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Physical Interaction between Heat Shock Proteins DnaK, DnaJ, and GrpE and the Bacterial Heat Shock Transcription Factor σ^{32}

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Summary

Genetic evidence indicates central roles for Hsp70 chaperones in the regulation of heat shock gene expression. This regulatory function has been postulated for Escherichia coli to rely on the direct association of DnaK (Hsp70) with the heat shock transcription factor σ^{32}. This report presents evidence for the physical association of DnaK, DnaJ, and GrpE chaperones with σ^{32} in vivo. Surprisingly, an interaction of DnaJ with σ^{32} exists that is distinguishable from an interaction of DnaK and GrpE with σ^{32}: addition of ATP disrupts the association of DnaK and GrpE with σ^{32}, but not the association of DnaJ with σ^{32}. Furthermore, DnaJ-σ^{32} and DnaK-σ^{32} associations occur independent of DnaK and DnaJ, respectively. These results suggest distinct regulatory functions of DnaJ and DnaK/GrpE.

Introduction

The heat shock response is an important homeostatic mechanism that enables cells to survive a variety of environmental stresses. Heat shock proteins (HSPs) appear to be constituents of the cellular machinery of protein-folding, degradation, and repair (Skowyra et al., 1990; Martin et al., 1991; reviewed in Morimoto et al., 1990; Rothman, 1989). The functions of members of the highly conserved Hsp90, Hsp70, and Hsp60 protein families in assisting protein folding processes led to their designation as molecular chaperones (Ellis, 1987). Given these fundamental biological functions of HSPs, it is not surprising that their cellular concentration is tightly coupled to the environmental and metabolic status of the cell and that altering their levels of expression results in cellular defects (Bukau and Walker, 1989a, 1989b, 1990).

The molecular principles that govern the regulation of expression of heat shock genes are only partially understood. In Escherichia coli, heat shock gene expression requires the heat shock–specific σ subunit of RNA polymerase, σ^{32}, which confers to core RNA polymerase the specificity to transcribe heat shock genes (Grossman et al., 1984; Cowing et al., 1985). Regulation of heat shock gene expression is mediated by controlling the cellular concentration as well as the activity (Straus et al., 1989) of σ^{32}. The cellular concentration of σ^{32} is controlled by regulation of the transcription and the translation of *rpoH* (Erickson et al., 1987; Grossman et al., 1987; Nagai et al., 1990; H. Nagai and T. Yura, personal communication), the gene encoding σ^{32}, and regulation of the stability of σ^{32} (Grossman et al., 1987; Straus et al., 1987, 1989, 1990; Tilly et al., 1989). In fact, one of the most remarkable features of σ^{32} is its extremely short half life ($t_{1/2}$ = 1 min) at steady-state growth conditions (Grossman et al., 1987; Straus et al., 1987).

Genetic evidence indicates key regulatory functions for the HSPs DnaK (a member of the Hsp70 protein family), DnaJ, and GrpE at the levels of synthesis, activity, and degradation of σ^{32}. Mutations in *dnaK*, *dnaJ*, and *grpE* cause partial stabilization of σ^{32} (Straus et al., 1990; Tilly et al., 1989); loss of repression of heat shock gene transcription normally found in wild-type cells after temperature downshift (Straus et al., 1989; Tilly et al., 1983); and deficiencies in posttranscriptional regulation of σ^{32} synthesis after heat shock (Grossman et al., 1987; Straus et al., 1990). The mechanism by which DnaK, DnaJ, and GrpE regulate the activity and stability of σ^{32} is assumed to rely on their concerted activity as chaperones. This activity involves the ATP-dependent binding to substrates of DnaK and the stimulation of hydrolysis of DnaK-bound ATP by DnaJ and GrpE (Liberek et al., 1991a, 1991b; Skowyra et al., 1990). It has been proposed that DnaK interacts with σ^{32} and dissociates it from RNA polymerase, thereby rendering it accessible to cellular proteases (Georgopoulos et al., 1990). Consistent with this model is the earlier observation that preparations of purified E. coli RNA polymerase holoenzyme exhibit slight cross-reactivities with antisera raised against DnaK (Skelly et al., 1988).

The signal transduction pathway that converts environmental stress to specific alterations in the transcription of heat shock genes remains unclear. There is some evidence to suggest that the intracellular concentration of aberrant proteins is a major determinant of the cellular concentration of HSPs in E. coli as well as in eukaryotic cells (Ananthan et al., 1986; Gaitanaris et al., 1990; Goff and Goldberg, 1985; Kozutsumi et al., 1988; Parsell and Sauer, 1989). An attractive general model is that by sequestering Hsp70 through its binding to aberrant proteins, induction of the heat shock response is triggered (Craig and Gross, 1991; Straus et al., 1990). Sequestering Hsp70 prevents the heat shock transcription factor (e.g., σ^{32}) from interacting with Hsp70, which in turn allows activation of heat shock gene transcription (Craig and Gross, 1991). Although genetic evidence supporting this model exists, its key prediction, the existence of physical interactions between Hsp70 proteins and heat shock transcription factors, remains to be demonstrated.

Here we report the association of the DnaK, DnaJ, and GrpE proteins with σ^{32} in vivo. Unexpectedly, we observed an association of DnaJ with σ^{32} that can be distinguished from an association of DnaK and GrpE with σ^{32}. Our results indicate distinct functions for DnaJ and DnaK/GrpE in regulating E. coli heat shock gene expression and indicate that the heat shock regulatory circuits proposed previously should be reevaluated.

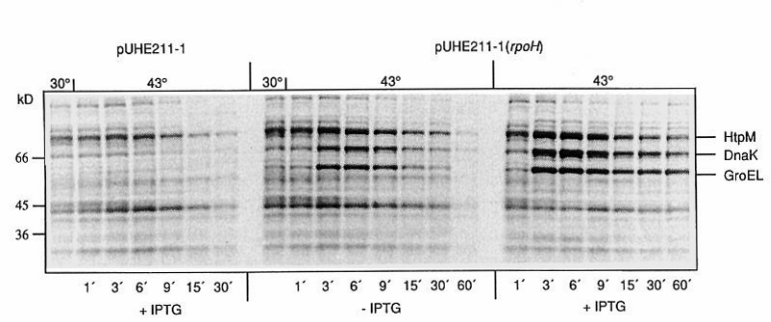

Figure 1. Heat Shock Response of *rpoH165(am)* Mutants Mediated by Histidine-Tagged σ^{32}

Cultures of *rpoH165(am)* mutants (BB2073) carrying pDMI,1 and pUHE211-1(*rpoH*) or pUHE211-1 were grown at 30°C and then shifted to 43°C in the presence (+IPTG) or absence (−IPTG) of IPTG. Aliquots were pulse-labeled with [^{35}S]methionine before or at the indicated times after heat shock. The proteins were separated by SDS–PAGE and autoradiographed. The migration positions of major HSPs (right) and molecular weight standards (left) are indicated.

