Ribosome–DnaK interactions in relation to protein folding

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Summary

Bacterial ribosomes or their 50S subunit can refold many unfolded proteins. The folding activity resides in domain V of 23S RNA of the 50S subunit. Here we show that ribosomes can also refold a denatured chaperone, DnaK, *in vitro*, and the activity may apply in the folding of nascent DnaK polypeptides *in vivo*. The chaperone was unusual as the native protein associated with the 50S subunit stably with a 1:1 stoichiometry *in vitro*. The binding site of the native protein appears to be different from the domain V of 23S RNA, the region with which denatured proteins interact. The DnaK binding influenced the protein folding activity of domain V modestly. Conversely, denatured protein binding to domain V led to dissociation of the native chaperone from the 50S subunit. DnaK thus appears to depend on ribosomes for its own folding, and upon folding, can rebind to ribosome to modulate its general protein folding activity.

Introduction

The involvement of molecular chaperones in protein folding and related phenomena has been a subject of extensive research (Radford, 2000; Frydman, 2001). The chaperones do not specify protein conformation but employ distinct mechanisms to prevent aggregation of a significant subset of newly synthesized polypeptide chains in both bacteria and eukaryotic cells. The bacterial ribosomes have also been shown to possess a general protein folding activity with distinct features (Das *et al*., 1992; 1996; Chattopadhyay *et al*., 1994; 1996; 1999; Kudlicki *et al*., 1997; Pal *et al*., 1997; 1999; Argent *et al*., 2000; Clark and King, 2001; Kramer *et al*., 2001; Chowdhury *et al*., 2002; Sanyal *et al*., 2002). The folding can be accomplished by the large subunit of bacterial ribosomes or simply by the domain V of 23S ribosomal RNA (rRNA) of the subunit. The process appears to be universal as proteins of diverse three-dimensional fold can be reactivated after denaturation using only the domain V RNA *in vitro*. We therefore have two classes of candidates in charge of protein folding: the chaperones and the ribosome, the protein synthesizing machine itself. The relationship of the two systems in the protein folding consortium is not known. These could be two independent systems running in parallel within the cell or these could be assisting each other to foster protein folding. The chaperones could very well be involved in the morphogenesis of the huge RNA–protein complex, the ribosome. In such a scenario, a close association between DnaK, the major *Escherichia coli* Hsp70, and the ribosomes is of particular interest.

The following characteristics of chaperones are of relevance to the present discussion. The ribosome–chaperone complexes are involved in protein trafficking in eukaryotic cells (Rothman, 1994; Schmid, 1997). In *E. coli*, thermosensitive DnaK mutants show ribosome assembly defects at a non-permissive temperature (45°C), and abnormal ribosomal particles (e.g. 45S, 35S and 25S) can be rescued if the mutant cells are returned to 30°C (Alix and Guerin, 1993). Thus, DnaK could be involved in ribosome morphogenesis. Also, the *E. coli* DnaK (Alix and Guerin, 1993) and the yeast Hsp70 (Nelson *et al*., 1992) are found in polysomes. Co-migration of DnaK with 50S ribosomal subunit (Vysokanov, 1995), and purified 5S rRNA (Okada *et al*., 2000) have also been reported. These findings on DnaK and *E. coli* ribosomes warrant a detailed study of their interactions. We report here some such studies in relation to the ribosome-assisted protein folding *in vitro* and *in vivo*.

