lacking DnaJ was also monophasic (k_d 6.2×10^{-3}/s). From these rates the K_d for DnaJ–σ^{32} complexes was calculated to be 1.9×10^{-8} M. Thus the affinity of DnaJ for σ^{32} is 260-fold higher than the affinity of DnaK for σ^{32} in the absence of ATP, due to a higher σ^{32} association rate.

DnaK and DnaJ do not stably bind to σ^{32}-containing RNA polymerase

We determined whether DnaK and DnaJ have affinity for σ^{32} bound to RNAP. Purified RNAP core enzyme was incubated for 20 min at 30°C with equimolar amounts of σ^{32}-C-his to allow holoenzyme assembly. Gel filtration analysis indicated that virtually all σ^{32}-C-his was assembled with RNAP (Figure 4A). DnaK or DnaJ were then added at a 4-fold molar excess and, after further incubation for 20 min, complex formation was analyzed. At these protein concentrations, which in separate experiments allowed formation of significant amounts of DnaK–σ^{32} and DnaJ–σ^{32} complexes, virtually no DnaK and DnaJ co-eluted with RNAP holoenzyme (Figure 4B and C). In the case of DnaJ a 10-fold excess over σ^{32}-C-his resulted in DnaJ–σ^{32}-C-his complex formation, but even at these concentrations no association of DnaJ with RNAP holoenzyme was observed (data not shown). These DnaJ–σ^{32}-C-his complexes most likely resulted from DnaJ binding to σ^{32}-C-his spontaneously dissociated from RNAP. DnaK and DnaJ thus have significantly higher affinity for free σ^{32} than for RNAP-bound σ^{32}.

ATP-dependent formation of DnaK–DnaJ–σ^{32} complexes

DnaK, DnaJ and σ^{32} form ternary complexes in the presence of ATP (Liberek and Georgopoulos, 1993; Liberek *et al.*, 1995). In agreement with this finding, in gel filtration experiments in the presence of ATP the majority of total σ^{32} or σ^{32}-C-his co-eluted with DnaJ and DnaK in fractions 6–8, indicating ternary complex formation (lower panel of Figure 2 for σ^{32}-C-his, Figure 5 for σ^{32}). These ternary complexes formed efficiently at σ^{32} concentrations (1 μM) below the K_d (5 μM) of DnaK–σ^{32} complexes (in the absence of ATP), suggesting that the K_d of ternary complexes is below 1 μM.

The stability of DnaK–DnaJ–σ^{32} complexes formed in the presence of ATP was determined by two chase experiments using different competitors. In the first experiment (Figure 5) complexes of [^{3}H]σ^{32} with DnaK and DnaJ were formed at 30°C in the presence of ATP. Then a 50-fold molar excess of unlabeled σ^{32}-C-his was added to compete with [^{3}H]σ^{32} dissociated from these complexes for rebinding to DnaJ and DnaK. Efficient competition occurred even when σ^{32}-C-his was added just prior to injection of the mixture into the column. We conclude that in the presence of ATP DnaK–DnaJ–σ^{32} complexes dissociate within minutes and re-associate rapidly. In the second experiment (Figure 2, lower panel) complexes of σ^{32}-C-his with DnaK and DnaJ were formed at 30°C in the presence of ATP and a 500-fold molar excess of peptide C to compete with σ^{32}-C-his for binding to DnaK (but not DnaJ, since peptide C has no detectable affinity for DnaJ). Peptide C did not compete with σ^{32}-C-his for formation of ternary complexes. We cannot completely

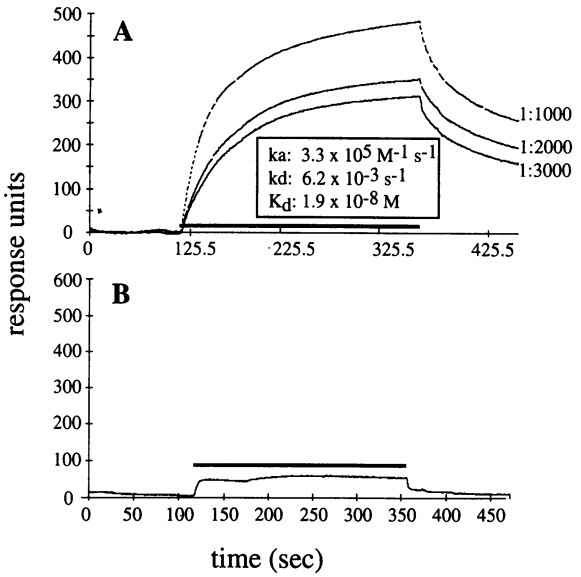

Fig. 3. Surface plasmon resonance analysis of DnaJ association with σ^{32}. The resonance signals are plotted as a function of time (s). The amount of protein associating/dissociating from the surface over time was measured in resonance units (RU). The bars in each panel display the time during which the protein was injected. (**A**) DnaJ in concentrations ranging from 1.1×10^{-7} to 3.7×10^{-8} M was injected over immobilized σ^{32}. Normalized curves for 1:1000, 1:2000 and 1:3000 dilutions of a 110 μM solution of DnaJ are plotted. (**B**) Bovine serum albumin was injected over immobilized σ^{32}.

exclude the possibility that very rapid DnaJ-mediated re-association of σ^{32}-C-his with DnaK and DnaJ after separation of peptide C during filtration also occurs. Both competition experiments together indicate that DnaJ, through binding to σ^{32}, activates DnaK for specific σ^{32} binding in the presence of ATP and competing DnaK substrates.