Results

Histidine-Tagged σ^{32} Is Functionally Active In Vivo

Short stretches of histidine residues attached to C- or N-termini of proteins allow the purification of these proteins from crude lysates by nickel–nitrilotriacetic acid affinity chromatography (Hochuli et al., 1987, 1988). We used this principle of immobilized metal ion affinity chromatography (IMAC) for the purification of σ^{32} and the identification of proteins associated with σ^{32}. The *rpoH* gene was cloned into pUHE21 plasmids such that, upon induction with isopropyl-β-D-thiogalactopyranoside (IPTG), they express recombinant σ^{32} proteins with six additional histidine residues at their C-termini (σ^{32}-C-his) or N-termini (σ^{32}-N-his). These histidine-tagged proteins were evaluated by the following criteria: attachment of histidine tags does not affect the in vivo activity of σ^{32}; the histidine tags are surface exposed and accessible for chelation of Ni^{2+}; and chelation of Ni^{2+} is not preventing specific protein–protein interactions of σ^{32}.

The in vivo activity of the σ^{32}-C-his and σ^{32}-N-his was determined by complementation analysis of *rpoH165(am)* mutants (BB2073). These mutants fail to produce functional σ^{32} at 42°C and therefore are unable to exhibit a heat shock response and to grow at elevated temperature (Yamamori and Yura, 1982). However, when σ^{32}-C-his or σ^{32}-N-his were provided by the plasmids pUHE211-1(*rpoH*) and pUHE212-1(*rpoH*), respectively, the temperature sensitivity of *rpoH165(am)* mutants was abolished. In pUHE211-1(*rpoH*) and pUHE212-1(*rpoH*), the expression of *rpoH* is controlled by a promoter–lac operator system, whereby the lac repressor is provided by pDMI,1 (Lanzer, 1988). In the absence of IPTG, the intracellular concentration of σ^{32}-C-his or σ^{32}-N-his is very low (less than 4-fold the level of σ^{32} in wild-type cells). The finding that under these conditions full complementation of the growth defects of *rpoH165(am)* mutants is observed indicates high functional activity of these proteins. Moreover, expression of σ^{32}-C-his can induce a mild heat shock response in *rpoH165(am)* cells even in the absence of IPTG and a strong heat shock response in the presence of low concentrations (50 μM) of IPTG (Figure 1). In contrast, a heat shock response was not observed in *rpoH165(am)* mutants carrying the *rpoH*-free pUHE211-1 plasmid (Figure 1). Thus, it is the activity of σ^{32}-C-his that is responsible for the observed heat shock response. Furthermore, the heat shock responses in σ^{32}-C-his-synthesizing *rpoH165(am)* mutants were transient and kinetically indistinguishable from heat shock responses in wild-type cells. This indicates that the DnaK-, DnaJ-, and GrpE-mediated modulation of the heat shock response is not affected by histidine tagging of σ^{32}. Taken together, our results show that σ^{32}-C-his and σ^{32}-N-his are functionally highly active in vivo as specific transcriptional activators of heat shock genes.

One-Step Purification of Native Histidine-Tagged σ^{32} by IMAC

We examined whether the histidine tag of σ^{32}-C-his is exposed at the surface of the native protein and would thereby allow complexation to Ni^{2+}. Cultures of wild-type cells carrying pDMI,1 and pUHE211-1(*rpoH*) were partially induced with IPTG, such that they contain elevated but not maximal levels of σ^{32}-C-his. Soluble extracts of these cells, obtained by mild, detergent-free lysis, were loaded onto a Ni^{2+} column. Proteins that were bound to the column were eluted by competing with the protein ligands, using 0–150 mM imidazole gradients and characterized by SDS–polyacrylamide gel electrophoresis (PAGE) (Figure 2A). The great majority of cellular proteins did not bind to the Ni^{2+} column and were recovered in the flow through. A small fraction exhibited weak affinity with the column and was eluted at low concentrations (10–30 mM) of imidazole (Figure 2A; fractions 5–7). In contrast, two major protein species with apparent molecular masses of 35 kd and 70 kd and five minor protein species, some of which are difficult to detect in the photograph of the Coomassie-stained SDS–PAGE gel (Figure 2A), were efficiently complexed to the Ni^{2+} column and specifically eluted at higher concentrations (up to 150 mM) of imidazole. The major 35 kd protein eluted with a peak at around 110 mM imidazole (Figure 2A; fraction 22). This protein was identified as σ^{32}-C-his by immunoblot analysis (Figure 2B). Three of the minor protein species (molecular mass of less than 35 kd) also reacted with anti-σ^{32} serum, indicating that they are proteolytic degradation products of σ^{32}-C-his. An efficient complexation to Ni^{2+} columns was also found for σ^{32}-N-his (data not shown). We conclude that histidine tags attached either to the N-terminus or the C-terminus of σ^{32} are exposed at the surface of the native proteins and are accessi-

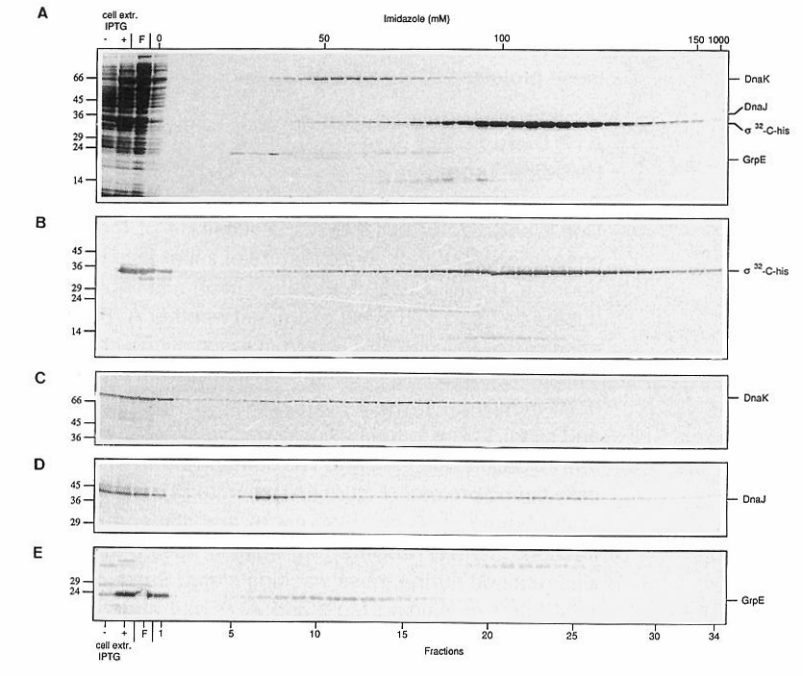

Figure 2. IMAC Purification of Histidine-Tagged σ32

Cultures of SG13009 cells carrying pDMI,1 and pUHE211–1(*rpoH*) were induced for 20 min with IPTG, and extracts of the cells were prepared for IMAC according to the standard procedure. A Ni^{2+} column was loaded with the extracts, washed with buffer A, and developed by imidazole gradients. Aliquots of the cell culture taken before (–) and after (+) IPTG induction, of the flow through (F), and of fractions of the imidazole gradient were separated by SDS–PAGE and visualized by Coomassie staining of the gel (A) or analyzed by immunoblots with antisera raised against σ32 (B), DnaK (C), DnaJ (D), and GrpE (E). The extracts used for Coomassie staining (A) and for immunoblots (B–E) were obtained by independent IMAC purifications. The slight shift of the peak elution fractions of the proteins seen in the Coomassie-stained gel (A) as compared with the proteins seen in the immunoblots (B–E) is due to some degree of experimental variation. The migration positions of relevant proteins (right), molecular weight standards (left), and imidazole elution fractions (bottom) are indicated. DnaJ and GrpE are hardly visible in the photograph of the Coomassie-stained gel (A). The 35 kd band that is seen in (E) is due to cross-reactivity of the GrpE-specific sera with the histidine tail of σ32-C-his (see Experimental Procedures).

ble to complexation with Ni^{2+}. Thus, IMAC allows the efficient purification of active σ32 from crude lysates in a single step.

