The EMBO Journal Vol.17 No.16 pp.4818–4828, 1998

Role of the DnaK and HscA homologs of Hsp70 chaperones in protein folding in *E.coli*

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Folding of newly synthesized cytosolic proteins has been proposed to require assistance by Hsp70 chaperones. We investigated whether two Hsp70 homologs of *Escherichia coli*, DnaK and HscA, have this role *in vivo*. Double mutants lacking *dnaK* and *hscA* were viable and lacked defects in protein folding at intermediate temperature. After heat shock, a subpopulation of pre-existing proteins slowly aggregated in mutants lacking DnaK, but not HscA, whereas the bulk of newly synthesized proteins displayed wild-type solubility. For thermolabile firefly luciferase, DnaK was dispensable for *de novo* folding at 30°C, but essential for aggregation prevention during heat shock and subsequent refolding. DnaK and HscA are thus not strictly essential for folding of newly synthesized proteins. DnaK instead has functions in refolding of misfolded proteins that are essential under stress.
Keywords: DnaK/heat-shock proteins/HscA/Hsc66/protein aggregation

Introduction

The primary amino acid sequence contains the entire information for the folding of proteins to the native structure *in vitro* (Anfinsen, 1973). This fundamental finding by Anfinsen had a major impact on our understanding of the *in vivo* process of protein folding. It has been assumed for a long time that protein folding in the cell also occurs spontaneously, except for organellar and exported proteins that require a cellular targeting and translocation machinery to reach their proper destination. This concept has been challenged by the discovery in the last decade of a cellular network of molecular chaperones and folding catalysts that assists a large variety of protein folding processes in virtually all compartments (Gething and Sambrook, 1992; Hartl, 1996). The activity of chaperones has been proposed to be essential for the folding of cytosolic proteins. In particular, the ubiquitous and abundant heat-shock protein 70 (Hsp70) chaperones, with their co-chaperones, have been proposed to be required for the co-translational folding of cytosolic proteins (Langer *et al.*, 1992; Hartl, 1996; Mayhew and Hartl, 1996), in addition to their demonstrated roles in other folding processes, including protein translocation across membranes (Schatz and Dobberstein, 1996), assembly and disassembly of protein oligomers (Chappell *et al.*, 1986; Alfano and McMacken, 1989), refolding of denatured proteins (Skowyra *et al.*, 1990; Langer *et al.*, 1992; Schröder *et al.*, 1993; Freeman and Morimoto, 1996; Ehrnsperger *et al.*, 1997), degradation of unstable proteins (Straus *et al.*, 1990; Sherman and Goldberg, 1992) and control of activity of regulatory proteins (Bohen and Yamamoto, 1994; Gamer *et al.*, 1996). These roles of Hsp70 proteins rely on their ability to associate with short hydrophobic segments of unfolded substrate polypeptides in an ATP-controlled fashion (Rüdiger *et al.*, 1997a; Bukau and Horwich, 1998).

The proposal for a key role for Hsp70 in the *de novo* folding of cytosolic proteins is based on several findings. Eukaryotic Hsp70 homologs are associated with translating ribosomes when prepared from cell cultures (Beckmann *et al.*, 1990; Nelson *et al.*, 1992; Beck and De Maio, 1994) or cell-free translation systems (Frydman *et al.*, 1994). This association of Hsp70 is puromycin sensitive and therefore depends on the presence of the nascent polypeptide chain (Nelson *et al.*, 1992; Beck and De Maio, 1994). Similarly, puromycyl fragments are associated with Hsp70 as judged by co-immunoprecipitation (Beckmann *et al.*, 1990; Frydman *et al.*, 1994; Hansen *et al.*, 1994; Frydman and Hartl, 1996; Eggers *et al.*, 1997). The best *in vitro* evidence for a role of Hsp70 in folding of newly synthesized polypeptides was obtained for firefly luciferase translated in reticulocyte lysates that were depleted of Hsp70 or its DnaJ co-chaperone, Hsp40 (Frydman *et al.*, 1994). Depletion resulted in a specific enzymatic activity of translated luciferase that was decreased by 70%. Restoration of full enzymatic activity was only possible when Hsp70 and Hsp40 were re-added before onset of translation, indicating a co-translational mode of action of Hsp70 and Hsp40 in the folding of firefly luciferase in this cell-free system (Frydman *et al.*, 1994).

A co-translational role in the folding of cytosolic proteins has also been proposed for the stress-inducible DnaK system of *Escherichia coli* (Langer *et al.*, 1992; Hendrick *et al.*, 1993; Gaitanaris *et al.*, 1994; Kudlicki *et al.*, 1994), consisting of the DnaK (Hsp70) chaperone and the DnaJ and GrpE co-chaperones (Georgopoulos, 1992; Bukau and Horwich, 1998). DnaJ, when added at high concentration to eukaryotic *in vitro* translation reactions, was cross-linked to nascent firefly luciferases, and blocked the folding of luciferase and chloramphenicol acetyltransferase until exogeneous DnaK and GrpE were added to allow continuation of the chaperone cycle and substrate folding (Hendrick *et al.*, 1993).

A different class of chaperones, the CCT (TRiC) and GroEL chaperonins, is known to assist the *de novo* folding of cytosolic proteins (Horwich *et al.*, 1993; Frydman and Hartl, 1996; Lewis *et al.*, 1996; Ewalt *et al.*, 1997; Farr

et al., 1997). For CCT of the eukaryotic cytosol, a role in folding for some proteins including actin and tubulin has been demonstrated. For GroEL of *E.coli*, a post-translational role in folding of a subset of ~5–15% of newly synthesized proteins has been shown (Bochkareva *et al.*, 1988; Horwich *et al.*, 1993; Gaitanaris *et al.*, 1994; Reid and Flynn, 1996; Ewalt *et al.*, 1997). In view of these data, and taking into account that the DnaK and GroEL systems can act in succession in the *in vitro* folding of chemically denatured rhodanese (Langer *et al.*, 1992), a model for the conserved role of both chaperone systems in the *de novo* folding of cytosolic proteins has been derived (Langer *et al.*, 1992; Hendrick *et al.*, 1993; Frydman *et al.*, 1994; Frydman and Hartl, 1996). Accordingly, DnaJ is the first chaperone to interact with the nascent polypeptide chain thereby preventing premature and incorrect folding and targeting Hsp70/DnaK to this substrate. Through successive ATP-controlled cycles of binding and release from Hsp70/DnaK, the partially folded polypeptide chain either reaches the native state directly, or is transferred to CCT or GroEL for final folding. This model implies that the Hsp70 chaperones and the chaperonins provide a protected folding environment for nascent chains (Frydman and Hartl, 1996). However, little *in vivo* evidence exists to support the proposed central role for Hsp70.

We investigated the role of two of three Hsp70 proteins in protein folding in *E.coli* cells, the heat-inducible DnaK and the recently discovered HscA (Hsc66) for which no cellular function is known (Seaton and Vickery, 1994; Lelivelt and Kawula, 1995). We constructed mutants lacking the function of DnaK and/or HscA, and analyzed the efficiency of protein folding in the mutant cells. We found that the HscA and DnaK chaperones are not strictly essential for folding of newly synthesized proteins at 30°C or heat-shock temperature. The DnaK chaperone, however, is essential for the repair of misfolded proteins which accumulate under stress.