GrpE binding to DnaK–DnaJ–σ^{32} complexes allows complex dissociation by ATP

The role of GrpE in the interaction of DnaJ and DnaK with σ^{32} was investigated by gel filtration. GrpE, in the absence of other proteins, eluted in fraction 10 with an apparent molecular weight of 170–190 kDa (Schönfeld *et al.*, 1995). GrpE was included during incubation of DnaK and DnaJ with σ^{32}-C-his in the presence of ATP, and complex formation was analysed with or without ATP in the running buffer. In the absence of ATP in the running buffer, free ATP present in the incubation mixture is expected to separate rapidly from protein during filtration. Under these conditions ~20% of total σ^{32}-C-his eluted early in fractions 4–5 (Figure 6A), together with a fraction of total DnaK, DnaJ and GrpE. This elution profile is consistent with formation of quaternary complexes containing all four proteins. In addition, complexes of σ^{32}-C-his with DnaK and/or DnaJ, as well as free σ^{32}-C-his, existed, as judged by the elution profiles of the different components, suggesting an equilibrium between different complexes. Formation of quaternary complexes was reproducible and was not observed in gel filtration experiments lacking GrpE. The shift in elution by 1–2 fractions compared with ternary complexes (fractions 6–8) is significant, as it corresponds to a gain in apparent molecular weight of ~90–180 kDa, consistent with binding

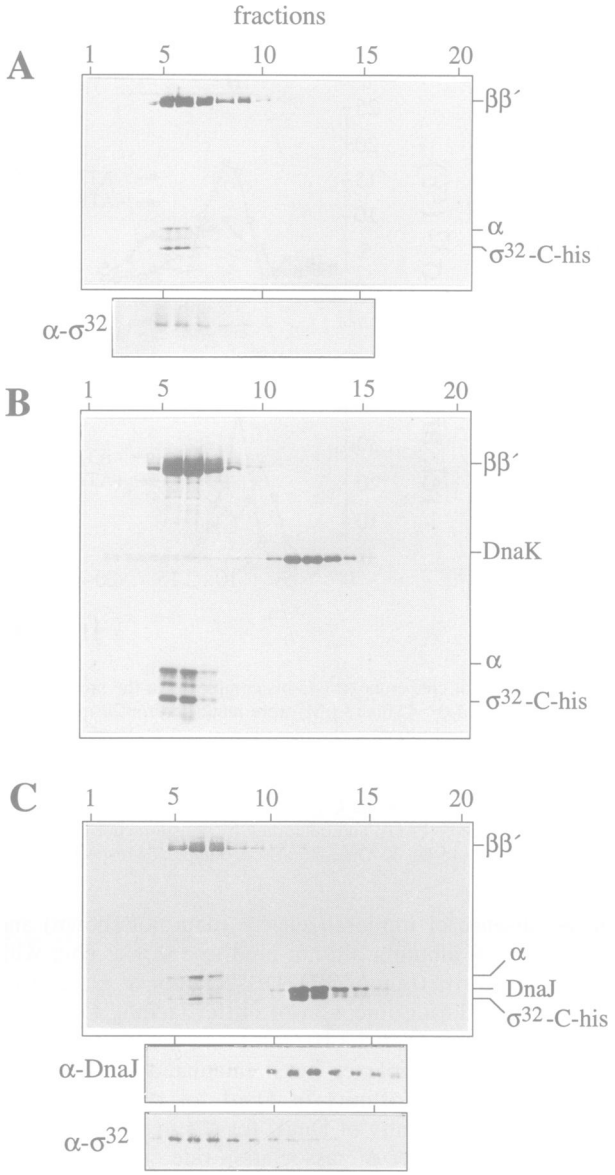

Fig. 4. DnaK and DnaJ do not associate with σ^{32}-C-his bound to RNA polymerase. Equimolar concentrations (1.5 μM final concentration) of RNAP core and σ^{32}-C-his were incubated in buffer T at 30°C for 10 min. The reactions were further incubated for 20 min at 30°C with no addition (**A**) or DnaK (6 μM) (**B**) or DnaJ (6 μM) (**C**), followed by Superdex 200 gel filtration. Silver stained SDS–PAGE gels with the indicated positions of the α, β and β′ subunits of RNA polymerase are shown. In addition, σ^{32}-C-his (A and C) and DnaJ (C) were identified by immunoblot analysis of eluted fractions using σ^{32}- or DnaJ-specific antisera.

of a GrpE monomer or dimer to a ternary complex. Furthermore, these quaternary complexes are distinct from DnaK–GrpE complexes observed to elute in fractions 7–9 (Figure 6B and data not shown) and they did not occur in the absence of DnaJ (Figure 6B). Finally, in the absence of DnaJ and without ATP in the running buffer a large fraction of σ^{32}-C-his elutes in its free form (Figure 6B), indicating a role for GrpE in shifting the equilibrium towards dissociated σ^{32}-C-his.

When complex isolation was performed with ATP in the running buffer neither quaternary nor ternary complexes containing σ^{32}-C-his were detected (Figure 6A). In addi-

tion, DnaK and GrpE eluted in fractions corresponding to their individual elution profiles in the absence of other proteins. σ^{32}-C-his eluted as free protein (fractions 15–16) and in complex with DnaJ (peak fractions 10–11). We also performed these experiments with authentic σ^{32} and observed no difference from the results described for σ^{32}-C-his. We conclude that GrpE can form quaternary complexes with DnaK, DnaJ and σ^{32}-C-his which are dissociated by ATP.

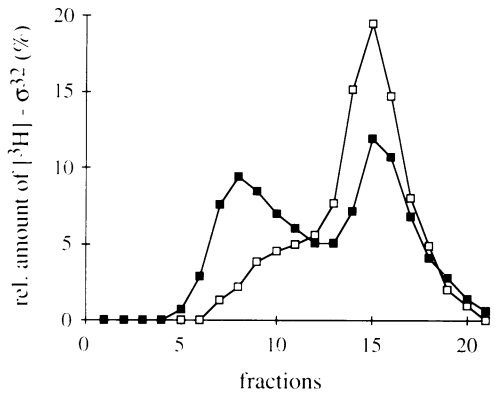

Fig. 5. Stability of DnaK–DnaJ–σ^{32} complexes in the presence of ATP. DnaK (8 μM), DnaJ (8 μM) and [^{3}H]σ^{32} (4 μM) were incubated in 20 μl buffer T at 30°C. After 30 min 180 μl buffer T without (■) or with σ^{32}-C-his (□, 20 μM final concentration) was added and immediately transferred onto ice and injected into a Superdex 200 gel filtration column. The [^{3}H]σ^{32} in each fraction was determined by scintillation counting and is represented as a percentage of total [^{3}H]σ^{32} of each run.