DnaK, GrpE, and DnaJ Physically Interact with Histidine-Tagged σ32

The three proteins that copurified with σ32-C-his during IMAC are candidates for proteins that are associated with σ32. The major copurifying 70 kd protein eluted between 30–120 mM imidazole with peak fractions at approximately 70 mM imidazole (Figure 2A; fractions 13–14). The molecular weight of this protein and preexisting genetic data (see Introduction) led us to hypothesize that this protein was DnaK. We therefore subjected the imidazole elution fractions to immunoblot analysis with DnaK-specific antisera. Clearly, the 70 kd protein is identical to DnaK (Figure 2C). The two minor copurifying proteins have apparent molecular masses of 40 kd and 20 kd and eluted with peaks at approximately 110 mM imidazole (fraction 22) and 60–70 mM imidazole (fractions 12–13), respectively. We considered that these proteins might be identical to the two other components of the DnaK chaperone machinery, DnaJ and GrpE. Immunoblot analysis of the imidazole elution fractions with DnaJ- and GrpE-specific antisera revealed that the 40 kd and 20 kd proteins are, in fact, DnaJ and GrpE, respectively (Figures 2D and 2E). We also noticed that the imidazole elution peaks of DnaK and GrpE were shifted by 8–10 fractions to lower concentrations relative to the imidazole elution peak of σ32-C-his, whereas the elution peak of DnaJ exactly matched that of σ32-C-his. This will be discussed below. Taken together, these experiments show that DnaK, DnaJ, and GrpE all copurify with σ32-C-his. Identical results were obtained with σ32-N-his (data not shown).

Although the copurification of DnaK, DnaJ, and GrpE and histidine-tagged σ32 during IMAC is likely to be due to association of these HSPs with σ32, our experiments do not exclude the possibility that DnaK, DnaJ, and GrpE are complexed to Ni^{2+} columns independent of histidine-tagged σ32. To test this possibility, we determined the affinities of DnaK, DnaJ, and GrpE to Ni^{2+} columns in the absence of histidine-tagged σ32. For this purpose, we cloned the *rpoH* gene into pUHE21–2 plasmids such that they encode untagged σ32. Wild-type cells carrying pUHE21–2(*rpoH*) and pDMI,1 plasmids were partially induced with IPTG such that the levels of DnaK, DnaJ, and GrpE were similar to the levels of these proteins in the σ32-C-his-synthesizing cells used for the experiment described in Figure 2. Soluble cell extracts were subjected to IMAC, and the proteins that were bound to the Ni^{2+} column were eluted by a 0–150 mM imidazole gradient and analyzed by SDS–PAGE and immunoblotting (Figures 3A–3D). The majority of the σ32, DnaK, DnaJ, and GrpE proteins did not bind to the Ni^{2+} column, and only a minor fraction was eluted at low concentrations of imidazole with peaks at approximately 20 mM (fractions 4–5). These elution peaks were shifted by 17 fractions for σ32 and DnaJ and 9 fractions for DnaK and GrpE to lower imidazole concentrations as compared with the elution peaks of these proteins in the presence of σ32-C-his (see Figures 2A–2E). Thus, DnaK, DnaJ, GrpE, and σ32 possess only weak intrinsic affinities to Ni^{2+} columns. We conclude that the specific binding of

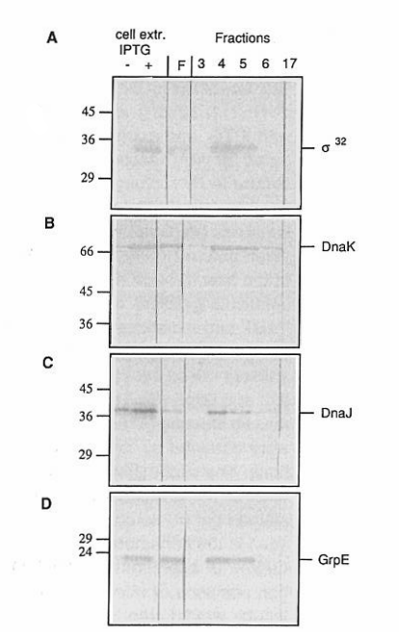

Figure 3. Affinity of DnaK, DnaJ, and GrpE to Ni^{2+} Columns in the Absence of Histidine-Tagged σ^{32}

A culture of SG13009 cells carrying pDMI,1 and pUHE21-1(*rpoH*), encoding untagged σ^{32}, was induced for 20 min with IPTG and prepared for IMAC. A Ni^{2+} column was loaded with the cell extract, washed with buffer A, and developed by an imidazole gradient. Aliquots of the cell culture taken before (−) and after (+) IPTG induction, of the flow through (F), and of the indicated imidazole gradient elution fractions were separated on SDS–PAGE and analyzed by immunoblots with antisera raised against σ^{32} (A), DnaK (B), DnaJ (C), and GrpE (D). The migration positions of HSPs and σ^{32} (right), molecular weight standards (left), and imidazole elution fractions (top) are indicated.

DnaK, DnaJ, and GrpE to Ni^{2+} columns in the presence of histidine-tagged σ^{32} results from physical interaction of these proteins with tagged σ^{32}.

ATP Disrupts the Binding of DnaK and GrpE to Histidine-Tagged σ^{32}

A plausible reason for the association of σ^{32} with DnaK, DnaJ, and GrpE is that σ^{32} is a substrate of the DnaK chaperone. A characteristic feature of substrates that are bound to DnaK is their dissociation upon addition of ATP (Liberek et al., 1991b). We examined whether ATP is also effective in releasing σ^{32}-C-his from association with DnaK, DnaJ, and GrpE. A Ni^{2+} column was loaded with extracts of IPTG-induced wild-type cells that carry pUHE211-1(*rpoH*) and pDMI,1. The loaded column was washed first with 30 mM imidazole and then with Tris buffer to elute nonspecifically bound proteins (Figure 4A, lanes 1–12). The majority of the bound σ^{32}-C-his (Figures 4A and 4B) and a major fraction of DnaK ranging from 50% to 80% (Figure 4C) was retained during these washing steps. Subsequently, Tris buffer containing Mg^{2+} and ATP was added to the column, and this treatment quantitatively eluted DnaK as well as GrpE (Figures 4C and 4D; lanes 13–19). In contrast, σ^{32}-C-his remained complexed to the Ni^{2+} column even in presence of ATP and was eluted only with a 30–150 mM imidazole gradient (Figure 4B; lanes 27–34). In control experiments in which ATP was omitted from the elution buffer, DnaK and GrpE remained bound to the column during all wash steps and were eluted together with σ^{32}-C-his by the final 30–150 mM imidazole gradient (data not shown). Thus, ATP efficiently disrupts the association of