Results

Cellular levels of DnaK and HscA

The chaperone activity of Hsp70 proteins relies on the shielding of hydrophobic patches in extended polypeptides by direct association (Zhu *et al.*, 1996; Rüdiger *et al.*, 1997a). DnaK-binding sites are frequent in protein sequences, occurring statistically every 30–40 residues (Rüdiger *et al.*, 1997b). A role for two *E.coli* Hsp70 homologs DnaK and HscA in shielding such sites in nascent polypeptide chains is expected to require high levels of these chaperones, given that in growing *E.coli* cells the concentration of nascent polypeptide chains (each containing several chaperone-binding sites) is estimated to be 35 μM (Ellis and Hartl, 1996).

We determined the levels of DnaK and HscA in two wild-type *E.coli* strains, MC4100 and C600 (Figure 1). DnaK constitutes ~1.2% (estimated 50 μM) of total soluble protein (estimated 340 mg/ml) at 30°C and ~3% at 1 h after upshift of the cells to 42°C, consistent with previous reports (Herendeen *et al.*, 1979). HscA is ~5-fold less abundant than DnaK at 30°C, has marginally increased levels at 6 h after downshift to 15°C and slightly reduced levels at 1 h after upshift to 42°C. DnaK is therefore considerably more abundant than HscA at all temperatures tested, with the strongest difference (~25-fold) at elevated temperature. These findings are inconsistent with two studies which in addition are in conflict with each other. The *hscA* mRNA was barely detectable at 37°C but was increased 11-fold 3 h after temperature downshift to 10°C (Lelivelt and Kawula, 1995). The HscA protein was found at constitutive high levels of ~1% of the total *E.coli* protein over a wide temperature range (Vickery *et al.*, 1997). The discrepancy between the latter finding and our data may be due to differences in the methods used to quantify the amount of HscA and the failure of that study to compare HscA and DnaK levels within one experiment.

Our data show that the combined levels of DnaK and HscA are roughly in the concentration range of nascent polypeptide chains in the *E.coli* cytosol. They are, however, probably lower than the combined concentration of exposed hydrophobic segments of nascent chains and misfolded proteins and, therefore, are likely to be too low systematically to shield the hydrophobic segments of the majority of nascent polypeptide chains.

Overproduction of HscA does not complement growth defects of ΔdnaK52 mutant cells

To elucidate the functional relationship between DnaK and HscA, we investigated whether DnaK and HscA can complement each other *in vivo*. Mutant cells carrying an insertionally inactivated *hscA* gene are viable at 30 and 42°C, and grow only slightly more slowly than wild-type (Kawula and Lelivelt, 1994). *hscA::cat* mutant cells do not appear to carry suppressor mutations affecting their growth behavior, as judged by comparing growth in liquid culture and on LB agar plates of the original strain and of fresh *hscA::cat* transductants. Mutant cells lacking *dnaK* grow slowly at 30°C in the absence of suppressor mutations (see below) and fail to grow at temperatures above 37°C (Paek and Walker, 1987; Bukau and Walker, 1989a). This latter phenotype allows us to test whether overexpression of *hscA* can suppress the temperature-sensitive growth of *dnaK* mutant cells. For this purpose, we used the well characterized Δ*dnaK52 sidB1* mutant strain, BB1553, which lacks DnaK and has reduced levels of DnaJ due to polar effects on the promoter-distal *dnaJ* gene (Bukau and Walker, 1990). In addition, BB1553 carries the *sidB1* suppressor mutation which allows Δ*dnaK52* mutant cells to grow at 30°C without apparent defects and to be genetically stable. This mutation maps in the *rpoH* gene encoding the heat-shock transcription factor of *E.coli*, σ^{32}, and partially compensates for the missing function of DnaK in negative modulation of the heat-shock response. This mutation is solely responsible for the suppressed phenotype as a recombinational replacement of the suppressor allele with wild-type *rpoH* causes reappearance of the growth defects at 30°C (Bukau and Walker, 1990). Despite the presence of the *sidB1* suppressor, however, Δ*dnaK52* mutant cells lose viability upon prolonged incubation at temperatures above 37°C.

The *hscA* and *dnaK* genes were cloned into expression vectors such that their transcription is controlled by the Lac repressor and isopropyl-β-D-thiogalactopyranoside (IPTG). The overproduction of DnaK, but not of HscA, allowed Δ*dnaK52 sidB1* cells to grow at 42°C (Table I). Overproduction of HscA did occur up to similar high

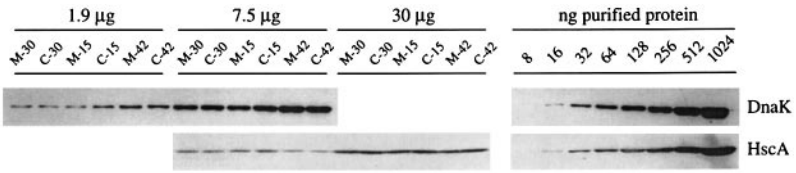

Fig. 1. Cellular levels of DnaK and HscA. MC4100 (M–) and C600 (C–) cells were grown logarithmically in LB medium at 30°C and aliquots were subjected to a temperature shift to 42°C for 1 h or 15°C for 6 h. Cells were harvested by centrifugation and lysed by a freeze–thaw/sonication procedure. A soluble fraction of the total *E.coli* protein was prepared by centrifugation at 25 000 *g* for 30 min. The indicated amounts of the soluble protein fraction were subjected to SDS–PAGE followed by immunoblot analysis with DnaK- and HscA-specific antisera. Serial dilutions of purified DnaK and HscA served as a standard. More than 95% of DnaK and HscA partitioned with the soluble protein fraction during centrifugation.

Table I. *In vivo* complementation of temperature-sensitive growth of Δ*dnaK52 sidB1* cells by plasmid-borne *hscA* and *dnaK* genes

Temperature (°C)	30					42				
IPTG (µM)	0	50	100	250	1000	0	50	100	250	1000
Δ*dnaK52* pUHE 21-2 fd Δ12	+	+	+	+	+	−	−	−	−	−
Δ*dnaK52* pUHE 21-2 fd Δ12 (*dnaK*$^+$)	+	+	+	+	+	±	±	+	+	±
Δ*dnaK52* pUHE 21-2 fd Δ12 (*hscA*$^+$)	+	+	+	+	+	−	−	−	−	−
dnaK$^+$ pUHE 21-2 fd Δ12 (*hscA*$^+$)	+	+	+	+	+	+	+	+	+	±

Cells of strains MC4100 (*dnaK*$^+$) and BB1553 (Δ*dnaK52*) were transformed with plasmids pUHE 21-2 fd Δ12, or derivatives expressing the *dnaK* and *hscA* genes in a Lac repressor-controlled fashion, and pDMI.1 (*lacI*$^{q+}$). Overnight cultures of transformants were serially diluted and spotted onto LB (Amp/Kan) plates containing IPTG as indicated. Plates were incubated overnight at 30 or 42°C and evaluated for cell growth.

levels as observed for DnaK (not shown) but did not inhibit growth of wild-type cells at 42°C, excluding the possibility that toxic effects may have masked a potential complementation activity. Furthermore, the presence of the multicopy plasmid pTHK201 encoding the entire *hscB hscA fdx* operon with its authentic transcriptional and translational control elements (Kawula and Lelivelt, 1994) also failed to suppress the temperature sensitivity of Δ*dnaK52 sidB1* cells (not shown). We conclude that HscA and DnaK functionally differ to such an extent that HscA cannot restore the missing function of DnaK required for cell growth at high temperature.