Status of the DnaK-bound nucleotide in chaperone–σ^{32} complexes

To characterize the σ^{32} binding/release cycle further we determined the type of nucleotide associated with chaperone–σ^{32}-C-his complexes. Complexes were formed in the presence of [α-^{32}P]ATP and, after complex isolation from free ATP by gel filtration, analyzed for the nucleotide associated with DnaK by thin layer chromatography (Figure 7). The nucleotide bound to DnaK in the absence of σ^{32}-C-his or co-chaperone was a mixture of ATP (~70%) and ADP (~30%). When σ^{32}-C-his was included during incubation DnaK–σ^{32}-C-his complexes eluted in fractions (8–10, peak fractions) overlapping with fractions containing free DnaK (fractions 10–12). The fractions associated with DnaK–σ^{32}-C-his complexes were enriched in ADP relative to those associated with free DnaK. Early fractions (6 and 7) contained only ADP. Inclusion of DnaJ during incubation with DnaK and σ^{32}-C-his (under conditions allowing efficient formation of DnaK–DnaJ–σ^{32}-C-his complexes) resulted in exclusive association of ADP with DnaK, including the fractions corresponding to ternary complexes (fractions 6–8). Inclusion of GrpE during incubation under conditions allowing formation of quaternary complexes of all three chaperones and σ^{32}-C-his (running buffer lacking ATP) resulted in complete displacement of nucleotide from DnaK. In this experiment

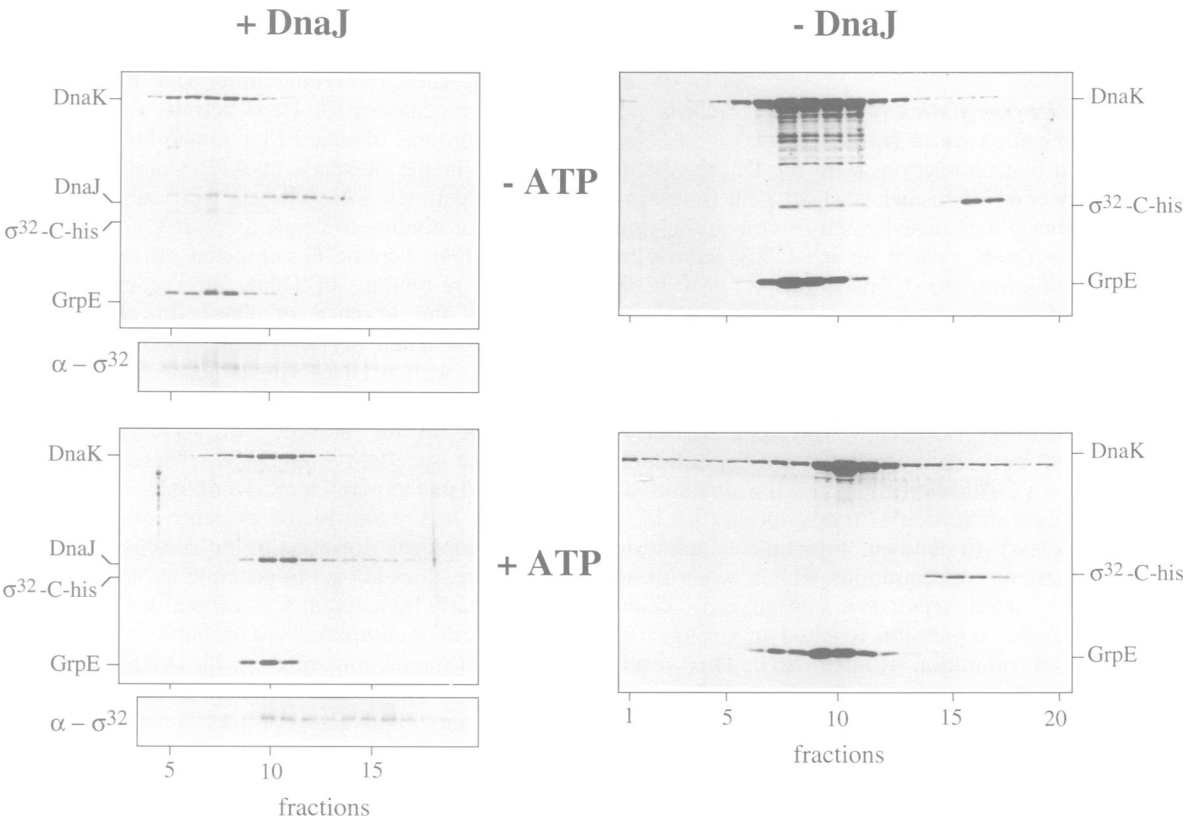

Fig. 6. GrpE allows formation and ATP-mediated dissociation of quarternary GrpE–DnaK–DnaJ–σ^{32}-C-his complexes. (Left) DnaK, DnaJ, GrpE and σ^{32}-C-his (final concentrations of 2, 4, 4 and 1 μM respectively) were incubated for 20 min at 30°C in buffer T containing ATP (1 mM), followed by gel filtration using a Superdex 200 column equilibrated in running buffer with or without 200 μM ATP. Eluted proteins were separated by SDS–PAGE and visualized either by silver staining or, for σ^{32}-C-his, immunoblot analysis with σ^{32}-specific antiserum. (Right) DnaK, GrpE and σ^{32}-C-his (9, 18 and 3 μM final concentrations respectively) were incubated and analyzed as described above. σ^{32}-C-his is poorly visualized by silver staining.

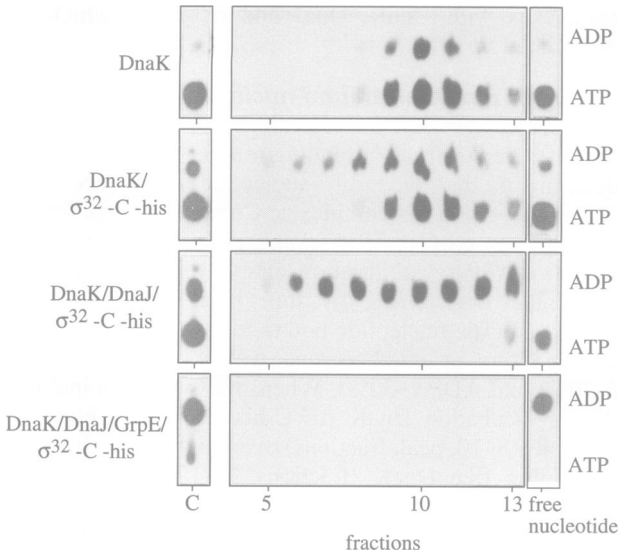

Fig. 7. Identification of nucleotide associated with chaperone–σ^{32}-C-his complexes. DnaK (2 μM), DnaJ (4 μM), GrpE (4 μM) and σ^{32}-C-his (1 μM) as indicated were incubated with [α-^{32}P]ATP for 7 min at 30°C and subjected to Superdex 200 gel filtration. Aliquots of the incubation mixture before injection (C) and the eluted fractions including free nucleotide (eluting in peak fraction 25) were analysed by TLC.

quaternary complex formation was verified by OD_{280} absorption. Together these experiments identified ATP in DnaK–σ^{32}-C-his and DnaK–DnaJ–σ^{32}-C-his complexes and demonstrate a lack of nucleotide in GrpE–DnaK–DnaJ–σ^{32}-C-his complexes.