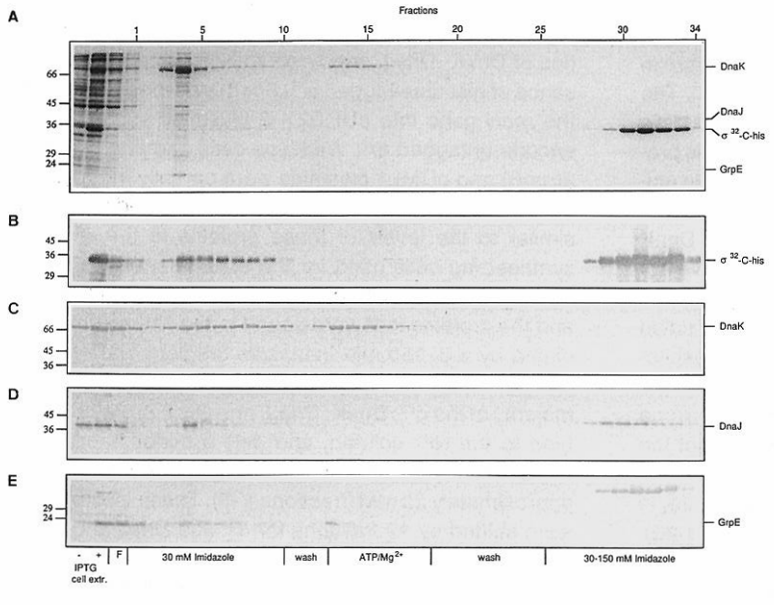

Figure 4. Effects of ATP on the Association of HSPs with Histidine-Tagged σ^{32}

Cultures of SG13009 cells carrying pDMI,1 and pUHE211-1(*rpoH*) were induced for 60 min with IPTG, and extracts were obtained and subjected to IMAC as described for Figure 2. The loaded Ni^{2+} column was washed consecutively with buffer A, buffer A containing 30 mM imidazole (20 ml), and buffer A (6 ml). Then the column was eluted by 12 ml of buffer A containing 5 mM ATP and 5 mM $MgCl_2$, washed with buffer A (20 ml), and further eluted by a 30–150 mM imidazole gradient. Aliquots of the cell culture taken before (−) and after (+) IPTG induction, of the flow through (F), and of the indicated imidazole and ATP/Mg^{2+} elution fractions were separated on SDS–PAGE and visualized by Coomassie staining of the gels (A) or analyzed by immunoblotting with antisera raised against σ^{32} (B), DnaK (C), DnaJ (D), and GrpE (E). The migration positions of HSPs and σ^{32}-C-his (right), molecular weight standards (left), and wash and elution steps (bottom) are indicated. The prominent 70 kd protein present in fraction 4 (A) is not DnaK; the 35 kd band that is seen in the 30–150 mM imidazole elution fractions in Figure 4E is due to cross-reactivity of the GrpE-specific sera with the histidine tail of σ^{32}-C-his (see Experimental Procedures).

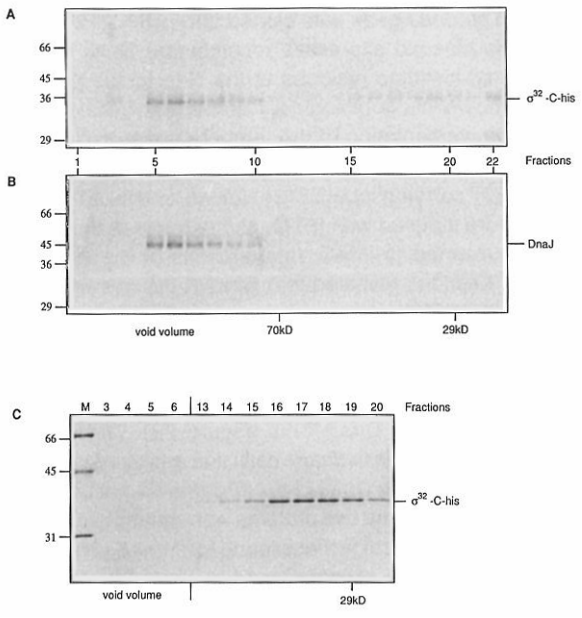

Figure 5. Gel Filtration of σ^{32}-C-his-Containing Imidazole Elution Fractions

Aliquots of fractions 32 (A and B) and 38 (C) of the ATP elution experiment shown in Figure 4 (up to fraction 34) were subjected to sizing chromatography using a Superdex 75 column. The column was eluted by buffer D, and aliquots (150 μl) of the collected fractions were subjected to SDS–PAGE and immunoblot analysis. (A) immunoblot with σ^{32}-specific antisera; (B) immunoblot with DnaJ-specific antisera; (C) Coomassie-stained protein gel. The positions of DnaJ and σ^{32}-C-his (right), molecular weight standards (left), and the Superdex 75 elution fraction numbers and sizes (bottom) are indicated.

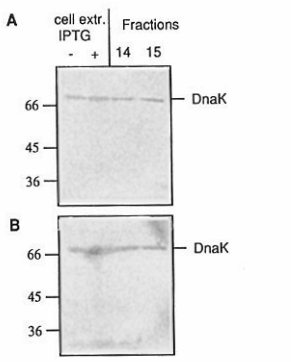

Figure 6. Association of DnaK with Histidine-Tagged σ^{32} in the Absence of DnaJ

Cultures of *dnaJ*$^+$ cells (SG13009) (A) and *dnaJ47(am)* mutants (BB2296) (B), each carrying pDMI,1 and pUHE211–1(*rpoH*), were induced for 30 min with IPTG and prepared for IMAC. A Ni^{2+} column was loaded with the extracts and then eluted by imidazole gradients according to the standard procedure. Aliquots of the cell cultures taken before (−) and after (+) IPTG induction and of the indicated imidazole gradient elution fractions were separated on SDS–PAGE and analyzed by immunoblots with antisera raised against DnaK. Fractions 14 and 15 correspond to 70–75 mM imidazole. The migration positions of DnaK (right), molecular weight standards (left), and imidazole elution fractions (top) are indicated.

σ^{32}-C-his with DnaK and GrpE, which provides evidence that σ^{32}-C-his is bound to DnaK (and GrpE) in a substrate-specific manner.

DnaJ Binds to Histidine-Tagged σ^{32} Independent of ATP

The behavior of DnaJ in the ATP elution experiment, shown in Figure 4, was surprising. In sharp contrast to DnaK and GrpE, DnaJ was not eluted from the Ni^{2+} column upon addition of ATP but instead was eluted together with σ^{32}-C-his by the subsequent 30–150 mM imidazole gradient (Figure 4D; fractions 27–34). This surprising result indicates that DnaJ itself, in the absence of DnaK and GrpE and independent of ATP, can efficiently and stably bind to σ^{32}-C-his.