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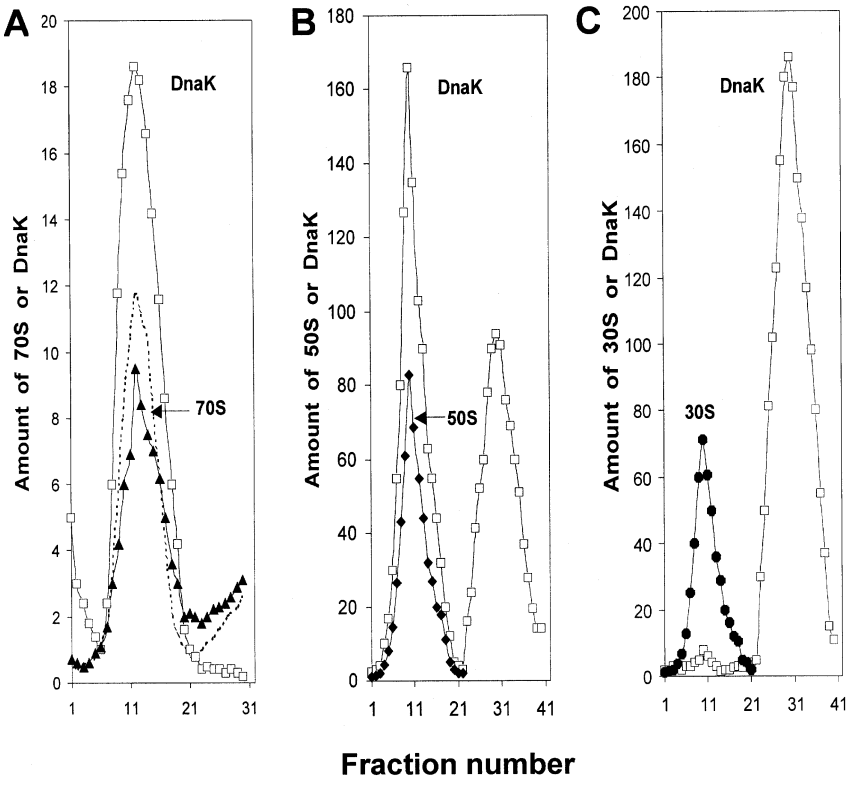

Fig. 1. Association of DnaK with 70S, 50S and 30S ribosomal particles.
A. A mixture of ^{32}P-labelled native DnaK and 70S ribosomal particle (300 nM each) was incubated in buffer A (*Experimental procedures*) at 25°C for 5 min. The sample was then loaded on to a 5–20% sucrose gradient (5 cm) prepared in buffer A, and centrifuged at 28 000 r.p.m. in a SW51 rotor for 1.5 h at 4°C. Fractions were collected from the bottom of the tube till its contents were exhausted. Migration profiles of 70S particle when present in the mixed sample (triangles) and when alone (dotted line) were obtained by plotting absorbance of the fractions at 260 nm against the fraction numbers. Note that when run in parallel 50S and 30S particles peak at fractions 18 and 25, respectively, as opposed to 12 for 70S (data not shown). Migration profile of DnaK (squares) was obtained by plotting ^{32}P counts. One unit in ordinate represents 0.09 absorbance at 260 nm (for 70S) or 2×10^3 c.p.m. (for DnaK).
B and C. FITC-labelled native DnaK was mixed with either 50S or 30S particle in buffer A with a DnaK (600 nM) to particle (300 nM) molar ratio of 2:1, incubated at 25°C for 5 min, and loaded on to a Sephadex G-200 gel filtration column equilibrated with buffer A. The column was eluted with buffer A and the fractions were collected. Elution profiles of either 50S (diamonds, B) or 30S (circles, C) were obtained as in A except that DnaK was measured by fluorescence emission. One unit in ordinate represents 0.014 absorbance at 260 nm (for 50S or 30S) or an arbitrary unit of fluorescence (for DnaK).

Results

Association of DnaK protein with ribosomal particles

The association of DnaK to ribosomal particles was determined either by gel filtration or sucrose gradient centrifugation. For ease of detection, DnaK was autophosphorylated with [γ-^{32}P]-ATP or labelled with a fluorescent dye, FITC. The autophosphorylated protein co-migrated in a 5–20% sucrose gradient with the 70S particle (Fig. 1A). The interaction with ribosomal subunits was determined by filtration through sephadex G-200 columns. The FITC-labelled DnaK co-eluted with the 50S subunit (Vysokanov, 1995) in the void volume (Fig. 1B), whereas it eluted in later fractions (Fig. 1C) in the presence of the 30S subunit that eluted in the void volume. The elution profiles were identical when autophosphorylated DnaK instead of FITC-labelled DnaK was used. These results suggest that DnaK protein binds to 70S ribosome and to its 50S subunit but not to the 30S subunit.