HscA and DnaK functions are not essential for viability at 30°C

We further investigated the functional relationship between DnaK and HscA and determined whether *E.coli* is viable in the absence of both DnaK and HscA. This was achieved by a genetic cross, performed at 30°C, in which a *hscA::cat* allele (Kawula and Lelivelt, 1994) and a closely linked tetracycline resistance marker (*zfh-208::Tn10*) were introduced into *dnaK*$^+$ and Δ*dnaK52* cells using P1 *vir* transduction. Tetracycline-resistant transductants were selected and screened for co-transduction of the *hscA::cat* allele by immunological detection of the HscA protein. The co-transduction frequency of the two loci was similar in *dnaK*$^+$ and Δ*dnaK52* cells (Figure 2A), providing genetic proof that a Δ*dnaK52 hscA::cat* double mutant is viable at 30°C.

Immunoblot analysis of wild-type and mutant cells (Figure 2B) confirmed the absence of DnaK and HscA in double mutant cells. In Δ*dnaK52* mutant cells, DnaJ has strongly reduced levels due to polar effects on the promoter-distal *dnaJ* gene, and other heat-shock proteins (e.g. GroEL, HtpG) have 2- to 3-fold increased levels due to regulatory defects. The presence of the *hscA::cat* allele did not affect the levels of these proteins, either in the *dnaK*$^+$ or the Δ*dnaK52* background, indicating that HscA does not have a role in heat-shock gene regulation. Furthermore, mutations in *hscA* and *dnaK* did not affect the levels of trigger factor, a chaperone that does not belong to the heat-shock regulon, and of the DnaJ homolog, HscB.

In liquid culture at 30°C, wild-type and mutant cells grew to identical optical densities albeit with different rates (Figure 2C). In rich medium, the doubling time of Δ*dnaK52* cells carrying the *sidB1* suppressor mutation was almost the same as for wild-type, while that of *hscA::cat* and Δ*dnaK52 hscA::cat* mutant cells was ~2- and 3-fold longer than wild-type, respectively. In minimal medium, double mutant cells grew more slowly than wild-type and single mutant cells, but the defects were less pronounced. The slow growth phenotype of the double mutant remained unchanged in fresh cultures inoculated with stationary phase cells, indicating that the cells did not accumulate suppressor mutations that improve growth. Plating cells taken from overnight cultures showed a uniform morphology and growth behavior on agar. Furthermore, upon continuous restreaking, we did not observe changes in growth and morphology of colonies formed by Δ*dnaK52 hscA::cat*C double mutants, and did not observe papillae of faster growing cells. The growth behavior of the double mutant suggests additive, rather than synergistic, growth defects and thus distinct cellular roles for HscA and DnaK that are not essential for viability at 30°C.

We also determined the viability of logarithmically growing Δ*dnaK52 hscA::cat* cells as colony-forming units per optical density (OD) unit. Although the number of colonies per OD unit was 4- to 6-fold reduced in the double mutant as compared with wild type, we found by microscopic observation that this reduction is not explained by reduced viability but by formation of cell filaments which change the cell to OD ratio (not shown). A filamentous phenotype, indicative of cell division defects, has also been reported for freshly transduced, non-suppressed Δ*dnaK52* cells (Bukau and Walker, 1989a,b).

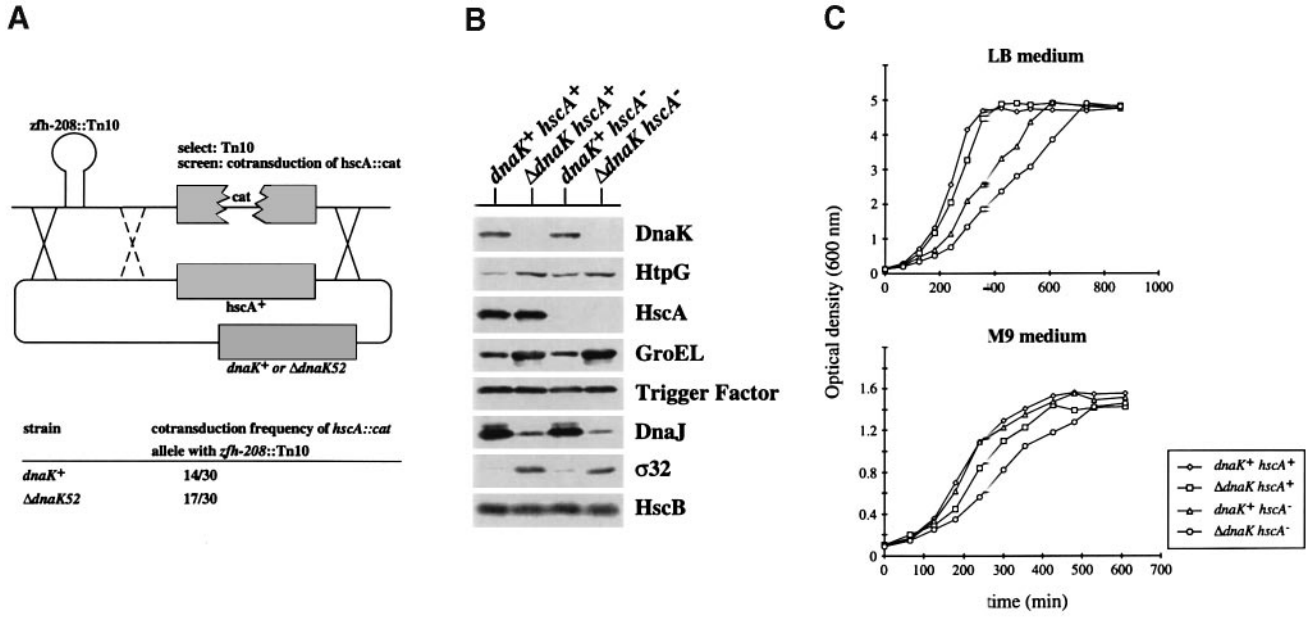

Fig. 2. Construction of mutants lacking *dnaK* and *hscA*. (**A**) Co-transduction frequency of *hscA::cat* and *zfh-208::*Tn*10* in *dnaK*$^+$ and Δ*dnaK52* backgrounds. A P1 *vir* lysate was prepared on MC4100 cells carrying the *hscA::cat* allele (from THK80) and the closely linked *zfh-208::*Tn*10* (from CAG 18481), and used to transduce MC4100 (*dnaK*$^+$) and BB1553 (Δ*dnaK52*) cells. Tetracycline-resistant transductants were selected, purified, transferred to liquid culture and subsequently analyzed for the presence or absence of HscA by immunoblot analysis with specific antiserum. (**B**) Chaperone content of mutants lacking *dnaK* and/or *hscA*. Cells of wild-type and mutant strains were grown logarithmically at 30°C in M9/glucose/casamino acids medium and harvested. Identical OD_{600} equivalents of the individual cultures were separated by SDS–PAGE and subjected to immunoblot analysis with antisera specific for DnaK, HtpG, HscA, GroEL, trigger factor, DnaJ, σ^{32} and HscB. (**C**) Growth of mutants lacking DnaK and/or HscA. Cells of wild-type and mutant strains were grown at 30°C in LB or M9/glucose/casamino acids medium. Growth curves were taken by measuring the optical density of the cultures at 600 nm.

