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).
Role of Hsp70 in protein folding in E.coli

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 reaches the native state directly, is transferred to CCT or GroEL for final folding. These 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.

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 insertional 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 ΔdnaK52 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 ΔdnaK52 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, σ32, 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, ΔdnaK52 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 ΔdnaK52 sidB1 cells to grow at 42°C (Table I). Overproduction of HscA did occur up to similar high
and screened for co-transduction of the DnaK and HscA served as a standard. More than 95% of DnaK and HscA partitioned with the soluble protein fraction during centrifugation.

More than 95% of DnaK and HscA partitioned with the soluble protein fraction during centrifugation. A soluble fraction of the total E. coli protein was 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.

The co-transduction frequency of the two loci was similar in E. coli strains MC4100 (M–) and C600 (C–) cells 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 ΔdnaK52 sidB1 cells by plasmid-borne hscA and dnaK genes

<table>
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<tr>
<th>Temperature (°C)</th>
<th>30</th>
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<td>IPTG (µM)</td>
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<td>ΔdnaK52 pUHE 21-2 fd Δ12</td>
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<tr>
<td>ΔdnaK52 pUHE 21-2 fd Δ12 (dnaK+)</td>
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<td>+</td>
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<tr>
<td>ΔdnaK52 pUHE 21-2 fd Δ12 (hscA')</td>
<td>+</td>
<td>+</td>
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<tr>
<td>dnaK+ pUHE 21-2 fd Δ12 (hscA'')</td>
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Cells of strains MC4100 (dnaK+) and BB1553 (ΔdnaK52) 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 (lacY'). 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.

**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 ΔdnaK52 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 cotransduction frequency of the two loci was similar in dnaK+ and ΔdnaK52 cells (Figure 2A), providing genetic proof that a ΔdnaK52 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 ΔdnaK52 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 ΔdnaK52 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 ΔdnaK52 cells carrying the sidB1 suppressor mutation was almost the same as for wild-type, while that of hscA::cat and ΔdnaK52 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 ΔdnaK52 hscA::catC 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 ΔdnaK52 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 ΔdnaK52 cells (Bukau and Walker, 1989a,b).
DnaK and HscA are not essential for folding of β-galactosidase and luciferase at 30°C

The ability of the ΔdnaK32 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 ΔdnaK32 or ΔhscAΔdnaK32 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 vitro 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 dnaKΔ 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, ΔdnaK32 and ΔdnaK32 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 ΔdnaK52 hscA::cat strains containing plasmids pDS12 pN25 O4/O3 (lac+) and pDMI.1 (lacP+) 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+, lacIq+/H11001) 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 ΔdnaK52 and ΔdnaK52 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 ΔdnaK52 and ΔdnaK52 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 ΔdnaK52 cells were pulse-labeled with [35S]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 ΔdnaK52 mutant cells (Figure 4B). After
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<sup>+</sup> and 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, activity was reactivated in dnaK<sup>+</sup> cells to >90% of its activity prior to inactivation, while in DnaK52 cells no reactivation occurred (Figure 6A). hscA::cat mutant cells did not show comparable luciferase refolding defects (data not shown).
containing plasmids pDS12, P N25, Lac O3/O4. Luciferase production was induced by addition of IPTG for 30 min, further protein synthesis was stopped 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.

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 temperatures. 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 mutant cells and mutants lacking dnaK and/or hscA. For ΔdnaK52 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 β32 (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 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.
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 ΔdnaK52 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 ΔdnaK52 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 ΔdnaK52 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 Lelieveld, 1994), it might be that HscA has a function related to DNA packaging.

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 ΔdnaK52 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 ΔdnaK52 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 t-aminoc acids (Sigma LAA-21) at 80 μg/ml/amin 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 [35S]methionine, cells were grown in M9 minimal medium containing glucose and all t-aminoc acids except methionine. Labeling was done as indicated in the figure legends by adding [35S]methionine (Amersham SH3151, 15 μCi/μmol, 100 Ci/mmol) to 10 μCi/ml cell culture for 60 s, followed, where indicated, by addition of unlabeled t-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 Pyrophis was expressed from plasmid pDS12 pH25 Lac O3/O4 (lacP+, βamp) (Schröder et al., 1993) which allows tight transcriptional repression by the lac repressor. lacP+ was provided by plasmid pDML1 (Kan+) (Lanzer, 1991). Induction of expression was achieved during early logarithmic growth (OD600 of 0.4) by addition of IPTG to 100 μM final concentration. In conclusion, lacP−β-galactosidase activity were performed as duplicates on a Berthold LB 9501 lumat by pipetting 10 μl culture aliquots into the test tube followed by automatic injection of luciferase activity were measured within a few minutes following injection of 200 μL of 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 (lacP−, lacP+ Amp+) (Lanzer, 1991) encoding lacZ under control of the pA1 Lac O3/O4 promoter/operator and lacP−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 MgSO4, 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 37°C. Reactions were stopped by addition of 500 μl of 1 M Na2CO3 solution and quantified photometrically at 420 nm. Millier units were calculated using the formula MU = 1000×Δ420/μl x(min)⁻¹ x ν(μl)⁻¹ x OD600⁻¹.

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 OD600 = 1) and frozen at −20°C. These samples were then thawed slowly at 0°C followed by addition of 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. 264, 261 (5E10) DNA (comprising the relevant 57 min area of the E.coli chromosome) as the template, the forward primer 5'-GGC Ctc gat cAT GGA GTC TTT ATT TAG ATA AAC GAT GGC CT-3' and the reverse primer 5'-GGC Cct gca gGT AAA CCA CGC CCG AAC AAT GAT GGC CT-3'. The engineered BamHI and PstI sites were used for subsequent cloning of the hscA gene fragment into the single BamHI and PstI 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 pDM11 (lacP−) 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 Mg2+ 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% (NH4)2SO4 saturation at 31515. 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 MgSO4, 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 HR 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 22/031146), the DFG (Bu176/4-1 and SFB388) and the Fonds der Chemischen Industrie, and a fellowship from the Boehringer Ingelheim Fonds to THI.

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Hesterkamp and Bukau


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