In the above study, we used a DnaK:50S molar ratio of 2:1 in the binding reaction. The gel elution profile (Fig. 1B) indicates that about half of the FITC-labelled native DnaK co-eluted with 50S in the void volume. The remaining half of DnaK eluted later. From these results it appears that FITC-labelled DnaK binds to 50S with a 1:1 stoichiometry. When we used an equimolar mixture of DnaK and 50S in the binding reaction, all of the DnaK co-eluted with 50S in the void volume indicating tight binding of the two (data not shown). The binding reaction between DnaK and any one of 70S, 50S or 30S particle was carried out for 5 min (Fig. 1). The results were same when the reaction time was increased to 1 h.

Protein folding by 50S in the presence of DnaK

The effect of bound DnaK on the protein folding activity of ribosomes was determined next. The activity of unfolded bovine carbonic anhydrase (BCA) increased to about 80% both with free and DnaK-bound 50S particles (top two curves, Fig. 2A). Although the final yield of refolded protein did not change, the binding of DnaK to 50S increased the rate of protein folding. The time to gain half of the increase in activity was 4.4 ± 0.18 min and 3.3 ± 0.22 min with free and DnaK-bound 50S particles, respectively, from four different experiments. The time was 4.0 ± 0.19 min when DnaK, 50S and unfolded BCA were added at the same time (50S, DnaK curve, Fig. 2A). In this case the initial activity (12%) and the maximal activity (47%) were both lower, most likely because of some trapping of unfolded protein by DnaK as determined