DnaK and HscA are not essential for folding of β-galactosidase and luciferase at 30°C

The ability of the Δ*dnaK52* and *hscA::cat* single and double mutants to grow at 30°C allows experiments to determine whether the lack of DnaK and/or HscA affects the efficiency of protein folding. This was tested for two reporter substrates, luciferase from *Photinus pyralis* and β-galactosidase from *E.coli*, for which activity assays allow rapid detection of the native and active state. The monomeric luciferase has been used widely as model substrate for Hsp70 chaperones, including *in vitro* translation experiments which supported the proposal of a co-translational role for Hsp70 and Hsp40 (DnaJ) in folding of newly synthesized proteins (Hendrick *et al.*, 1993; Frydman *et al.*, 1994; Frydman and Hartl, 1996). Luciferase produced at 30°C in wild-type cells and Δ*dnaK52* or Δ*dnaK52 hscA::cat* mutants accumulated to similar amounts and yielded similar activities in time-resolved assays performed *in vivo* (Figures 3A and 6) and in corresponding cell extracts (not shown). At this temperature, the majority of (>50%), though not all luciferase molecules are active as judged by comparison with commercially available enzyme. β-galactosidase is a large homotetramer composed of multidomain subunits that is difficult to refold *in vitro* from the denatured state. A role for human Hsp40 and Hsp70 in *in vitro* refolding of denatured β-galactosidase has been reported (Freeman and Morimoto, 1996). To test whether the *in vivo* folding of β-galactosidase requires DnaK and/or HscA, the plasmid-encoded *lacZ* gene was expressed for 20 min in wild-type and mutant cells, followed by rapid determination of β-galactosidase activity and levels. No differences existed between the strains concerning β-galactosidase activity and protein content (Figure 3B), implying that the folding of newly synthesized β-galactosidase can occur in the absence of DnaK and HscA. Taken together, these findings rule out a strictly essential role for these two *E.coli* Hsp70s in the *de novo* folding of two reporter enzymes at intermediate temperature.

DnaK is required to prevent aggregation of proteins after heat shock

In another approach, the role of DnaK and HscA in folding of the bulk of the *E.coli* proteins was investigated at intermediate and heat-shock temperatures. Aggregation of proteins was used as a criterion for misfolding in accordance with related studies (Gragerov *et al.*, 1991; Horwich *et al.*, 1993; Herrmann *et al.*, 1994). Aggregation was analyzed by a lysis and centrifugation protocol (25 000 *g*, 30 min) that is more rigorous compared with published procedures (Gragerov *et al.*, 1991; Horwich *et al.*, 1993; Herrmann *et al.*, 1994). Cultures of wild-type and mutant cells were grown at 30°C and then shifted to 42°C. Cell growth was monitored as optical density. Accordingly, cells of the two *dnaK*$^+$ strains continued to grow at 42°C (Figure 4A). In the case of the *hscA::cat* strain, the temperature upshift was accompanied by a transient lag in growth. Thereafter, growth resumed, consistent with the fact that *hscA::cat* cells can form colonies on plates at 42°C. In contrast, Δ*dnaK52* and Δ*dnaK52 hscA::cat* cells were severely impaired in growth upon temperature upshift (Figure 4A). The manifestation of growth defects upon temperature upshift was a slow process, taking up to 30 min in rich medium (not shown),

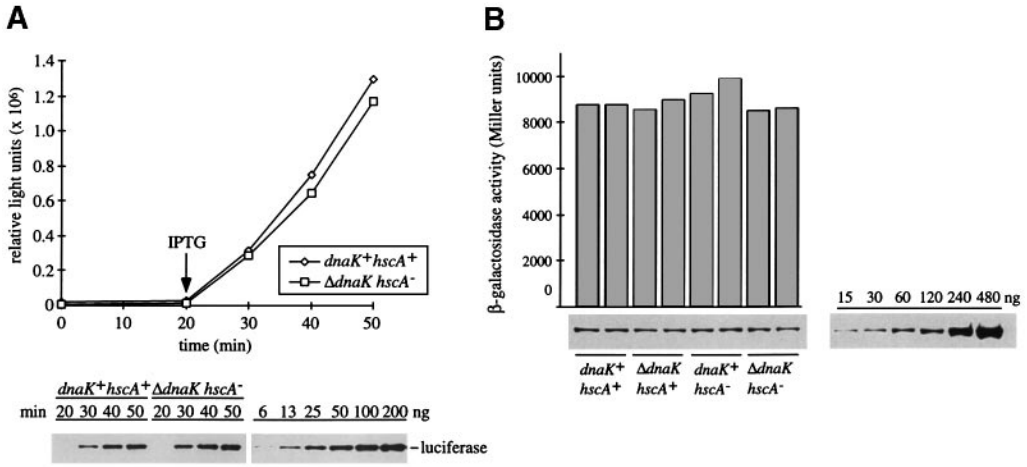

Fig. 3. *De novo* synthesis and folding of firefly luciferase and β-galactosidase in mutants lacking DnaK and/or HscA. (**A**) Cells of *dnaK*$^+$ *hscA*$^+$ and Δ*dnaK52 hscA::cat* strains containing plasmids pDS12 pN25 O4/O3 (*luc*$^+$) and pDMI.1 (*lacI*$^{q+}$) were grown in M9/glucose/casamino acids at 30°C to early logarithmic phase. IPTG was added to 100 μM final concentration to induce synthesis of luciferase, followed by determination of the *in vivo* luciferase activity and the cellular amounts of luciferase by immunoblot analysis. Purified luciferase (Sigma) served as a standard for the quantification of luciferase. Specific luciferase activity in the two strains was estimated to be ~14 000 relative light units/ng luciferase. (**B**) Cells of wild-type and mutant strains, as indicated, containing plasmid pML3 pA1 O4/O3 (*lacZ*$^+$, *lacI*$^{q+}$) were grown in LB medium at 30°C to early logarithmic phase followed by addition of IPTG (100 μM final concentration) to induce production of β-galactosidase. After 20 min, aliquots were removed for determination of β-galactosidase activity and protein content using immunoblot analysis. Purified β-galactosidase (Boehringer Mannheim) served as a standard for quantification. The specific β-galactosidase activities were calculated to be 570–650 Miller units/ng β-galactosidase. Two independent measurements for each strain are shown.

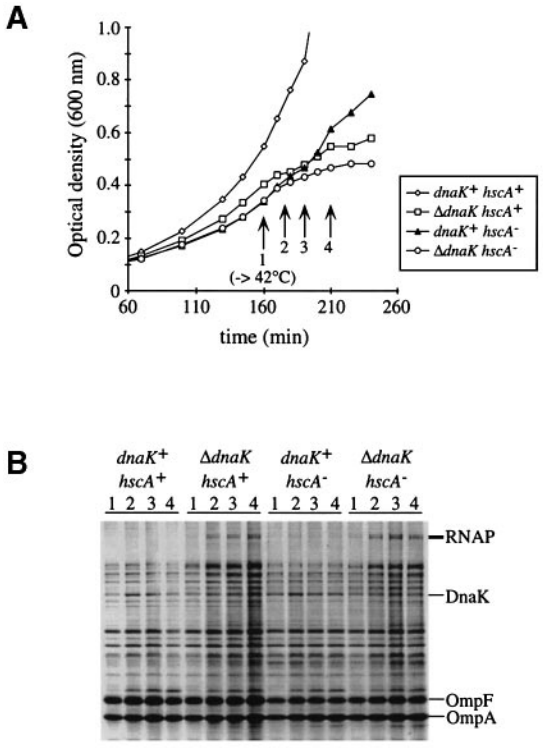

Fig. 4. Solubility of pre-existing proteins before and after heat shock in mutant cells lacking DnaK and/or HscA. Cultures of wild-type and mutant strains, as indicated, were grown in M9/glucose/casamino acids at 30°C to logarithmic phase, shifted to 42°C (time point 1) and analyzed for their growth rate by optical density measurements (**A**). At the indicated time points, 1–4 culture aliquots were withdrawn and analyzed for the presence of insoluble protein by cell lysis and centrifugation. Insoluble fractions were normalized to the optical density of the cultures and applied to SDS–PAGE followed by staining with Coomassie Brilliant Blue (**B**). Selected proteins were identified by immunoblot analysis using specific antisera. RNAP, ββ′ subunits of RNA polymerase.

while it occurred within a few minutes in minimal medium (Figure 4A).