DnaK and DnaJ synergistically repress σ^{32}-C-his activity in heat shock gene transcription

We hypothesized that interactions with the DnaK system affect the activity of σ^{32}-C-his in heat shock gene transcription. This hypothesis was investigated *in vitro* by testing the effects of the DnaK system on σ^{32}-C-his activity in run-off transcription from the σ^{32}-dependent P2 heat shock promoter of *dnaK* (Figure 8). Transcription from P2 was absolutely dependent on the presence of σ^{32}-C-his and the RNAP core enzyme in the transcription mixture. Addition of increasing amounts of DnaK, up to a 40-fold molar excess with respect to σ^{32}-C-his, or GrpE to the transcription mixture did not significantly affect transcription activity (Figure 8A). Addition of increasing amounts of DnaJ caused a slight inhibition of transcription (3-fold at 10-fold molar excess). In contrast, simultaneous addition of DnaJ and DnaK at concentrations which, when these chaperones were added separately, caused only slight (DnaJ) or no (DnaK) repression resulted in strong (10-fold) transcription inhibition (Figure 8B). This result indicates a synergistic action of DnaK and DnaJ in repression of σ^{32}-C-his activity. Addition of increasing amounts of GrpE to a transcription mixture containing DnaK and DnaJ resulted in partial reversal of repression (Figure 8B). The repression by DnaJ and DnaK is specific for σ^{32}-C-his, since activity of the *E.coli* housekeeping protein σ^{70} in transcription from a σ^{70}-dependent promoter ($P_{N25/O4/O3}$) was unaffected by DnaJ and/or DnaK at varying concentrations (Figure 8C). Together the effects of chaperones on σ^{32}-C-his activity are well correlated with the ability of these chaperones to bind and release σ^{32}-C-his in the presence of ATP. Accordingly, DnaJ and DnaK binding to σ^{32}-C-his inactivates σ^{32}-C-his.

Discussion

Our analysis of the interactions of DnaK, DnaJ and GrpE with σ^{32} identified an ATP-controlled σ^{32} binding/release cycle regulating σ^{32} activity in heat shock gene transcription (Figure 9). This cycle is proposed to constitute a central element in the chaperone-dependent regulation of the *E.coli* heat shock response. Furthermore, the presented data provide mechanistic insights into the functional cycle of the DnaK chaperone system.

DnaK and DnaJ bind independently to σ^{32}. The K_d of DnaK–σ^{32} complexes formed in the absence of ATP was ~5 μM, which is within the K_d range of other DnaK substrates. DnaK–σ^{32} complexes formed slowly and were highly stable ($t_{1/2}$ 40 min at 30°C). ATP destabilized these complexes, presumably by increasing the dissociation rate (Schmid *et al.*, 1994). The K_d of DnaJ–σ^{32} complexes was 19 nM, a value ~260-fold lower than the K_d of DnaK–σ^{32} complexes. We note that the k_a and k_d values for the DnaJ–σ^{32} complex (3.3×10^5/M/s and 6.2×10^{-3}/s respectively) are 5900-fold and 20-fold higher respectively than the approximate values for the DnaK–σ^{32} complex (56/M/s and 2.8×10^{-4}/s respectively). To our knowledge, these binding constants are the first reported for DnaJ and DnaK interactions with protein substrates.

DnaJ increases the efficiency of DnaK binding to σ^{32} in the presence of ATP and leads to formation of DnaK–DnaJ–σ^{32} complexes (this study; Liberek and Georgopoulos, 1993) containing ADP. Further information on the mechanism of DnaJ activity in ternary complex formation was obtained by competition experiments performed in the presence of ATP. Complexes of σ^{32} with the chaperones were chased with substrates competing either for binding to DnaK (peptide C) or DnaK and DnaJ (σ^{32}-C-his). Peptide C competed efficiently with σ^{32}-C-his for re-binding of DnaK after complex dissociation only in the absence of DnaJ. In contrast, σ^{32}-C-his competed efficiently with (labeled) σ^{32} for re-binding of DnaK as well as DnaJ. This indicates that binding of DnaJ to σ^{32} allows DnaK to specifically associate with this substrate in the presence of ATP, thereby conferring substrate specificity on DnaK. These experiments also revealed that ternary DnaK–DnaJ–σ^{32} complexes are significantly less stable in the presence of ATP than DnaK–σ^{32} complexes formed in the absence of ATP. DnaJ, therefore, does not act to generate ternary complexes with particularly high stability as generally assumed. However, these ternary complexes are probably more stable than the transient interactions of σ^{32} with DnaK·ATP (see below).

The ATP-dependent formation of DnaK–DnaJ–σ^{32} complexes may involve several mechanisms. Binding of ATP to DnaK induces conformational changes in its substrate binding region (Buchberger *et al.*, 1995) and leads to destabilization of DnaK–substrate complexes (Schmid *et al.*, 1994; McCarty *et al.*, 1995). However, it is important to note, and perhaps essential for DnaK association with σ^{32}, that DnaK·ATP exhibits a significantly higher association rate for substrate binding than DnaK·ADP (Schmid *et al.*, 1994). DnaJ stimulates hydrolysis of DnaK-bound