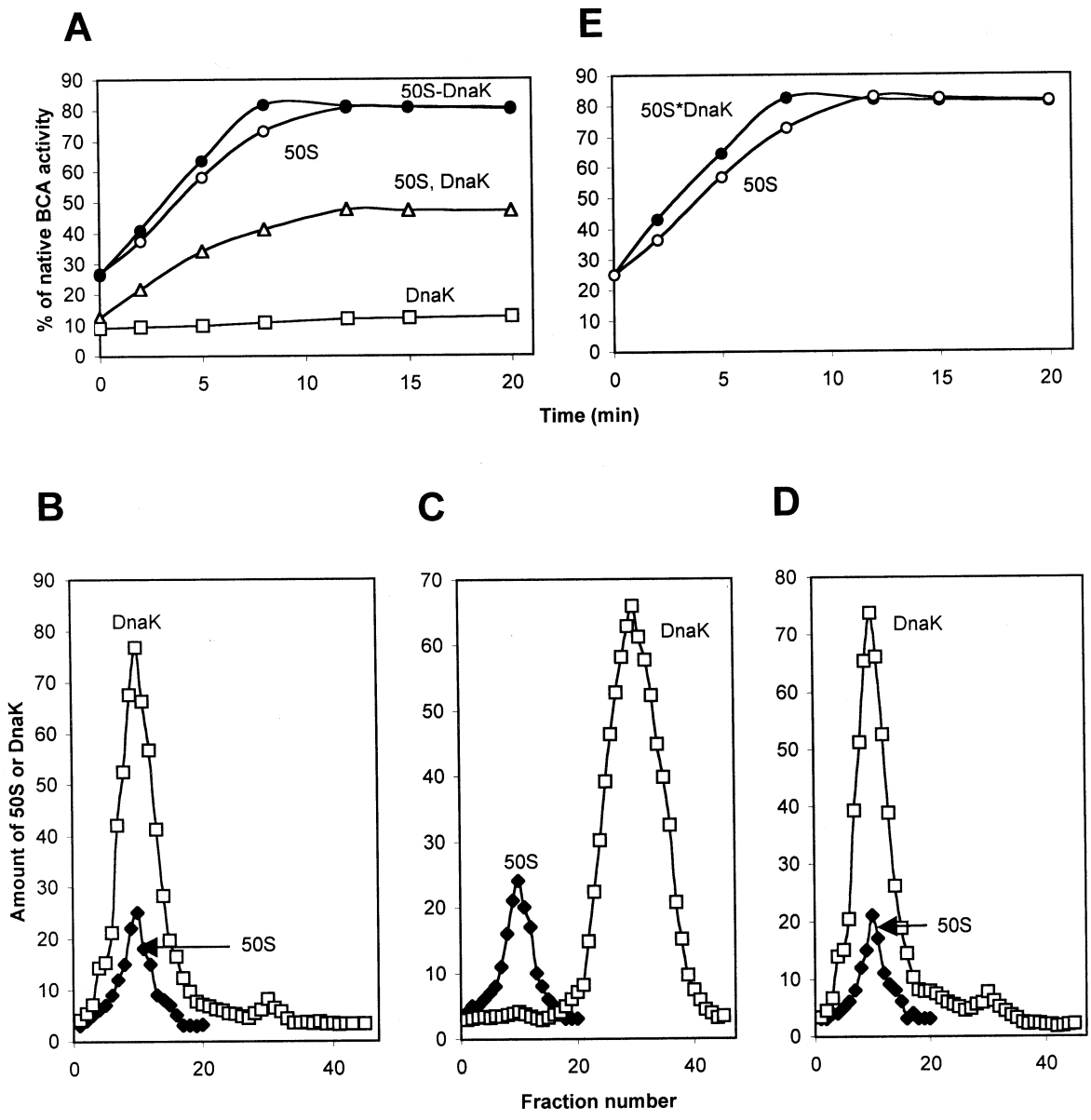

Fig. 2. Time course of refolding of bovine carbonic anhydrase (BCA) as modulated by 50S, DnaK and 50S–DnaK complex.
A. BCA was allowed to refold in presence of preformed 50S-DnaK complex (filled circles), or 50S (open circles), or 50S and DnaK added at the same time without preincubation (triangle), or DnaK (squares). The concentration of the modulators was equimolar to BCA (300 nM) in all cases. Per cent of BCA activity at each time point was expressed taking the activity of the same amount of native protein as 100%.
B–E. Effect of cross-linking of 50S-DnaK complex.
B. 50S and RITC-labelled DnaK were allowed to interact in equimolar proportion (300 nM each) for 5 min at 25°C, cross-linked with UV (250 mJ, 5 min, 25°C), incubated in 2 M ammonium chloride for 30 min and then subjected to gel filtration as in Fig. 1. One unit in ordinate represents 0.06 absorbance at 260 nm (for 50S) or 2 arbitrary units of fluorescence (for DnaK).
C. Same as B but without the UV exposure.
D and E. D is same as B except that after the UV exposure, instead of ammonium chloride, unfolded BCA (300 nM final) was added. After 2 min, a part of the mixture was subjected to gel filtration (D) as in B, and another was used to assay for recovery of the BCA activity (E). Uncrosslinked 50S was used as a control in E. For D, one unit in ordinate represents 0.03 absorbance at 260 nm (for 50S) or 1 arbitrary unit of fluorescence (for DnaK).