At 30°C, the insoluble protein fraction was quantitatively and qualitatively similar in wild-type and mutant cells, and was dominated by the outer membrane proteins OmpA and OmpF that sediment during centrifugation as part of membrane vesicles (Figure 4B). After shift to 42°C, the amount of insoluble protein remained unchanged in wild-type and *hscA::cat* cells, but slowly increased in Δ*dnaK52* and Δ*dnaK52 hscA::cat* cells. About 10% of the total cytosolic protein became insoluble in these cells within 50 min at 42°C. The amount and composition of aggregated protein were virtually identical in Δ*dnaK52* and Δ*dnaK52 hscA::cat* mutant cells, demonstrating that it is the lack of DnaK which is solely responsible for the observed protein folding defects. Among the aggregated proteins, we identified the β and β′ subunits of RNA polymerase. This finding is consistent with the reported heat sensitivity of RNA polymerase and its tendency to aggregate at high temperature (Skowyra *et al.*, 1990; Blaszczak *et al.*, 1995). Interestingly, in *dnaK*$^+$ cells, DnaK itself became transiently part of the insoluble protein fraction (Figure 4B). This may be due to its reversible association with mildly aggregated protein that is resolubilized with time. Together, our findings indicate an essential role for DnaK in preventing aggregation of denatured proteins that slowly accumulate during heat treatment.

Pre-existing proteins constitute the bulk of the proteins aggregated after heat shock

We investigated whether the heat-induced aggregation of proteins in cells lacking DnaK is caused by folding defects of newly synthesized proteins. *dnaK*$^+$ and Δ*dnaK52* cells were pulse-labeled with [^{35}S]methionine either during growth at 30°C or at 25 min after temperature upshift to 42°C, where aggregation of pre-existing proteins was observed in Δ*dnaK52* mutant cells (Figure 4B). After

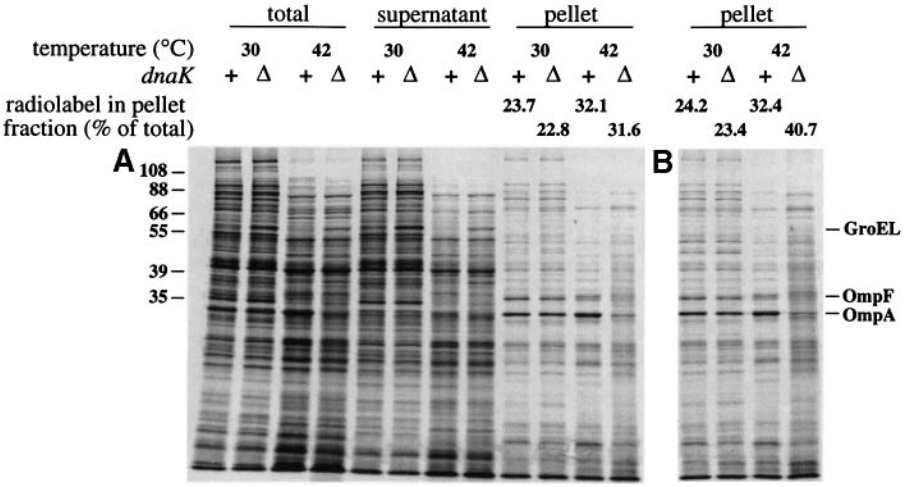

Fig. 5. Solubility of newly synthesized proteins in *dnaK*$^+$ and Δ*dnaK52* cells. *dnaK*$^+$ and Δ*dnaK52* cells were grown at 30°C in M9/glucose/19 L-amino acids medium to early logarithmic phase. Cultures were split and grown further at 30 or 42°C, followed by labeling for 1 min with [^{35}S]methionine at 30°C or at 25 min after temperature upshift. Cells were cooled rapidly, lysed and analyzed for soluble and insoluble protein by centrifugation. Soluble and insoluble proteins were precipitated and subjected to liquid scintillation counting. Subsequent SDS–PAGE loadings were normalized to the total incorporated radiolabel of the cells. At 42°C, the total incorporation of [^{35}S]methionine by Δ*dnaK52* cells was 53 ± 5% of the corresponding incorporation by *dnaK*$^+$ cells. (**A**) Pulse-labeling only; (**B**) pulse-labeling and an additional 8 min chase (at 42°C) with excess unlabeled L-methionine, insoluble protein fractions only. All labeling and fractionation steps were carried out in duplicate. The relative amounts of incorporated [^{35}S]methionine in soluble and insoluble fractions differed by <2% in the two data sets. The absence of labeling of heat-shock proteins in the two strains at 42°C is explained by the fact that the heat-shock response, which leads to only transient induction of heat-shock gene expression, is already in the shut-off phase after 25 min at 42°C. Immunoblotting of culture aliquots before and after heat treatment with GroEL- and HtpG-specific antisera confirmed the occurrence of a regular (*dnaK*$^+$) or small (Δ*dnaK52*) increase in the heat-shock protein levels (not shown).

labeling, the cultures were split and analyzed for soluble and insoluble protein, either immediately or after a chase for 8 min with unlabeled L-methionine. This chase allows for completion of trafficking and folding of those proteins that were synthesized during the pulse.

Figure 5 shows total lysates and the corresponding soluble and insoluble protein fractions before (Figure 5A) and after chase (Figure 5B). The general synthesis pattern of proteins at 30°C is indistinguishable in *dnaK*$^+$ and Δ*dnaK52* cells, except for an increased synthesis of GroEL in the mutant strain. Upon shift to 42°C, the general protein synthesis pattern of both strains is changed compared with at 30°C, but is still highly similar in *dnaK*$^+$ and Δ*dnaK52* cells, except for an ~2-fold increased synthesis of OmpA in *dnaK*$^+$ cells contrasted by a shut off of its synthesis in the Δ*dnaK52* mutant (Figure 5A). This effect could be due to differences in *ompA* mRNA stability in the two strains. Degradation of OmpA mRNA is a function of RNase E (Lundberg *et al.*, 1990) which was recently isolated in a complex with DnaK (Miczak *et al.*, 1996) that may have functional implications for *ompA* mRNA stability.

Under conditions without chase, 23.7 and 22.8% of protein labeled at 30°C, and 32.1 and 31.6% of protein labeled at 42°C partitioned to the insoluble fraction in *dnaK*$^+$ and Δ*dnaK52* cells, respectively (Figure 5A). With respect to protein composition, the lack of labeled OmpA in the insoluble fraction of Δ*dnaK52* cells is the single major difference between both strains. Except for this difference and an unknown protein running just above the OmpF band, the amount and composition of the insoluble fraction of newly synthesized protein are indistinguishable in *dnaK*$^+$ and Δ*dnaK52* cells at 30 and 42°C (Figure 5A). This indicates that the lack of DnaK does not cause extensive misfolding and aggregation of the majority of newly synthesized polypeptide chains in *E.coli* at intermediate and heat-shock temperatures. We cannot exclude, however, that rare proteins aggregated but escaped our detection.