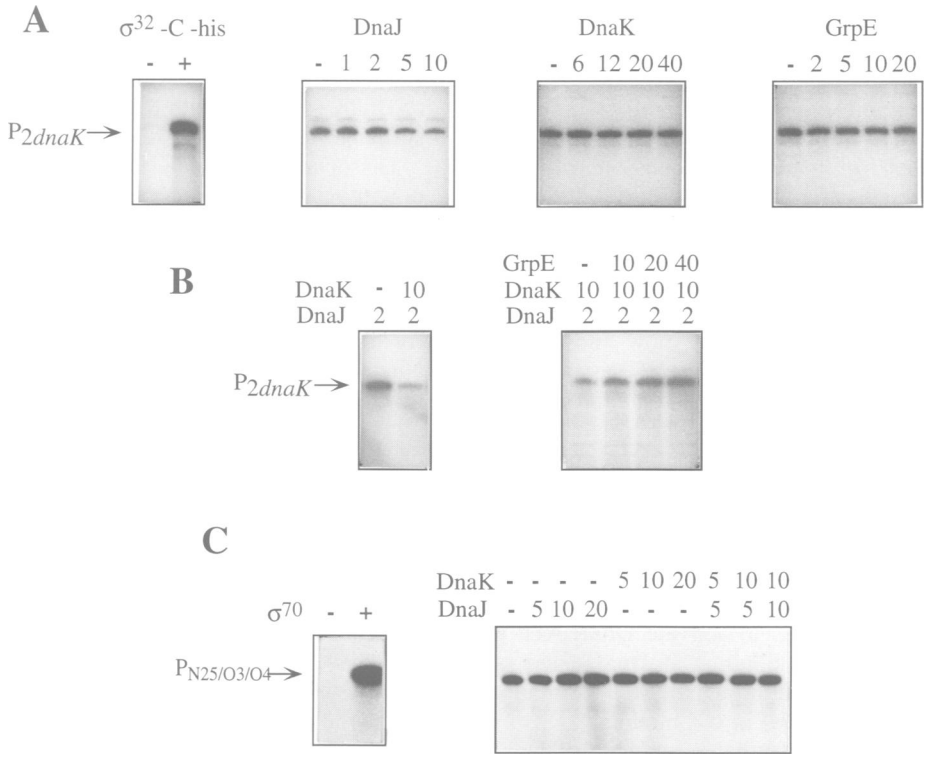

Fig. 8. Control of σ^{32}-C-his activity by DnaK, DnaJ and GrpE. *In vitro* run-off transcriptions are shown from linear DNA templates containing the σ^{32}-dependent P2 promoter of the *dnaK* gene (**A** and **B**) and the σ^{70}-dependent $P_{N25/O4/O3}$ promoter (**C**). To transcription assay mixtures containing RNAP core and σ^{32}-C-his (A and B) or σ^{70} (C) were added DnaK, DnaJ and GrpE at the indicated molar excesses with respect to σ^{32}-C-his or σ^{70}. Transcripts were analyzed by polyacrylamide–urea gel electrophoresis followed by autoradiography.

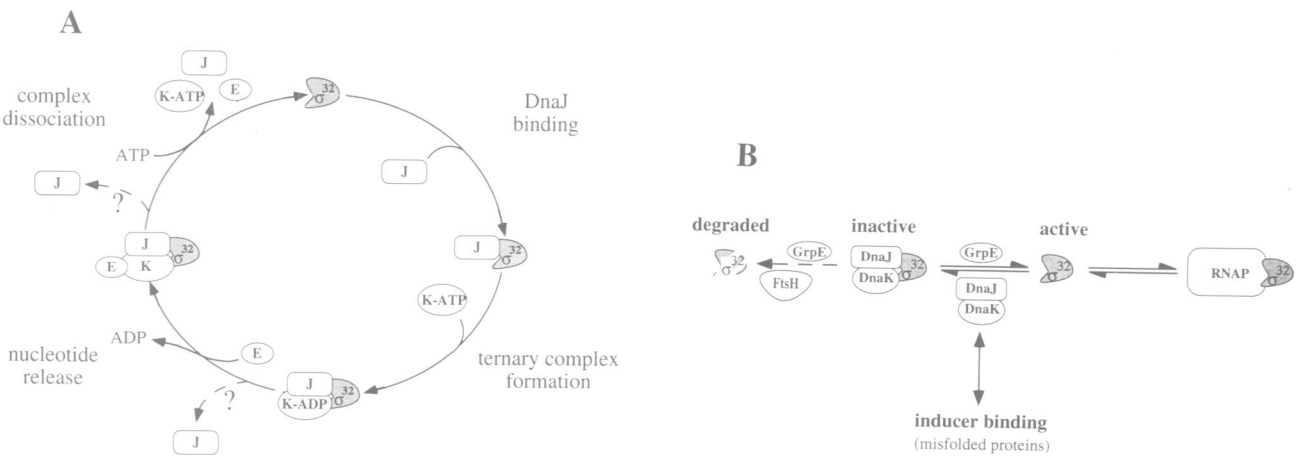

Fig. 9. Homeostatic control of σ^{32} activity and stability by a chaperone binding/release cycle. (**A**) Model of the chaperone binding/release cycle. The cycle starts with rapid association of DnaJ (J) with σ^{32}. DnaK (K), in its ATP-bound form, interacts with σ^{32} only transiently. DnaJ associated with σ^{32} interacts with DnaK·ATP and, through rapid stimulation of ATP hydrolysis by DnaK, targets DnaK to σ^{32}, promotes DnaK binding to σ^{32} and mediates efficient formation of DnaK–DnaJ–σ^{32} complexes containing ADP. GrpE (E) can associate with the DnaK component of this complex, thereby triggering nucleotide release and forming quaternary GrpE–DnaK–DnaJ–σ^{32} complexes lacking nucleotide. Spontaneous nucleotide release in the absence of GrpE might also occur, but is considered less important, given that GrpE has an essential role in σ^{32} regulation *in vivo*. Binding of ATP induces complex dissociation. It is possible that DnaJ dissociates from ternary or quaternary complexes independently of DnaK, thereby allowing it in principle to act in this cycle in substoichiometric amounts. (**B**) Model of chaperone-mediated homeostatic control of σ^{32} activity and stability. σ^{32} exists either as an active free form that can assemble with RNAP to direct heat shock gene transcription or as an inactive form bound to DnaJ and DnaK. Inactive σ^{32} can be activated by chaperone dissociation mediated by GrpE-stimulated nucleotide exchange. σ^{32} can be eliminated through DnaK-, DnaJ- and GrpE-dependent degradation by FtsH (Herman *et al.*, 1995; Tomoyasu *et al.*, 1995) and perhaps additional proteases. During induction of the heat shock response the equilibrium is shifted towards active σ^{32} through sequestering of the DnaK system by binding to misfolded proteins. Upon protein damage repair the DnaK system becomes available and the homeostatic balance is re-established. Elements of this model, in particular the sequestering of chaperones through binding to misfolded proteins, have been described earlier (Craig and Gross, 1991; Bukau, 1993; Yura *et al.*, 1993).