by fluorescence energy transfer (data not shown). DnaK alone in the absence of 50S could not assist the folding of denatured BCA (DnaK curve, Fig. 2A). The maximal activity in the presence of DnaK alone (12%) was lower than the values at time zero (27%) obtained with 50S or 50S–DnaK complex, indicating that DnaK alone could be preventing self-folding of BCA. We also checked that the activity of BCA, when the unfolded protein was prebound to equimolar DnaK for 30 min, was not enhanced on further incubation with equimolar 50S (data not shown). These results show that DnaK only when it is present as a complex with 50S can help in 50S-mediated protein refolding.

Because DnaK did not interfere with the protein folding activity of ribosomes, the binding site of DnaK does not appear to overlap with the exposed parts of the domain V region of 23S rRNA that is responsible for protein folding. This is consistent with the finding that DnaK can bind to 5S rRNA (Okada *et al*., 2000). To confirm the notion that DnaK binding leaves the domain V region accessible, we first cross-linked DnaK to 50S by exposure to ultraviolet light (Fig. 2B–D) and then used the cross-linked complexes (50S*DnaK) to refold denatured BCA (Fig. 2E). The recovery of activity was identical to that of the 50S–DnaK complexes before cross-linking (Fig. 2A). These results indicate that DnaK and the folding substrate can be present simultaneously on 50S.