The chase for 8 min allowed us to investigate whether the relative amount of insoluble protein changes due to slow denaturation and aggregation. The only difference in the pattern occurred in Δ*dnaK52* cells at 42°C. Here, 40.7% of the incorporated label became insoluble during the chase period as compared with 31.6% before the chase (Figure 5B). Judged from the autoradiography, many proteins covering the entire molecular weight range were affected by aggregation. These findings further support that pre-existing, rather than newly synthesized proteins constitute the bulk of the proteins affected by aggregation in Δ*dnaK52* cells at 42°C.

Prevention of aggregation by the DnaK system is essential for refolding of misfolded proteins

The ability of DnaK to prevent the aggregation of proteins in heat-shock conditions may be a prerequisite for their refolding under permissive conditions. This was tested using the thermolabile luciferase as test substrate. Cultures of *dnaK*$^+$ and Δ*dnaK52* cells were grown at 30°C to early logarithmic phase. Expression of the plasmid-encoded luciferase gene was then induced followed by activity measurements *in vivo* (Figure 6A). After 30 min, further protein synthesis was blocked by tetracycline and the cultures were exposed to an 8 min heat shock at 42°C to inactivate the heat-labile protein. During the following 45 min recovery period, again at 30°C, heat-inactivated luciferase was reactivated in *dnaK*$^+$ cells to >90% of its activity prior to inactivation, while in Δ*dnaK52* cells no reactivation occurred (Figure 6A). *hscA::cat* mutant cells did not show comparable luciferase refolding defects (data

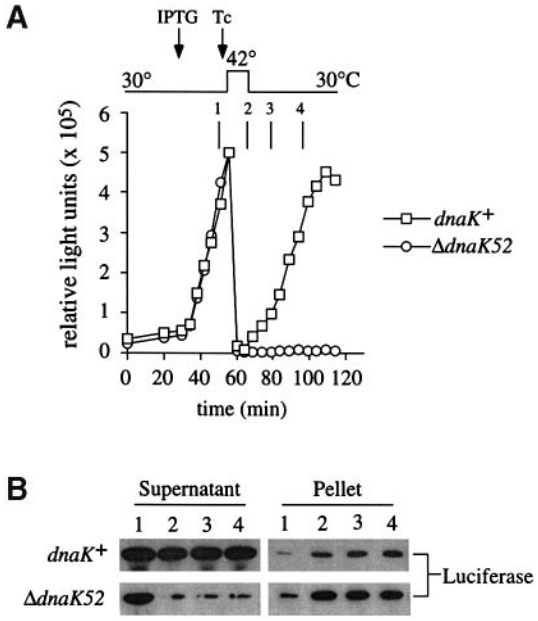

Fig. 6. Role of DnaK in prevention of aggregation and refolding of heat-denatured luciferase. Cells of wild-type and Δ*dnaK52* strains containing plasmids pDS12 P_{N25} Lac O3/O4 (*luc*$^+$) and pDMI.1 (*lacIq*$^+$) were grown in LB medium at 30°C to early logarithmic phase. Luciferase production was induced by addition of IPTG followed by *in vivo* determination of luciferase activity (**A**). After 30 min, further protein synthesis was stopped by addition of tetracycline to 50 μg/ml final concentration. The cultures were then exposed to an 8 min heat shock to inactivate luciferase, then transferred back to 30°C and assayed for luciferase reactivation. At the indicated time points (1–4), culture aliquots were withdrawn, lysed and centrifuged to investigate solubility of luciferase (**B**). Luciferase was detected in identical aliquots of the soluble and insoluble fractions by immunoblotting with specific antiserum.

not shown). This requirement for DnaK for refolding of thermally inactivated luciferase, as already observed before (Schröder *et al.*, 1993), is in strict contrast to the lack of a defect in *de novo* folding of the protein. We performed two controls to rule out that the reactivation deficiency of the mutant cells was an indirect effect caused by metabolic deficiencies of the Δ*dnaK52* mutant upon heat-shock treatment. First, viability plating of Δ*dnaK52* cells after washing out of tetracycline showed a >90% survival rate compared with the pre-heat-shock control. Secondly, cultures of Δ*dnaK52* and wild-type cells were subjected to a heat shock at 42°C for 8 min followed by induction of luciferase expression after temperature downshift back to 30°C. While the rate of synthesis of luciferase initially was 2-fold reduced in Δ*dnaK52* mutant cells compared with wild-type, there was no decrease in specific activity of the newly synthesized protein (not shown).

To investigate the folding status of luciferase in wild-type and Δ*dnaK52* cells before and after heat treatment, we determined its solubility. Soluble and aggregated protein were separated by the lysis and centrifugation protocol outlined above. The majority of luciferase in both strains was soluble prior to heat shock (Figure 6B). Heat-shock treatment caused only a mild aggregation of luciferase in wild-type cells while it caused extensive aggregation in Δ*dnaK52* cells. The aggregates formed during heat treatment did not re-solubilize during the recovery phase at 30°C. DnaK and probably wild-type amounts of DnaJ are thus essential to keep inactive and aggregation-prone luciferase in a soluble state that is competent for refolding under permissive conditions.

Discussion

We investigated the role of DnaK and HscA in protein folding in the *E.coli* cytosol. We have shown that they are not strictly required for the correct folding of newly synthesized proteins. Instead, DnaK is essential for the prevention of aggregation of misfolded proteins accumulating under heat shock conditions and for the subsequent refolding of these proteins.

This conclusion is supported genetically by the finding that double mutants lacking the *dnaK* and *hscA* genes and having low DnaJ levels due to polar effects of the *dnaK* deletion on the *dnaJ* gene remain viable at intermediate temperature. The slower growth of the double mutants compared with the single mutants and wild-type may be due to additive, rather than synergistic, defects. Further evidence comes from analysis of the folding status of reporter enzymes and the bulk of cellular proteins. Deletion of *dnaK* and/or *hscA* does not affect the specific enzymatic activity of β-galactosidase, and the specific activity and apparent folding kinetics of firefly luciferase at 30°C. Furthermore, DnaK and HscA are not strictly essential for *de novo* folding of the bulk of *E.coli* proteins since newly synthesized polypeptide chains were equally soluble at 30 and 42°C in *dnaK*$^+$ cells and mutants lacking *dnaK* and/or *hscA*. For Δ*dnaK52* mutants, we obtained similar results at temperatures up to 45°C, which is close to the upper growth temperature limit of *E.coli*, and at different time points after temperature upshift of the cells to 42°C. Furthermore, similar results were obtained in a strain in which DnaK synthesis was shut off by use of a tightly regulatable promoter/operator system to control the expression of chromosomally encoded *dnaK* (T.Tomoyasu, T.Hesterkamp and B.Bukau, unpublished results). All our data show that newly synthesized polypeptide chains are not particularly aggregation prone in mutant cells lacking DnaK and/or HscA, even under prolonged heat-shock conditions where pre-existing proteins aggregate (see below). It should be emphasized though that our data do not exclude that DnaK/HscA can associate with nascent polypeptide chains. In cases where such an interaction exists, it may have an auxiliary role in improving the efficiency of folding of some proteins against the dangers of misfolding, but such a role is not strictly essential for the folding of the majority of *E.coli* proteins.