The existence of complexes between DnaJ and σ^{32}-C-his was further demonstrated by sizing chromatography of two fractions of an ATP elution experiment that was performed as described in Figure 4. Fraction 32 of the 30–150 mM imidazole elution gradient contained σ^{32}-C-his and DnaJ but lacked DnaK and GrpE because of their previous elution by ATP; fraction 38 (not shown in Figure 4) is the last fraction of the 30–150 mM imidazole elution gradient and contained σ^{32}-C-his but no detectable DnaJ, DnaK, or GrpE. The σ^{32}-C-his contained in fraction 32 eluted from Superdex 75 columns in two peaks, one at 30–40 kd, which corresponds to the expected position of monomeric σ^{32}-C-his and another at the exclusion limit of the column (around 80 kd; Figure 5A). The DnaJ contained in fraction 32 eluted in only one peak at the exclusion limit of the Superdex 75 column, together with the high molecular weight fraction of σ^{32}-C-his (Figure 5B). In contrast, the σ^{32}-C-his present in fraction 38 was quantitatively eluted from the Superdex 75 column in only one peak at 30–40 kd (Figure 5C). These results indicate that in the presence of DnaJ, σ^{32}-C-his forms stable complexes having a molecular mass of at least 80 kd, while in the absence of DnaJ, DnaK, and GrpE, σ^{32}-C-his is monomeric.

DnaK and DnaJ Bind Independently to σ^{32}

The experiments described in the previous sections raise the question whether both DnaJ and DnaK bind directly to σ^{32}. Alternatively, only DnaJ may bind σ^{32}, and DnaK and GrpE may then associate with preformed DnaJ–σ^{32} complexes. Furthermore, it may well be that the binding of any of the three HSPs to σ^{32} requires cooperation by the other two HSPs. We first asked whether DnaK alone, in the absence of DnaJ, can bind to σ^{32}. To do this, σ^{32}-C-his was produced in *dnaJ47(am)* mutants (BB2296) that completely lack DnaJ function, and extracts of these cells were subjected to IMAC. In addition to σ^{32}-C-his (Figure 6B), a fraction of DnaK was specifically bound to the Ni^{2+} column and eluted at 40–70 mM imidazole with the peak at approximately 60 mM (Figure 6B; fractions 14–15). This elution profile of DnaK was qualitatively similar to the elution profile of DnaK in σ^{32}-C-his-producing *dnaJ*$^+$ cells (Figure 6A). Although there were no dramatic differences in the relative amounts of σ^{32}-C-his-associated DnaK observed comparing *dnaJ47(am)* and *dnaJ*$^+$ cells, the absolute amounts of DnaK and σ^{32}-C-his bound to the Ni^{2+} column were lower for *dnaJ47(am)* mutants. These differences are reflected

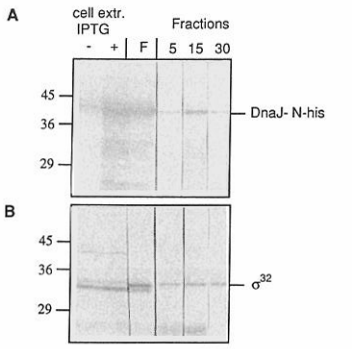

Figure 7. Association of σ^{32} with Histidine-Tagged DnaJ in the Absence of DnaK

A culture of *ΔdnaK52* mutants (BB1553) carrying pDMI,1 and pUHE212-1(*dnaJ*) were induced for 30 min with IPTG, and extracts were obtained and subjected to IMAC. A Ni^{2+} column was loaded with the extracts and then eluted by imidazole gradients according to the standard procedure. Aliquots of the cell culture taken before (−) and after (+) IPTG induction, of the flow through (F), and of the indicated imidazole gradient elution fractions were separated on SDS-PAGE and analyzed by immunoblots with antisera raised against DnaJ (A) and σ^{32} (B). Fractions 5, 15, and 30 correspond to approximately 20 mM, 70 mM, and 130 mM imidazole, respectively. The migration positions of DnaJ-N-his and σ^{32} (right), molecular weight standards (left), and imidazole elution fractions (top) are indicated.

in the concentrations of both proteins in extracts of *dnaJ47(am)* and *dnaJ*$^+$ cells. We were unable to detect GrpE in the imidazole elution fractions, which may be due to a low concentration of GrpE in the extracts of *dnaJ47(am)* cells.

We then determined whether the formation of DnaJ–σ^{32} complexes requires DnaK. The most direct experiment to show this is to identify DnaJ–σ^{32}-C-his associations in *ΔdnaK52* mutants that lack DnaK. However, this experiment was unsuccessful because *ΔdnaK52* mutants have very low levels of DnaJ (Sell et al., 1990), and the expression of σ^{32}-C-his proteins causes severe cellular defects in *ΔdnaK52* mutants even in the uninduced state (unpublished data), which is in agreement with earlier reports on *ΔdnaK52* mutants (Bukau and Walker, 1990). As an alternative approach, we examined whether untagged σ^{32} associates with histidine-tagged DnaJ in *ΔdnaK52* mutants. The *dnaJ* gene was cloned into pUHE212-1 such that this plasmid expresses recombinant DnaJ with six additional histidine residues at the N-terminus (DnaJ-N-his). DnaJ-N-his is biologically active, as revealed by in vivo complementation of the temperature sensitivity and λ resistance of *ΔdnaJ47* mutants. *ΔdnaK52* mutants (BB1553) carrying pUHE212-1(*dnaJ*) and pDMI,1 plasmids were induced with IPTG, and extracts of these cells were subjected to IMAC. Immunoblots of the imidazole elution fractions revealed that DnaJ-N-his was efficiently bound to the Ni^{2+} column and eluted at 40–120 mM imidazole with an elution peak at 70 mM (Figure 7A; fraction 15). A significant fraction of σ^{32} was specifically bound to the column and exhibited exactly the same imidazole elution characteristics as DnaJ-N-his (Figure 7B). We then expressed DnaJ-N-his in *dnaK*$^+$ cells and analyzed the copurification of σ^{32} with DnaJ-N-his (Figures 8A and 8B). The elution profiles of the two proteins were quantitatively and qualitatively identical to those found for *ΔdnaK52* mutants, indicating that the association of σ^{32} with DnaJ-N-his is as efficient in *ΔdnaK52* mutants as in *dnaK*$^+$ cells. Taken together, these experiments provide evidence that under our conditions, the formation of DnaJ-N-his–σ^{32} complexes occurs independently of DnaK, and the formation of DnaK–σ^{32}-C-his complexes occurs independently of DnaJ.

Discussion

This report presents evidence for the direct physical association of the HSPs DnaK, DnaJ, and GrpE with σ^{32}, the heat shock–specific transcription factor of E. coli. We demonstrate the specific copurification of these HSPs with σ^{32}. The purification strategy relied on the tagging of both σ^{32} and DnaJ with histidines that allowed isolation of σ^{32}–HSP complexes from crude E. coli lysates using IMAC. Since the histidine tagging did not affect the in vivo functions of either protein, this method may prove useful in complementing existing methods for studying protein–protein interactions.