Transient release of DnaK during protein folding by 50S

To determine the fate of bound (uncross-linked) DnaK during the folding reaction, the products of the reaction containing 50S, refolded BCA and DnaK were subjected to gel filtration. The DnaK protein, bound to 50S before the reaction, eluted separately from 50S after the reaction (Fig. 3A). Apparently, the process of refolding by domain V led to conformational changes in 50S that caused the release of bound DnaK. If the products of this reaction were left in the test tube for longer times (e.g. 20 min), the released DnaK protein rebound to 50S and co-eluted with it during gel filtration (Fig. 3B). We also note that DnaK appears to dissociate earlier from 50S than BCA, implying that 50S rather than DnaK is responsible for the refolding of BCA (Fig. 3C). The above observation was true when lactate dehydrogenase and malate dehydrogenase were used instead of BCA but in all cases the release of DnaK required the presence of denatured proteins. The experiments described so far were done in the absence of ATP (Figs 1–3). The results were same when ATP was included in the reaction.

When instead of native DnaK guanidium hydrochloride denatured DnaK was used, it first interacted with 50S and then dissociated from 50S (Fig. 3D and E). The binding of unfolded DnaK to 50S was further confirmed by UV-crosslinking (Fig. 3F). These experiments also showed that if DnaK is allowed to self-fold first and then treated with 50S, little complex formation occurs (Fig. 3G). In other words, as opposed to denatured DnaK, self-folded DnaK is no longer recognized by 50S, most likely because it is misfolded. Note that although the native DnaK reproducibly elutes around fraction #30, it eluted around fraction #35 in panels F and G, indicating that the conformation of the self-folded is significantly altered compared to the native protein.

The measurement of autophosphorylation activity of DnaK immediately after separation from 50S using gel filtration (Fig. 3E) revealed that the protein has become active. This indicates that denatured DnaK underwent refolding and then dissociated from 50S. On longer incubation, the refolded DnaK bound back to the 50S particle as was seen earlier (Fig. 3B). In summary, denatured DnaK behaves like other denatured proteins tested but the refolded DnaK is different as it rebinds to 50S as is characteristic of the native DnaK.

Conformation of domain V upon binding of DnaK to 50S

We showed above that interaction of denatured substrates with 50S–DnaK complex releases DnaK indicating changes in 50S conformation (Fig. 3). In the 50S–DnaK complex, the conformation in the domain V region could also be altered, as the kinetics of BCA refolding was somewhat faster with the 50S–DnaK complex compared to 50S alone (Fig. 2A). To gain further support for the DnaK-mediated conformational change of 50S, we determined its protein folding activity in the presence of antibiotics chloramphenicol and lincomycin that bind to and inactivate domain V of 23S RNA. Earlier we showed that the antibiotics inhibited protein folding by purified 23S rRNA but not (or only weakly in the case of lincomycin) by intact 50S (Chattopadhyay *et al*., 1996; 1999; Pal *et al*., 1997; Sanyal *et al*., 2002). However, in the presence of DnaK, the folding of BCA by 50S became more sensitive to the antibiotics (Fig. 4A and B). The 50S–DnaK complex remains intact in the presence of the antibiotic (Fig. 4C). Also, the antibiotics by themselves did not affect the activity of native or refolded BCA. It appears that DnaK binding alters 50S so that it becomes more accessible to the antibiotics that interact with domain V.