The role of chaperones in protein folding in the *E.coli* cytosol has been analyzed previously with *E.coli* mutants which have reduced levels of major chaperones due to a mutation in the *rpoH* gene encoding σ^{32} (Gragerov *et al.*, 1991). In these mutants, proteins aggregate extensively after 60 min exposure to 42°C. Since aggregation was reduced when *rpoH* mutant cells were treated during heat exposure with antibiotics that block protein synthesis, the authors concluded that particularly the newly synthesized proteins are endangered by heat-induced aggregation (Gragerov *et al.*, 1991). However, in those experiments, the *rpoH* mutant cells continued to grow and retain 100% viability for at least 1 h at 42°C. It is difficult to envisage how cell growth can be accomplished without correct

folding of nascent polypeptide chains. An interpretation that most of the aggregation resulted from folding defects of pre-existing proteins cannot be excluded and would be consistent with our findings.

With respect to firefly luciferase, our data contrast with the role found for Hsp70 in its *de novo* folding in reticulocyte lysates (Hendrick *et al.*, 1993; Frydman *et al.*, 1994) and show that results obtained in cell-free translation systems cannot be generalized. In these experiments, the observed association of Hsp70 and Hsp40 (Frydman *et al.*, 1994) and prokaryotic DnaJ (Hendrick *et al.*, 1993) with nascent luciferase may be caused by the stalling of translation and thus folding of the nascent polypeptide chain (for discussion see Bukau *et al.*, 1996). Alternatively it is possible that the Hsp70 requirements for folding differ between the bacterial and the eukaryotic cytosol, implying that an essential role for Hsp70 in folding of newly synthesized proteins is not conserved throughout evolution. An important parameter which may contribute to such a difference is the speed of translation, which is ~14-fold faster in *E.coli* (22 amino acids/s) compared with mammalian cells (for discussion see Bukau *et al.*, 1996). It has indeed been reported for *Saccharomyces cerevisiae* that two Hsp70 homologs, Ssb1 and Ssb2, specifically associate with translating ribosomes (Nelson *et al.*, 1992) and nascent polypeptide chains (E.Craig, personal communication), whereas *E.coli* DnaK did not associate specifically with ribosomes translating β-galactosidase *in vitro* (Hesterkamp *et al.*, 1996), and only minor amounts of DnaK co-fractionated with translating polysomes (Gaitanaris *et al.*, 1994).

In sharp contrast to the lack of observed defects in folding of newly synthesized proteins, major defects exist in Δ*dnaK52* mutants in the folding of proteins after heat shock. Predominantly pre-existing proteins aggregate in these mutants at 42°C. Only a fraction (~10%) of proteins aggregate within 1 h, as expected in view of the fact that 42°C is a temperature well within the growth range of *E.coli*. The essential role of the DnaK system in preventing aggregation of heat-denatured proteins is best exemplified by thermolabile luciferase. This protein aggregated extensively in Δ*dnaK52* cells at 42°C while in *dnaK*$^+$ cells aggregation was suppressed, thus allowing efficient refolding under permissive conditions. The small fraction of luciferase that remained soluble after shift of Δ*dnaK52* cells to 42°C may be prevented from aggregation by GroEL since this chaperonin was shown *in vitro* to interact with heat-denatured luciferase, without permitting refolding, however (Buchberger *et al.*, 1996). We conclude from these experiments that the DnaK chaperone system is essential for refolding of misfolded proteins accumulating under stress conditions. This conclusion agrees well with the stress inducibility of its synthesis in *E.coli*, which is a reflection of the increased cellular requirements for DnaK function at high temperature (Bukau, 1993; Gross, 1996).

With regard to HscA, we have no evidence for a general role in protein folding. Lack of HscA does not lead to thermosensitivity and detectable protein folding defects. Furthermore, HscA is unable to replace DnaK in the chaperone-assisted refolding of unfolded luciferase *in vitro* (D.Brehmer, T.Hesterkamp and B.Bukau, unpublished results) and *in vivo*. We assume that HscA has a rather specialized function, perhaps involving the ferredoxin protein that is encoded by the *fdx* gene located immediately downstream of *hscA* (Seaton and Vickery, 1994). In addition, since *hscA* had been identified genetically as a mutation that suppresses gene regulation defects associated with mutations in the *hns* gene (Kawula and Lelivelt, 1994), it might be that HscA has a function related to DNA packaging.

Table II. Bacterial strains

Strain	Relevant genetic marker(s)	Source or reference
MC4100	*dnaK*$^+$ *hscA*$^+$	Casadaban (1976)
C600	*dnaK*$^+$ *hscA*$^+$	laboratory collection
BB1553	MC4100 Δ*dnaK52::cat sidB1*	Bukau and Walker (1990)
THK80	*hscA::cat*	Kawula and Lelivelt (1994)
CAG18481	*zfh-208::*Tn*10*	C.Gross; CGSC
BB4542	MC4100 *hscA::cat*	this study
BB4515	MC4100 *zfh-208::*Tn*10*	this study
BB4513	MC4100 *hscA::cat zfh-208::*Tn*10*	this study
BB4517	BB1553 *zfh-208::*Tn*10*	this study
BB4514	BB1553 *hscA::cat zfh-208::*Tn*10*	this study

It cannot be excluded that other cytosolic chaperones of *E.coli* can substitute for the missing function of Hsp70 proteins in *dnaK*/*hscA* mutants in preventing aggregation and assisting folding of newly synthesized proteins. In particular, an open reading frame (f556) exists in *E.coli* which encodes a putative further Hsp70 homolog of unknown function. Potential back-up systems cannot rely on the compensatory overproduction of other major chaperone systems since in Δ*dnaK52* mutant cells the levels of the remaining known chaperones GroEL, HtpG (Hsp90) and trigger factor were not strongly altered compared with wild-type. Furthermore, potential back-up systems are inefficient at 42°C at preventing aggregation and, as shown for luciferase, allowing the refolding of misfolded proteins in Δ*dnaK52* mutant cells.

The results of this study rule out an essential role for the DnaK system in the *de novo* folding of the majority of *E.coli* proteins, although auxiliary roles that increase the efficiency of folding of some proteins are not excluded. It is intriguing that the only *E.coli* protein found to associate efficiently with and cross-link to nascent chains of a variety of cytosolic proteins is trigger factor (Valent *et al.*, 1995, 1997; Hesterkamp *et al.*, 1996). Trigger factor may assist the co-translational folding of polypeptide chains by virtue of its ribosome-binding domain, a peptidyl–prolyl *cis/trans*-isomerase activity (Stoller *et al.*, 1995; Hesterkamp *et al.*, 1996) and an additional chaperone-like function (Scholz *et al.*, 1997). Besides the possible assistance by trigger factor, it is known that the GroEL chaperonin is required for the *de novo* folding of a subset of *E.coli* proteins, estimated to be 5–15% of total protein (Horwich *et al.*, 1993; Ewalt *et al.*, 1997). It remains to be determined whether, besides the subpopulation of GroEL-dependent substrates, the *de novo* folding of cytosolic proteins requires assistance by additional factors or occurs spontaneously.