ATP (Liberek *et al.*, 1991) by affecting the rate-limiting γ-phosphate cleavage step of DnaK ATPase (McCarty *et al.*, 1995). This stimulation is very rapid (McCarty *et al.*, 1995) and therefore has the potential to be a key initial event in ternary complex formation. We propose the following model for chaperone association with σ^{32}. Initially DnaJ associates at a high rate with σ^{32}, followed by DnaK·ATP association at a high rate with DnaJ–σ^{32} complexes. The role of ATP in DnaK association remains unclear. The interaction of DnaJ with DnaK, which has to be rapid since DnaJ acts rapidly to stimulate ATP hydrolysis, is ATP dependent (Wawrzynów and Zylicz, 1995) and might, therefore, drive DnaK·ATP association. The ability of ATP to increase the association rate of DnaK binding to substrates could also be a determining factor. During these initial interactions DnaJ mediates rapid conversion of DnaK·ATP to DnaK·ADP, thereby lowering the dissociation rate of the DnaK–substrate complex. This DnaJ-mediated mechanism of rapid complex formation would increase the affinity of DnaK for σ^{32} in the presence of ATP, consistent with the experimental data. In this scheme DnaJ has to activate DnaK·ATP at σ^{32} to allow efficient DnaK binding. If DnaJ acted on DnaK·ATP in solution the resulting DnaK·ADP formation would be very slow (k_a 56/M/s) in σ^{32} binding.

It has been reported recently that a fragment of DnaJ, deficient in substrate binding but proficient in DnaK ATPase stimulation, has retained the ability, when provided in excess, to increase the efficiency of DnaK binding to σ^{32} in the presence of ATP (Wall *et al.*, 1994, 1995; Liberek *et al.*, 1995). Our proposal is not in direct conflict with these data. We predict that this DnaJ fragment is less efficient in mediating DnaK association with σ^{32} and fails to target DnaK to σ^{32} in a competitive situation. Testing the latter prediction experimentally, however, is complicated by the possibility that ATP and DnaJ (wild-type and fragment) alter the association and dissociation rates of DnaK–substrate complexes differently, dependent on the particular substrate. Such differences may result in substrate-specific differences in the efficiency of complex isolation. Furthermore, slight overproduction of this DnaJ fragment in Δ*dnaJ* mutant cells was reported to restore the defects of these cells in regulation of HSP synthesis at 30°C (Wall *et al.*, 1995). However, the defects of Δ*dnaJ* mutants in regulation of the heat shock response after shift to 42°C were not rescued (D.Wall, personal communication), indicating limitations in the activity of the DnaJ fragment in heat shock gene regulation. In addition, as this DnaJ fragment is predicted to be non-functional in targeting DnaK to many cellular substrates, the pool of free DnaK available for σ^{32} binding might be increased to levels sufficient to cause the observed 2-fold decrease in basal expression of heat shock genes at 30°C.

The proposed role for DnaJ in chaperone association with σ^{32} has physiological implications. DnaJ mediates chaperone–σ^{32} complex formation even at low levels of available chaperone and σ^{32}. In addition, an ability of DnaJ to allow rapid formation of DnaK–DnaJ–σ^{32} complexes might confer on this process a kinetic advantage over the association of σ^{32} with RNAP. This advantage might be important, since DnaK and DnaJ do not stably interact with σ^{32} associated with RNAP. Our data do not support a regulation model in which the DnaK chaperone system 'strips' σ^{32} from RNAP (Georgopoulos *et al.*, 1990; Liberek and Georgopoulos, 1993), although we cannot exclude the possibility that transient interactions of DnaK and DnaJ with RNAP-bound σ^{32} exist which are sufficient to trigger σ^{32} release.

Dissociation of DnaK from complexes with σ^{32} (with or without DnaJ) is proposed to be controlled by nucleotide exchange. It is possible that DnaJ dissociates from DnaK–DnaJ–σ^{32} complexes independently of DnaK. Binding of ATP to DnaK triggers substrate release (Palleros *et al.*, 1993; McCarty *et al.*, 1995) and probably also accounts for the ATP-induced dissociation of DnaK from σ^{32}. Nucleotide exchange may occur spontaneously within a few minutes, as observed for Hsc70 (Ha and McKay, 1994), and thus account for the observed instability of ADP-containing DnaK–DnaJ–σ^{32} complexes in the presence of ATP. Alternatively, nucleotide exchange may be stimulated by GrpE. We found that quaternary GrpE–DnaK–DnaJ–σ^{32} complexes lacking nucleotide dissociate on addition of ATP. This indicates that GrpE functions to increase the rate of nucleotide release, thereby allowing subsequent ATP binding and complex dissociation. GrpE may have the same function for DnaK–σ^{32} complexes, since GrpE prevented detectable association of DnaK with σ^{32} in the presence of ATP. GrpE has the potential to act as a timing factor that speeds up chaperone–σ^{32} association/dissociation cycles. We note that these cycles are rapid even without GrpE. However, the fact that GrpE is essential for regulation of σ^{32} activity and stability *in vivo* suggests that the role of GrpE in the σ^{32} binding/release cycle is physiologically important.

The mechanism driving the σ^{32} binding/release cycle (Figure 9A) might also drive the functional cycle of the DnaK system in protein folding (Szabo *et al.*, 1994; McCarty *et al.*, 1995). In particular, the activity of DnaJ in targeting DnaK to specific substrates and allowing rapid formation of DnaK–DnaJ–substrate complexes might be essential for this chaperone system to compete successfully with folding reactions leading to aggregation. Consistent with this possibility is that DnaJ binds to thermally inactivated firefly luciferase, thereby preventing its aggregation (Schröder *et al.*, 1993), whereas DnaK does not prevent its aggregation and requires DnaJ to form complexes with luciferase. Furthermore, DnaJ fragments deficient in luciferase binding are also deficient in mediating DnaK system-dependent folding of denatured luciferase (unpublished results). Our findings indicate that σ^{32} acts as a regular substrate in the functional cycle of the DnaK system. With respect to regulation of the stress response, the apparent similarity of the binding/release cycles of σ^{32} and other folding substrates suggests that competition between σ^{32} and other substrates for the same function of the DnaK system may occur.