Refolding of DnaK in vitro *with 23S rRNA and its components*

The domain V RNA can be split into two regions, namely RNA1 and RNA2 (lengths 337 and 425 nucleotides, respectively; *Experimental procedures*), which together at equimolar concentration with respect to denatured protein, reconstitute the refolding activity of domain V (Pal

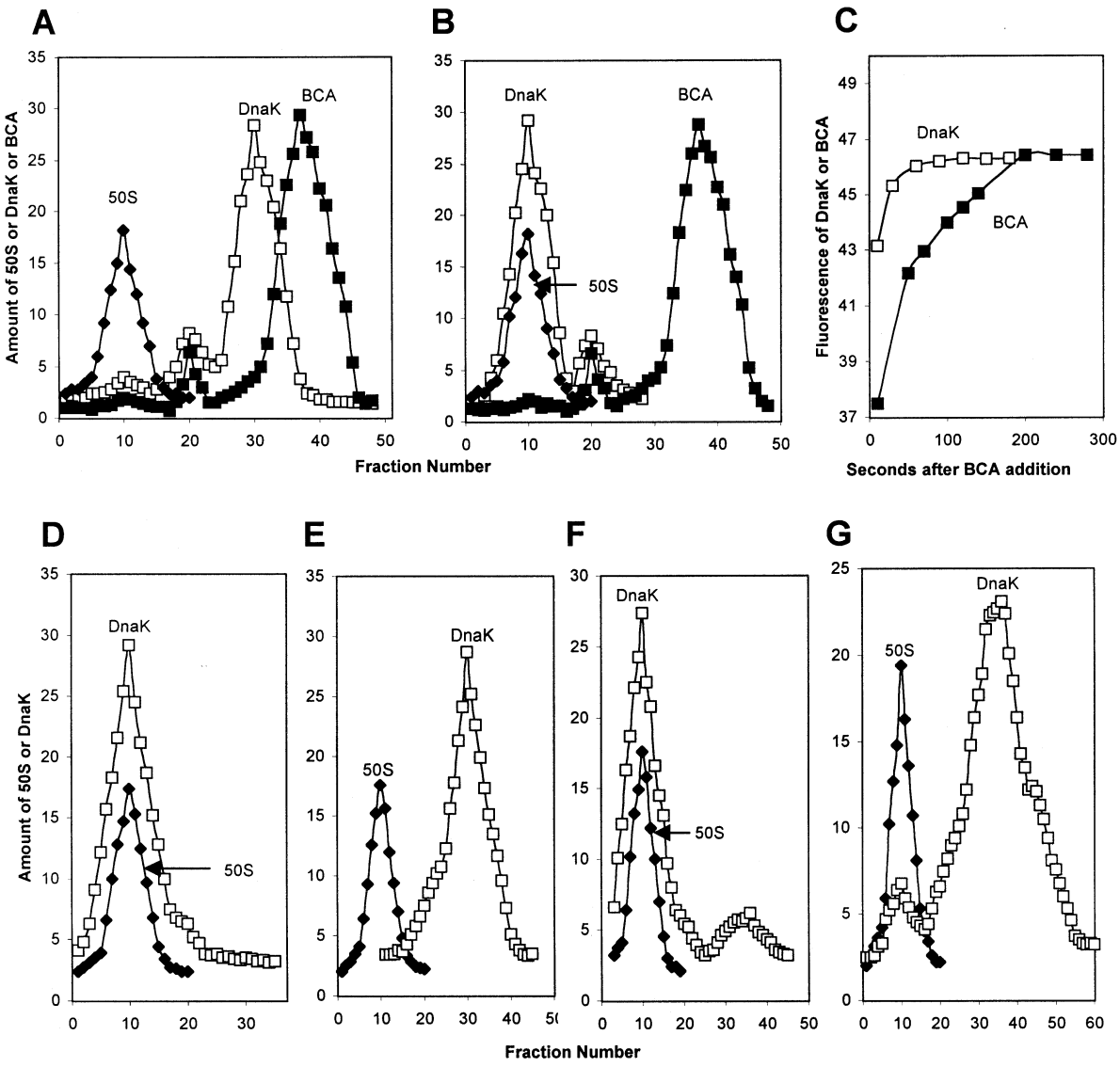

Fig. 3. Interaction of unfolded proteins with 50S or 50S-DnaK complex.
A and B. Denatured BCA (FITC-labelled, 300 nm) was added to equimolar 50S–DnaK complex in buffer A where DnaK was labelled with 32P (phosphorylated with [γ-^{32}P]-ATP). The mixture was incubated at 25°C for 2 min (A) or 20 min (B) and then subjected to gel filtration as in Fig. 1. One unit in ordinate represents 0.06 absorbance (for 50S) or 103 c.p.m. (for DnaK) or 10 arbitrary units of fluorescence (for BCA).
C. RITC-labelled DnaK (200 nm) was allowed to bind to equimolar 50S in buffer A at 25°C for 5 min. To this 300 nm denatured BCA was added and the fluorescence was measured with time (open squares). The experiment was repeated with unlabelled DnaK and FITC-labelled BCA. One unit in ordinate represents one arbitrary unit of fluorescence at 575 nm (for DnaK) or 2.8 arbitrary units of fluorescence at 520 nm (for BCA). The recoveries of fluorescence are due to release of the labelled proteins from 50S (fluorescence of these proteins are quenched when bound to 50S).
D and E. Same as A and B, respectively, except that instead of BCA denatured DnaK (RITC-labelled, 220 nm) was added to equimolar 50S. One unit in ordinate represents four arbitrary units of fluorescence.
F. Denatured DnaK (RITC-labelled) is diluted 27.2-fold in the presence of equimolar 50S in buffer A and immediately chilled in ice. After 5 min the mixture is cross-linked in UV (250 mJ, 5 min) during which it is kept in ice. The resulting product is subjected to gel-filtration in G-200 equilibrated in buffer A. The column is eluted with buffer A, fractions collected and measured for O.D. at 260 nm (for 50S) or RITC-fluorescence (for DnaK).
G. Self folded DnaK (RITC-labelled) is allowed to bind to equimolar 50S in buffer A at 25°C for 5 min, and then subjected to gel filtration in G-200 equilibrated in buffer A. The column is eluted with buffer A, fractions collected and measured for absorbance at 260 nm (for 50S) or RITC-fluorescence (for DnaK).