Materials and methods

Strains and culture conditions

Routinely, the bacterial strains listed in Table II were cultured at 30°C in Luria broth (LB) medium or M9 minimal medium supplemented with

0.2% (w/v) glucose as a carbon source, and 0.2% (w/v) casamino acids (Difco Laboratories) or 20 L-amino acids (Sigma LAA-21) at 80 μg/ml/amino acid. The antibiotics tetracycline, chloramphenicol, kanamycin and ampicillin were purchased from Sigma and used at final concentrations of 10, 20, 40 and 100 μg/ml, respectively. Temperature shift experiments were performed in orbital shaking water baths. For pulse-labeling with [^{35}S]methionine, cells were grown in M9 minimal medium containing glucose and all L-amino acids except methionine. Labeling was done as indicated in the figure legends by adding [^{35}S]methionine (Amersham SJ1515, 15 mCi/ml, 1000 Ci/mmol) to 10 μCi/ml cell culture for 60 s, followed, where indicated, by addition of unlabeled L-methionine to 200 μg/ml. Cells were then chilled rapidly and treated as detailed below.

Production and activity measurements of firefly luciferase and β-galactosidase

The luciferase gene from *P.pyralis* was expressed from plasmid pDS12 pN25 Lac O3/O4 (*luc*$^+$, Amp^r) (Schröder *et al.*, 1993) which allows tight transcriptional repression by the Lac repressor. *lacI*^{q} was provided by plasmid pDMI,1 (Kan^r) (Lanzer, 1991). Induction of expression was achieved during early logarithmic growth (OD_{600} of 0.4) by addition of IPTG to 100 μM final concentration. *In vivo* measurements of luciferase activity were performed as duplicates on a Berthold LB 9501 lumat by pipeting 10 μl culture aliquots into the test tube followed by automatic injection of 200 μl of D-luciferin (AppliChem, 0.25 mM in water). Where indicated, further production of luciferase was stopped by addition of tetracycline to 50 μg/ml final concentration. For heat inactivation of luciferase *in vivo*, the cultures were transferred to a 42°C shaking water bath for 8 min. For subsequent recovery, cultures were transferred back to a 30°C shaking water bath.

Escherichia coli β-galactosidase was produced from plasmid pML3 (*lacZ*$^+$, *lacI*$^{q+}$ Amp^r) (Lanzer, 1991) encoding *lacZ* under control of the pA1 Lac O3/O4 promoter/operator and *lacI*^{q}. Cultures were grown to early logarithmic phase at 30°C followed by addition of IPTG to 100 μM final concentration and continuation of growth for 20 min. β-galactosidase activities of chilled cultures were then measured within a few minutes in duplicate as follows: 700 μl of Z-buffer (100 mM Na-phosphate, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol), 60 μl of chloroform, 40 μl of 0.1% (w/v) SDS and 5 μl of the cultures were mixed and vortexed for 20 s at room temperature. Then, 200 μl of ONPG substrate solution (4 mg/ml in Z-buffer) was added followed by incubation of the tubes at 30°C. Reactions were stopped by addition of 500 μl of 1 M Na_2CO_3 solution and quantified photometrically at 420 nm. Miller units were calculated using the formula $MU = 1000 \times \Delta A_{420} \times t\ (\text{min})^{-1} \times \text{vol}\ (\text{ml})^{-1} \times OD_{600}{}^{-1}$.

Extract preparation and solubility of proteins

Aliquots (10 ml) of bacterial cultures were cooled rapidly to 0°C in an ice–water bath and within a few minutes centrifuged for 10 min at 5000 *g* and 4°C to harvest the cells. Pellets were resuspended in 10× lysis buffer [100 mM Tris–HCl pH 7.5, 100 mM KCl, 2 mM EDTA, 15% (w/v) sucrose, 1 mg/ml lysozyme] according to their optical density (50 μl lysis buffer for 10 ml culture of OD_{600} = 1) and frozen at –20°C. These samples were then thawed slowly at 0°C followed by addition of a 10-fold volume of ice-cold water and mixing. The viscous, turbid solution was sonicated with a Branson Cell Disruptor B15 (microtip, level 6, 50% duty cycle, five pulses) while cooling. Insoluble material was pelleted by centrifugation at 25 000 *g* for 30 min at 4°C. Supernatants were taken off and subjected to precipitation by trichloroacetic acid (TCA, 10% final concentration), and pellets were re-extracted with 1× lysis buffer followed by TCA precipitation. Aliquots of soluble and insoluble fractions were analyzed by SDS–PAGE followed by immunoblotting, staining with Coomassie Brilliant Blue or autoradiography.

Cloning of hscA and purification of HscA

The *hscA* gene from *E.coli* was amplified by PCR using native *Pfu* proofreading DNA polymerase (Stratagene), Kohara λ phage No. 430 (5E10) DNA (encompassing the relevant 57 min area of the *E.coli* chromosome) as the template, the forward primer 5′-GGC Cgg atc cAT GGC CTT ATT ACA AAT TAG TGA AC-3′ and the reverse primer 5′-GGC Cct gca gTT AAA CCT CGT CCA CGG AAT GGC CT-3′. The engineered *Bam*HI and *Pst*I sites were used for subsequent cloning of the *hscA* gene fragment into the single *Bam*HI and *Pst*I restriction sites of the *E.coli* expression vector pUHE21-2 fd Δ12 (Buchberger *et al.*, 1994) followed by sequencing of the entire gene. The resulting plasmid pUHE21-2 fd Δ12 (*hscA*$^+$) was used for *in vivo* complementation analysis and purification of HscA. Briefly, 500 ml of DH5α cells transformed with plasmids pUHE21-2 fd Δ12 (*hscA*$^+$) and pDMI.1 (*lacI*$^{q+}$) were grown at 30°C in LB medium to mid-logarithmic phase. Then, expression of the *hscA* gene was induced by addition of IPTG to 0.5 mM. After 4 h, the cells were chilled, harvested by centrifugation and lysed by the freeze–thaw/sonication protocol outlined above except that the free Mg^{2+} concentration was permanently maintained at 1 mM. The cleared lysate was subjected to a fractionated ammonium sulfate precipitation with the bulk of HscA precipitating at 40% $(NH_4)_2SO_4$ saturation at 4°C. The resuspended protein fraction was passed over a preparative Superdex 200 gel filtration column (Pharmacia) in buffer A (20 mM Tris–HCl pH 7.5, 1 mM $MgSO_4$, 5 mM 2-mercaptoethanol) containing 50 mM NaCl. HscA peak fractions, migrating with ~100 kDa native molecular weight, were pooled and subjected to anion exchange chromatography using a preparative Protein Pak Q 8HR column (Waters) and a linear salt gradient from 50 to 500 mM NaCl in buffer A. Pure HscA was concentrated and stored in small aliquots at –80°C.

Miscellaneous

P1 *vir* transductions were done according to standard procedures (Silhavy *et al.*, 1984). SDS–PAGE was carried out according to Laemmli (1970). Immunoblots were developed with alkaline phosphatase-conjugated secondary antibodies from goat (Dianova) and the substrate mix BCIP/NBT.

Acknowledgements

We thank T.Kawula for *hscA* mutant strains and *hscA* expression plasmids; L.Vickery, M.Ehrmann and U.Henning for antisera specific for HscA, HscB, OmpF and OmpA; and H.Bujard for plasmids expressing luciferase and β-galactosidase. We thank S.Rüdiger for comments on the manuscript. This work was supported by grants from the BMBF (Project BEO 22/0311146), the DFG (Bu617/6-4 and SFB388) and the Fonds der Chemischen Industrie, and a fellowship from the Boehringer Ingelheim Fonds to T.H.

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Received May 4, 1998; revised June 16, 1998;
accepted June 17, 1998