The conditions leading to chaperone–σ^{32} complex formation in the presence of ATP correlate well with the conditions leading to σ^{32} inactivation. DnaJ slightly inactivates σ^{32} in transcription and cooperates with DnaK to inactivate σ^{32} efficiently. Inactivation is reversed in part by GrpE. Our data indicate an equilibrium between free active σ^{32} and DnaK/DnaJ-bound inactive σ^{32}. We propose that this equilibrium constitutes an important element for homeostatic regulation of heat shock gene expression (Figure 9B). This equilibrium is affected by availibility

of DnaK and DnaJ. Sequestering of these chaperones through binding to inducers of the heat shock response (e.g. misfolded proteins) shifts the equilibrium to active σ^{32}. In addition, chaperone sequestering might inhibit degradation of σ^{32}.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains are MC4100 [*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 deoC1 ptsF25 rpsR flbB301*], W3110 (F^-, λ^-, ATCC 27325), XL-1-blue [*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac* F'(*proAB lacI*^{q} *lacZ ΔM15* Tn*10*)] and BB2296 [MC4100, *dnaJ47*(*am*) Sp^R] (Gamer *et al.*, 1992). Cells were grown in Luria Broth or 2× YT (Sambrook *et al.*, 1989) supplemented with kanamycin (Km; 40 μg/ml final concentration) or ampicillin (Ap; 100 μg/ml final concentration) as indicated.

DNA manipulations and plasmid constructions

Plasmids pDMI.1 (Lanzer, 1988), pDS10 (Bujard, 1987) and pUHE211-1(*rpoH*) (Gamer *et al.*, 1992) have been described. For construction of pDS10(P2*dnaK*) the region −65 to +6 relative to the transcription start site of the P2 promoter of the *dnaKdnaJ* operon of MC4100 was amplified by PCR according to instructions provided by the supplier of *Taq* polymerase (Perkin-Elmer Cetus). As primers we used oligonucleotides (31mers) carrying *Xho*I and *Eco*RI restriction sites at their 5'-ends. The 73 bp amplified fragment was cloned into the *Xho*I and *Eco*RI sites of pDS10 to generate pDS10(P2*dnaK*). pUHE21-2fdΔ12(*rpoH*), encoding authentic σ^{32}, was constructed by cloning a *Bam*HI–*Pst*I fragment from pUHE211-1(*rpoH*) containing the 5'-end of the *rpoH* coding region and a *Pst*I–*Hin*dIII fragment from pUHE212-1(*rpoH*) (Gamer *et al.*, 1992) encoding the 3'-end of *rpoH* into pUHE21-2fdΔ12 (Buchberger *et al.*, 1994b).

In vitro transcription

Run-off transcription experiments were performed with a 360 bp *Xho*I–*Ase*I fragment of pDS10(P2*dnaK*) containing the P2 promoter of the *dnaKdnaJ* operon and a 120 bp *Xho*I–*Bam*HI fragment of pUHE212-1(*rpoH*) containing the $P_{N25/04/O3}$ promoter as templates for σ^{32}- and σ^{70}-dependent transcription respectively. The recessed 3'-termini of both fragments were filled in with Klenow fragment. σ^{32} or σ^{70} (each 120 nM) was mixed on ice with core RNAP (120 nM) and the appropriate DNA template (40 nM) in buffer T (20 mM Tris–HCl, pH 7.9, 200 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 5% glycerol) to give a total volume of 21 μl. DnaK, DnaJ and GrpE (in 1 μl) were added at concentrations as indicated and the samples were immediately shifted to 30°C. After pre-incubation for 10 min transcription was started by addition of 3 μl of a nucleotide mix [0.2 mM each of GTP, ATP and CTP, 0.02 mM UTP, 10 μM [α-^{32}P]UTP (3000 Ci/mmol)]. The reaction was stopped after 10 min by addition of 2 vol phenol/chloroform. After extraction of the aqueous phase 50 μl 95% formamide were added and the transcription products were analyzed by electrophoresis through 7% polyacrylamide–8 M urea gels and autoradiography using Fuji RX films.

Purifiation of σ^{32}-C-his

Cultures of BB2296 cells containing pDMI.1 and pUHE211-1(*rpoH*) were grown aerobically at 30°C in 2× YT containing Ap and Km. At an OD_{600} of 0.8, IPTG (500 μM final concentration) was added for 30 min followed by collection of the cells by centrifugation (6000 *g*, 10 min, 4°C). All following manipulations were done at 4°C. Wet cell paste (100 g) was resuspended in 170 ml cold lysis buffer (50 mM Tris–HCl, pH 7.0, 100 mM KCl, 5 mM β-mercaptoethanol, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1% lysozyme) and stirred for 30 min, followed by sonication (5×10 s, level 7; Branson sonifier) and centrifugation (35 000 *g*, 30 min). Soluble proteins were precipitated with ammonium sulfate (0.4 g/ml), resuspended and dialyzed for 12 h in buffer A (50 mM Tris–HCl, pH 7.0, 200 mM KCl, 5 mM β-mercaptoethanol). After centrifugation (100 000 *g*, 1 h) the supernatant was loaded onto a Ni^{2+}–nitrilotriacetic acid–agarose column (20 ml; Diagen) prepared according to the supplier's instructions and equilibrated with buffer A. Chromatography of the extract was performed using a FPLC system from Pharmacia LKB (flow rate 2 ml/min). Loaded columns were washed with 100 ml buffer B (buffer A plus 20 mM imidazole). To remove bound DnaK and GrpE 50 ml buffer C (buffer A plus 5 mM ATP and 5 mM $MgCl_2$) was added, followed by a 20 ml wash with buffer A. Bound protein was then eluted with a linear 20–200 mM imidazole gradient and fractions containing σ^{32}-C-his pooled and dialyzed for 20 h in buffer D (40 mM HEPES–KOH, pH 7.6, 100 mM KCl, 0.1 mM EDTA, 1 mM β-mercaptoethanol, 2.5% glycerol). After centrifugation (100 000 *g*, 1 h) the supernatant was loaded onto a Superose 12 gel filtration column (1.6×60 cm; Pharmacia LKB) equilibrated with buffer D. Fractions containing σ^{32}-C-his were pooled, dialyzed for 16 h in buffer E (40 mM HEPES–KOH, pH 7.6, 100 mM KCl, 0.1 mM EDTA, 1 mM β-mercaptoethanol, 10% glycerol) and stored at −70°C.