When E. coli lysates containing σ^{32} tagged with a histidine hexamer were subjected to IMAC, three HSPs, DnaK, DnaJ, and GrpE, were retained with σ^{32} on a Ni^{2+} column. Moreover, IMAC of lysates containing authentic σ^{32} and

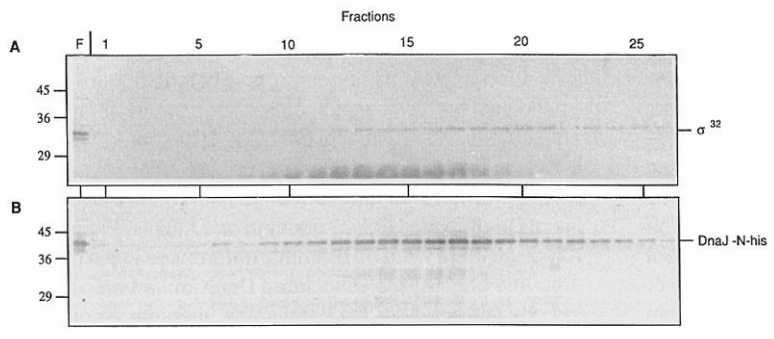

Figure 8. Association of σ^{32} with Histidine-Tagged DnaJ in the Presence of DnaK

A culture of SG13009 cells carrying pDMI,1 and pUHE212-1(*dnaJ*) was IPTG-induced and subjected to IMAC as described for Figure 7. A Ni^{2+} column was loaded with the cell extract, washed with buffer A, and developed by an imidazole gradient. Aliquots of the flow through (F) and of the indicated imidazole gradient elution fractions were separated on SDS-PAGE and analyzed by immunoblots with antisera raised against σ^{32} (A) and DnaJ (B). The imidazole concentrations of the elution fractions are similar to those of the elution fractions of the experiment shown in Figure 2. The 20 kd band present in fractions 10–20 (A) is a degradation product of σ^{32}. The migration positions of DnaJ-N-his and σ^{32} (right), molecular weight standards (left), and imidazole elution fractions (top) are indicated.

histidine-tagged DnaJ revealed the retention of σ^{32} together with DnaJ on a Ni^{2+} column. These findings provide strong evidence for the physical association of σ^{32} with DnaK, DnaJ, and GrpE in vivo. Together, our results allow us to exclude several potential experimental artifacts. Firstly, the tagging of σ^{32} and DnaJ with histidines did not affect the properties of these proteins in vivo. Furthermore, for histidine-tagged σ^{32} purified by Ni^{2+}-affinity chromatography, we have proved that it is active in in vitro transcription (unpublished data); thus, the histidine tag appears to interfere neither with the interaction between the σ^{32} subunit and core RNA polymerase nor with the recognition of the heat shock promoter sequence by the tagged σ factor. Together, this is strong evidence that the integrity of the structure and function of both proteins is maintained. Secondly, formation of σ^{32}–DnaJ complexes occurred regardless of whether the histidine tag is fused to the σ^{32} or the DnaJ moiety. This unequivocally excludes that the interaction of DnaJ with σ^{32} is mediated via the histidine tag of the σ^{32} protein.

Thirdly, formation of σ^{32}–DnaK complexes occurred regardless of whether σ^{32} was tagged at the N- or C-terminus. This largely excludes two formally possible artifacts. The first artifact is, should DnaK bind to the hexamer histidine moiety itself, it would be expected to affect the high affinity binding of σ^{32} to the Ni^{2+} chelating column. This is not observed, independently of whether the histidine tags are located at the N- or C-terminus of σ^{32}. The second artifact is, should DnaK bind to conformational distortions of σ^{32} induced by the terminal runs of histidines, one would have to predict a comparable effect of this moiety in both the N- and C-terminal portions of the molecule. This, however, appears highly unlikely. Fourthly, the following lines of evidence exclude the possibility that the σ^{32}–HSP complexes are protein aggregates that result from misfolding of σ^{32} due to its overproduction. First, the cells that were used for our experiments were only partially induced by IPTG (50 μM IPTG for 10–60 min) and therefore contain only intermediate levels of tagged σ^{32}. These levels of tagged σ^{32} did not affect continuous growth of the cells (data not shown). Second, DnaJ–σ^{32} complexes were present in cells that contained wild-type levels of untagged σ^{32} and histidine-tagged DnaJ. Third, under the inducing conditions used, tagged σ^{32} did not self-aggregate but rather existed as a monomeric species as long as DnaK, DnaJ, and GrpE were absent (see Figure 5C). Taken together, the demonstration of the physical association of σ^{32} with DnaK, DnaJ, and GrpE is strong support for genetic data indicating roles for these three HSPs in heat shock gene regulation in E. coli (see Introduction). Since Hsp70 proteins also appear to be involved in heat shock gene regulation in eukaryotes (Craig and Jacobsen, 1984), direct interactions between Hsp70 and heat shock transcription factors may play a role in these cells as well.

While the association of σ^{32} with DnaK may have been expected from genetic data, that of σ^{32} with DnaJ is surprising. It is quite interesting that both associations occur independently and have distinctly different biochemical properties. This conclusion is based on the findings that σ^{32}–DnaJ complexes existed in extracts of *ΔdnaK52* mutants, and σ^{32}–DnaK (and possibly GrpE) complexes existed in extracts of *dnaJ47(am)* mutants; that σ^{32}–DnaK/GrpE complexes were disrupted by ATP, whereas σ^{32}–DnaJ complexes persisted in the presence of ATP as well as after ATP elution of DnaK and GrpE; and that σ^{32}–DnaJ complexes were more stable in the presence of imidazole than the complexes between σ^{32} and DnaK/GrpE. The quantity of σ^{32}–DnaJ and σ^{32}–DnaK/GrpE complexes was not dramatically altered in extracts of *ΔdnaK52* mutants or *dnaJ47(am)* mutants as compared with extracts of wild-type cells. However, our experimental approach has limitations in the detection of less obvious quantitative differences in complex formation, and therefore we cannot rule out a possible cooperativity between DnaK and DnaJ in complex formation. Furthermore, our data provide no information on the relative affinities of DnaJ and DnaK for σ^{32}. For instance, it cannot be excluded that in wild-type cells that contain only 10–30 molecules per cell of σ^{32}, the number of σ^{32}–DnaK complexes is considerably smaller than the number of σ^{32}–DnaJ complexes, even though DnaK (approximately 5000 molecules per cell) is in a large excess over DnaJ (less than 200 molecules per cell) (Neidhardt and Van Bogelen, 1987). The ATP-dependent release of σ^{32} from DnaK is a property that is common to all known substrates of Hsp70 chaperones, and thus our data indicate that σ^{32} is bound to DnaK as a substrate. The concomitant ATP-mediated release of GrpE suggests that GrpE is associated with DnaK–σ^{32} complexes. The known ability of GrpE to interact physically with DnaK (Johnson et al., 1989) and to stimulate its ATPase activity (Liberek et al., 1991a) is likely to account for its coelution with DnaK. The lower abundance of GrpE as compared with DnaK in these complexes (as well as in total cell extracts) is consistent with a catalytic role of GrpE. The instability of σ^{32}–DnaK/GrpE complexes in the presence of imidazole is revealed by the shift of the DnaK/GrpE elution peaks by approximately eight fractions to lower imidazole concentrations as compared with the elution peaks of σ^{32} and DnaJ. Imidazole groups readily react with electrophilic groups, and this may disrupt ionic protein–protein interactions, especially if acidic proteins such as DnaK and GrpE are involved. In addition, imidazole has hydrophobic properties that may also account for the destabilization of σ^{32}–DnaK/GrpE complexes.