Purification and labeling of σ^{32}

Cultures of 20 l of BB2296 cells containing pDMI.1 and pUHE21-2fdΔ12(*rpoH*) were grown aerobically at 30°C in 2× YT containing Ap and Km. The first steps of purification of σ^{32}, including lysis of cells, ammonium sulfate precipitation and ion exchange chromatography using DEAE, were as described (Liberek *et al.*, 1992). All purification steps were at 4°C. After DEAE chromatography σ^{32}-containing fractions were pooled, precipitated with ammonium sulfate (0.4 g/ml), dialysed for 15 h in buffer I (20 mM imidazole, pH 7.0, 100 mM KCl, 0.1 mM EDTA, 1 mM β-mercaptoethanol, 2.5% glycerol) and loaded on an Affi Gel heparin column (5 ml; BioRad). After column loading (1 ml/ min) the column was successively washed with several volumes of buffer I and buffer II (buffer I plus 175 mM KCl), one volume of buffer III (buffer I plus 5 mM ATP and 5 mM $MgCl_2$) to remove bound DnaK and GrpE and one volume of buffer I. Bound σ^{32} was eluted with a linear gradient of 100–500 mM KCl in buffer I. Pooled fractions were dialyzed in buffer D (40 mM HEPES–KOH, pH 7.6, 100 mM KCl, 0.1 mM EDTA, 1 mM β-mercaptoethanol, 2.5% glycerol) and loaded on a Mono Q HR 5/5 column (1 ml; Pharmacia LKB) equilibrated with buffer D. The column was washed with buffer D and proteins were eluted with a linear gradient of 100–500 mM KCl in buffer D. Fractions containing σ^{32} were directly loaded on a Superdex 75 gel filtration column (1×40 cm; Pharmacia LKB) equilibrated in buffer E (40 mM HEPES–KOH, pH 7.6, 100 mM KCl, 0.1 mM EDTA, 1 mM β-mercaptoethanol, 10% glycerol). Fractions containing σ^{32} were pooled and stored at −70°C.

σ^{32} was labeled with *N*-succinimidyl[2,3-^{3}H]proprionate (NSP; 40 Ci/mmol; Amersham) as described (Begley and Chain, 1984). After incubation of σ^{32} (1 nmol) with [^{3}H]NSP (1 nmol) for 3 h at room temperature, free [^{3}H]NSP was separated from [^{3}H]σ^{32} by Sephadex G25 gel filtration.

Purification of other proteins and protein concentration determination

DnaK (Buchberger *et al.*, 1994b), GrpE (Schönfeld *et al.*, 1995), core RNAP and σ^{70} (Lowe *et al.*, 1978) were purified as described. DnaJ was purified as described (Zylicz *et al.*, 1985) with modifications to be published elsewhere (Schönfeld *et al.*, manuscript in preparation). Purity of all proteins was >95% as judged by SDS–PAGE. σ^{32}, DnaK, DnaJ and GrpE concentrations were determined by amino acid analysis. These determinations were used to calibrate BioRad assays. RNAP and σ^{70} concentrations were determined by BioRad assays using IgG as standard. For all proteins the molar concentrations indicated in the experiments are based on monomers.

Gel filtration

For all gel filtration experiments involving DnaK appropriate aliquots of DnaK stock solutions were diluted in 130 μl transcription assay buffer T and pre-incubated for 2 h at 30°C. This treatment allowed efficient conversion of DnaK oligomers (Schönfeld *et al.*, 1995) to monomers competent for substrate binding (J.S.McCarty, H.-J.Schönfeld, H.Schröder and B.Bukau, manuscript in preparation). Mixtures of DnaK, DnaJ, GrpE and σ^{32} were prepared in buffer T (130 μl total volume) as indicated, incubated at 30°C for 20 min followed by injection into the FPLC system (Pharmacia LKB) connected to an analytical Superdex 200 gel filtration column (1×20 cm; Pharmacia LKB) equilibrated in buffer T. All chromatographic steps were performed at 4°C with a flow rate of 0.4 ml/min. Eluted proteins were collected in 500 μl fractions, precipitated with acetone and subjected to SDS–PAGE, followed by silver staining or immunoblotting. Molecular weight standards and their peak elution fraction numbers were: apoferritin (443 kDa), fraction 5; alcohol dehydrogenase (200 kDa), fraction 8; β-amylase (150 kDa), fraction 9; transferrin (81 kDa), fraction 12; ovalbumin (45 kDa), fraction 14; carbonic anhydrase (29 kDa), fraction 17; ADP (0.5 kDa), fraction 25.

SDS–PAGE and immunological techniques

For SDS–PAGE protein samples/precipitates were resuspended in sample buffer (50 mM Tris–HCl, pH 6.8, 10 mM EDTA, 10% glycerol, 2% SDS, 5% β-mercaptoethanol), boiled for 5 min and subjected to electrophoresis in 12.5% polyacrylamide–SDS gels (Laemmli, 1970).

Immunoblots were developed with alkaline phosphatase-conjugated anti-rabbit IgG as secondary antibodies (Promega) (Harlow and Lane, 1988) as described. As primary antibodies we used 1:7000 and 1:50 000 dilutions of polyclonal rabbit antisera raised against histidine-tagged forms of DnaJ and σ^{32} respectively.

Determination of DnaK-bound nucleotide

DnaK, DnaJ, GrpE and σ^{32}-C-his as indicated were incubated for 7 min at 30°C in buffer T containing 10 µCi [α-^{32}P]ATP (800 Ci/mmol) and 100 µM unlabeled ATP (total reaction volume 130 µl). An aliquot of 0.1 µl of the mixture was spotted immediately before injection (indicated as C) and aliquots of 5 µl of the fractions immediately after elution were spotted onto thin layer chromatography plates (Merck) and dried. The plates were developed as described (Liberek *et al.*, 1991). and analyzed by autoradiography with Fuji RX films.

Surface plasmon spectroscopy

Surface plasmon resonance analysis (BIAcore system, Pharmacia Biosensor) of DnaJ binding to σ^{32} was done at 30°C in 1× HBS buffer [10 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% Surfactant P-20 (Pharmacia)] at a flow rate of 5 µl/ min according to standard protocols provided by the manufacturer. Authentic σ^{32} (90 ng in 10 mM NaOAc, pH 3.2) was coupled to the CM5 sensor chip via standard NHS/EDC activation chemistry (BIAcore amine coupling kit) and yielded 2200–3500 response units (RU). DnaJ at the indicated concentrations (in 1× HBS) was injected for 4 min. The sensor chip was cleaned of DnaJ bound to immobilized σ^{32} by injection of 30 µl regeneration solution (20 mM HCl). Binding parameters of the DnaJ–σ^{32} interaction were calculated using BIAevaluation version 2.1 (Pharmacia). The baselines for the curves shown in Figure 3 were adjusted to zero (12 385 RU absolute response before adjustment, 3564 RU relative response for σ^{32}) because the dissociation model equation represents an exponential decrease in the signal to a value of zero at $t = \infty$. The model calculated the k_d by fitting data to the equation $R = R_0\, e^{-Kd\,(t - to)}$.

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