While our data clearly demonstrate specific σ^{32}–DnaK/GrpE and σ^{32}–DnaJ complexes, they do not exclude the existence of additional complexes that involve σ^{32} and all three proteins, DnaK, DnaJ, and GrpE. For instance, the possibility exists that σ^{32}–DnaK/GrpE complexes also contain DnaJ which, after ATP elution of DnaK and GrpE, either remains bound to σ^{32} or elutes and rebinds to other, monomeric σ^{32} molecules that are complexed to the column. Moreover, the stoichiometric relationships of the individual components of each complex have yet to be determined.

The demonstration that two HSPs independently bind to σ^{32} has major implications concerning the principles that govern the signal transduction pathway of the heat shock regulon. Our data are consistent with two alternative scenarios that may account for the roles of DnaK, DnaJ, and

GrpE in heat shock gene regulation at 30°C. According to one scenario, the first step is the binding of DnaJ to σ^{32}, which then targets DnaK and GrpE to bind to σ^{32}. The subsequent association of all three proteins, DnaJ, DnaK, and GrpE, renders σ^{32} biologically inactive and susceptible to degradation. To account for the stoichiometric differences of the DnaK and DnaJ bound to σ^{32}, it has to be postulated that the σ^{32}–HSP complexes either are very large and contain a large molar excess of DnaK, or DnaJ has a catalytic function and readily dissociates upon binding of DnaK to the σ^{32}–DnaJ complex. In addition to the DnaJ-mediated binding of DnaK to σ^{32}, a DnaJ-independent binding of DnaK to σ^{32} exists that might be nonspecific and eventually nonfunctional. A quite similar situation has been described for the binding of DnaJ and DnaK to the nucleoprotein complex that is formed at the origin of replication of bacteriophage λ during the initiation of DNA replication (Alfano and McMacken, 1989). According to a second scenario, DnaJ and DnaK compete for binding to σ^{32} and form independent σ^{32}–DnaJ and σ^{32}–DnaK/GrpE complexes. However, the biological consequences of such competition are unclear, especially since genetic data indicate that DnaK, DnaJ, and GrpE coordinately affect stability and activity of σ^{32} at 30°C (Straus et al., 1989, 1990; Tilly et al., 1989). For either proposed scenario, it remains unclear whether it is free σ^{32} or RNA polymerase–bound σ^{32} that is the substrate for binding to DnaJ and DnaK.

The mechanism for the transduction of temperature effects into the signal pathway of the heat shock regulon remains to be determined. It is tempting to speculate that the cellular thermometer that receives intracellular stress signals is not primarily DnaK, but rather the coordinate activity of DnaJ and DnaK. One of several possible signal pathways is that upon stress induction both DnaJ and DnaK get increasingly bound to damaged proteins and thus become less available for binding to σ^{32} (Craig and Gross, 1991). This could account for the transient stabilization of σ^{32} during the heat shock response. An alternative signal pathway is that temperature differentially affects the availability of DnaJ and DnaK for binding to σ^{32}. Upon heat shock, DnaK might become bound to damaged proteins and thereby be less available for associating with σ^{32}, while DnaJ might retain its availability for binding to σ^{32}. Association of σ^{32} with DnaJ in the absence of DnaK after heat shock, as opposed to the presence of DnaK at 30°C, may in fact increase the stability of σ^{32} by preventing its degradation. However, the only evidence suggesting divergent regulatory roles of DnaJ at different temperatures is the puzzling finding that *dnaJ* missense mutations increase stability of σ^{32} at 30°C but not after heat shock (Straus et al., 1990). An additional consequence of this signal pathway is that during stress induction, DnaJ might become unavailable for the more unspecific chaperone functions of DnaK because it is bound to σ^{32}. This transient lack of DnaJ function might temporarily freeze DnaK in a substrate-bound form with an ATPase activity that is lower than in the presence of DnaJ. Retardation of the release of DnaK-bound substrates might be biologically significant, since recent results indicate that chaperone-mediated refolding of some denatured proteins not only requires DnaK, DnaJ, and GrpE, but also GroEL and GroES (Martin et al., 1991; F.-U. Hartl and T. Langer, personal communication). These proteins, however, appear limiting in the initial phase of heat stress and must be newly synthesized. In a more general context, DnaJ-directed specific chaperone functions of DnaK, such as the regulation of σ^{32}, might be quick, while nonspecific chaperone functions of DnaK might be slow.

Our finding that DnaJ associates with σ^{32} increases the number of substrates that are known to bind to DnaJ in the absence of DnaK. DnaJ stably associates with plasmid P1-encoded RepA (Wickner, 1990), E. coli DnaB helicase (Liberek et al., 1990; Wold et al., 1982; Georgopoulos et al., 1990), and phage λ–encoded P protein (Georgopoulos et al., 1990). In these cases, DnaJ appears to target DnaK to bind to the preformed DnaJ–substrate complexes. A reasonable speculation is that DnaJ provides specificity to the DnaK chaperone, which, by itself, has a broad, degenerative substrate specificity. However, in the case of σ^{32}, it remains to be tested whether DnaJ indeed has such a targeting function for DnaK. In this context it will be interesting to examine the relationship between the members of the eukaryotic DnaJ and Hsp70 protein families.

Experimental Procedures

Bacterial Strains and Plasmids

Bacterial strains are listed in Table 1. Strain BB2064 was constructed by P1*vir* transduction as follows. We introduced into strain GW4701 first a *malPQ*$^+$ allele by selection for Mal$^+$ transductants, then a *recA* allele by selection for a linked Tc^r marker (*zfi*::Tn*10*) and screening for ultraviolet sensitivity. Strain BB2296 carries an amber stop codon at nucleotide 202 of the coding sequence of *dnaJ* and therefore lacks DnaJ$^+$ function. This mutant will be described in detail elsewhere. Plasmids pDMI,1 (*lacI*^{q}, Km^r) (Lanzer, 1988) and pUHE21–2 (Ap^r) (Bujard et al., 1987) have been described earlier. pUHE211–1 (for construction of C-terminal histidine fusions) and pUHE212–1 (for construction of N-terminal histidine fusions) closely resemble the previously described pDS56 RBSII-6XHis plasmids (Ap^r) (Stüber et al., 1990).

Media and Growth Conditions

Bacteria were grown aerobically at 30°C or at temperatures as indicated in 2 × YT or M9 minimal media (Miller, 1972) supplemented with glucose (0.4%), thiamine (0.001%), and appropriate amino acids (50 μg/ml). Antibiotics were used in the following concentrations: tetracycline (Tc), 10 μg/ml; kanamycin (Km), 40 μg/ml; ampicillin (Ap), 100 μg/ml.

Genetic Techniques and DNA Manipulations

P1*vir* transductions and transformations were done using standard techniques (Miller, 1972; Maniatis et al., 1989; Silhavy et al., 1984). Preparations of plasmid DNA and cloning of PCR-amplified fragments were carried out as described (Maniatis et al., 1989). The PCR amplification of the *rpoH* and *dnaJ* genes of strain MC4100 was done according to the instructions provided by the supplier of Taq polymerase (Perkin-Elmer Cetus). As primers, we used oligonucleotides (31–64 mers) that at their 3′ end were homologous to *rpoH* or *dnaJ* sequences, and at their 5′ end carried recognition sites for restriction enzymes. Whenever required, appropriate translational signals were included. Plasmids pUHE211–1(*rpoH*) and pUHE21–2(*rpoH*), encoding σ^{32}-C-his and untagged σ^{32}, respectively, were constructed by cloning a PCR-generated BamHI–EcoRI fragment carrying the *rpoH*$^+$ gene into the EcoRI and BamHI sites of the vectors. Plasmid pUHE212–1(*rpoH*), encoding σ^{32}-N-his, was constructed by cloning a PCR-generated BamHI–HindIII fragment carrying the *rpoH*$^+$ gene into the HindIII and BamHI sites of the vector. pUHE212–1(*dnaJ*$^+$), encoding DnaJ-N-his,

Table 1. Bacterial Strains

Strain	Markers	Source or Reference
BB1553	MC4100, *ΔdnaK52, sidB1*	Bukau and Walker, 1990
BB2073	GW4701, *recA, mal⁺, zfi*::Tn*10*	this study
BB2296	MC4100, *dnaJ47(am), Sp^R*	lab collection
GW4701	*recA441, sulA11, Δ(argF-lac)U169, thr-1, leu-6, his-4, argE3, ilv(ts), galK2, rpsL31, rpoH165(am), malPQ*::Tn*5*	Krüger and Walker, 1984
MC4100	*araD139, Δ(argF-lac)U169, rpsL150, relA1, deoC1, ptsF25, rpsR, flbB301*	Casadaban, 1976
SG13009	MC4100, *his pyrD Δlon-100 rpsL*	Gottesmann et al., 1981

was constructed by cloning a PCR-generated BamHI–HindIII fragment carrying the *dnaJ*⁺ gene into the BamHI and HindIII sites of the vector.

Complementation Assays

Complementation tests for temperature-sensitive growth of *rpoH165(am)* mutants were carried out in BB2073 cells transformed with pDMI,1 and pUHE211-1(*rpoH*), pUHE212-1(*rpoH*), or their parental plasmids. Cell viability at 43°C was determined by plating appropriate dilutions of 30°C-overnight cultures on LB agar plates containing 0–500 μM IPTG, or by growing the cells in LB liquid media containing 0–500 μM IPTG. Complementation of *dnaJ47(am)* mutants was done in BB2296 cells transformed with pDMI,1 and pUHE212-1(*dnaJ*). Cell viability at 43°C was determined as described for *rpoH165(am)* mutants. The ability to grow λ phages was determined at 30°C by λ*vir* plating assays as described (Silhavy et al., 1984).

Pulse-Labeling Experiments

Cell cultures were grown at 30°C in M9 minimal medium to an OD_{600} of 0.6, induced with IPTG (final concentration 50 μM), and, after addition of IPTG, immediately incubated at 43°C. At the indicated times, aliquots of 1 ml were labeled with 10 μCi of L-[^{35}S]methionine (specific activity >800 Ci/mmole, Amersham Corp.) for 2 min, followed by a chase for 5 min with unlabeled methionine (2 mM final concentration). The labeled cells were transferred to ice, washed in minimal medium and lysed by boiling in cracking buffer (see below). Aliquots of 10 μl were subjected to electrophoresis in 12.5% SDS–polyacrylamide gels. Gels were autoradiographed using Fuji RX films.

Purification of Histidine-Tagged Proteins Using IMAC

Cells producing histidine-tagged σ^{32} and DnaJ were grown at 30°C in 1 liter of 2 × YT/Ap/Km medium to an OD_{600} of 1. At this point, IPTG (50 μM final concentration) was added, and after further growth of the culture for 10–60 min, the cells were collected by centrifugation (6,000 × g, 10 min, 4°C) and resuspended in 20 ml of cold lysis buffer A (50 mM Tris–HCl [pH 7], 100 mM KCl, 1 mM EDTA, lysozyme). Thereafter, all manipulations were done at 4°C. Phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM, and soluble cell extracts were obtained by stirring the suspension for 30 min followed by sonification (5 × 10 s, level 7; Branson sonifier) and centrifugation (35,000 × g, 30 min). For IMAC, cell extracts were supplemented with $MgCl_2$ (1 mM, final concentration) and KCl (200 mM, final concentration) and loaded onto 4 ml Ni^{2+}–nitrilotriacetic acid–agarose columns (prepared according to the manufacturer's instructions [Diagen] and equilibrated with buffer A). Chromatography of the extracts was performed at 4°C using an FPLC system from Pharmacia LKB (flow rate of 0.5 ml/min). Loaded columns were first washed with 20 ml of buffer A, then developed by linear 0–150 mM imidazole gradients (50 ml, in buffer A), followed by 1 M imidazole step gradients, and fractions of 1.5 ml were collected.

SDS-PAGE and Immunological Techniques

Protein samples were mixed with equal volumes of 2 × cracking buffer (40 mM EDTA, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 100 mM Tris–HCl [pH 6.8]), boiled for 5 min and subjected to electrophoresis in 12.5% SDS–polyacrylamide gels as described (Laemmli, 1970). Immunoblots were developed with alkaline phosphatase–conjugated anti-rabbit IgG as secondary antibodies (Promega), as described by Harlow and Lane (1988). Polyclonal antisera were raised against histidine-tagged DnaK, DnaJ, GrpE, and σ^{32} that had been purified under denaturing conditions by IMAC according to Hochuli et al. (1987, 1988). Dilutions for use in immunoblot experiments were as follows: σ^{32}-C-his antiserum, 1:10,000; DnaK-N-his and DnaJ-N-his antisera, 1:7,000; GrpE-N-his antisera, 1:6,000.

Gel Filtration

Protein samples were centrifuged (14,000 × g, 5 min), and aliquots of 200 μl were loaded onto a Pharmacia Superdex 75 column (flow rate 0.5 ml/min). As mobile phase, a buffer containing 50 mM Tris–HCl (pH 7), 200 mM KCl, 100 mM imidazole was used (flow rate 0.5 ml/min). Fractions of 300 μl were collected, and proteins were precipitated with trichloroacetic acid. The precipitate was prepared for SDS–PAGE. The molecular mass standards were apoferritin (440,000), bovine serum albumin (66,000), and carboanhydrase (29,000) (Sigma).

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