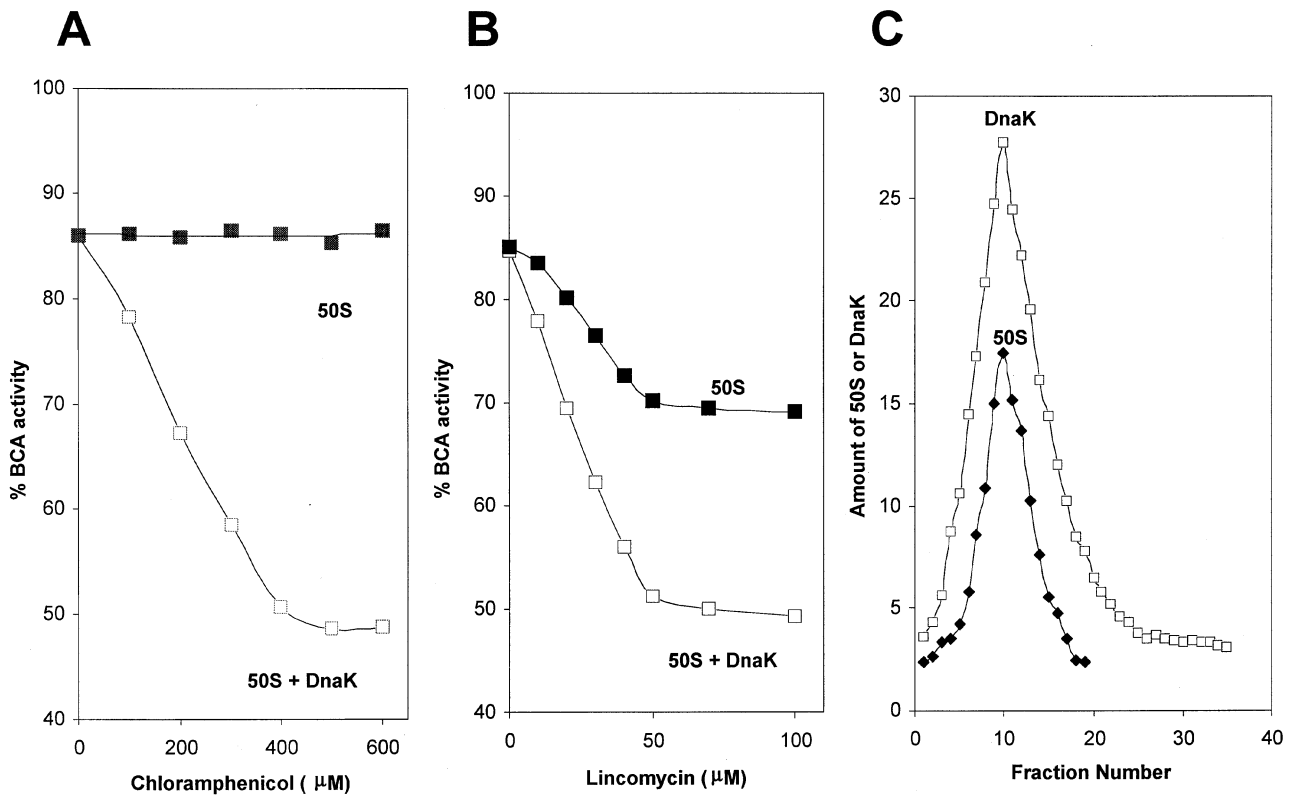

Fig. 4. A and B. Effect of chloramphenicol and lincomycin on refolding of BCA with 50S or 50S-DnaK complex. Unfolded BCA (300 nM) was allowed to refold in the presence of equimolar 50S (filled squares) or 50S-DnaK complex (empty squares) in buffer A that also contained increasing concentrations of chloramphenicol (A) or lincomycin (B). 50S or 50S-DnaK complex was incubated with antibiotics for 30 min on ice before adding to BCA.
C. 50S-DnaK (RITC-labelled) complex was incubated with 500 μM chloramphenicol as above and then subjected to gel filtration as in Fig. 2B.

et al., 1999). We studied the refolding of DnaK in the presence and in the absence of these folding modulators. The refolding was monitored by the autophosphorylation activity of the protein. As before, DnaK was denatured with guanidium hydrochloride that led to complete loss of secondary structure as shown by the far UV CD spectrum (Fig. 5A). The activity of unfolded DnaK increased with increasing concentration of 23S rRNA, domain V RNA or equimolar mixture of RNA1 and RNA2 (Fig. 5B and C). As the ratio of modulator to unfolded protein was increased from 0.25 to 1, the activity of DnaK increased up to about 80% of activity of the native protein. In the absence of any folding modulator the activity was about 15–20%. When the ratio was increased further, the refolding decreased gradually as was seen earlier with other proteins but the reason remains unexplored (Pal *et al.*, 1997; 1999). The 23S rRNA assisted folding was sensitive to 1 mM EDTA as was found earlier for lactate dehydrogenase and glucose 6-phosphatase (Das *et al.*, 1996).

The gain of tertiary structure of DnaK during refolding was monitored by measuring the intrinsic tryptophan fluorescence. Whereas the intensity of the emission peak of the unfolded protein was 54% of that shown by the native protein, it increased up to 63% in the case of spontaneous folding and up to 75% in the case of refolding with modulators (Fig. 5D). The latter increase was reproducibly biphasic, the basis of which remains to be determined. In the case of spontaneous as well as RNA assisted folding, the maximum fluorescence was reached in about 5 min.

The role of domain V in the folding of DnaK *in vitro* was tested using four antibiotics at concentrations that inhibit protein synthesis in *E. coli* completely. Two of them, chloramphenicol and lincomycin, are specific to domain V. The other two, kasugamycin and streptomycin, are specific to 30S and were used as controls. None of the antibiotics had any effect on spontaneous folding of DnaK *in vitro* when the autophosphorylation activity of the protein was measured (data not shown). However, increasing the concentration of chloramphenicol and lincomycin increasingly inhibited 23S rRNA assisted folding *in vitro* until it was brought down to the level of spontaneous folding (Fig. 6). The control antibiotics, streptomycin and kasugamycin, had no effect on 23S rRNA assisted folding. The specific effect of chloramphenicol and lincomycin

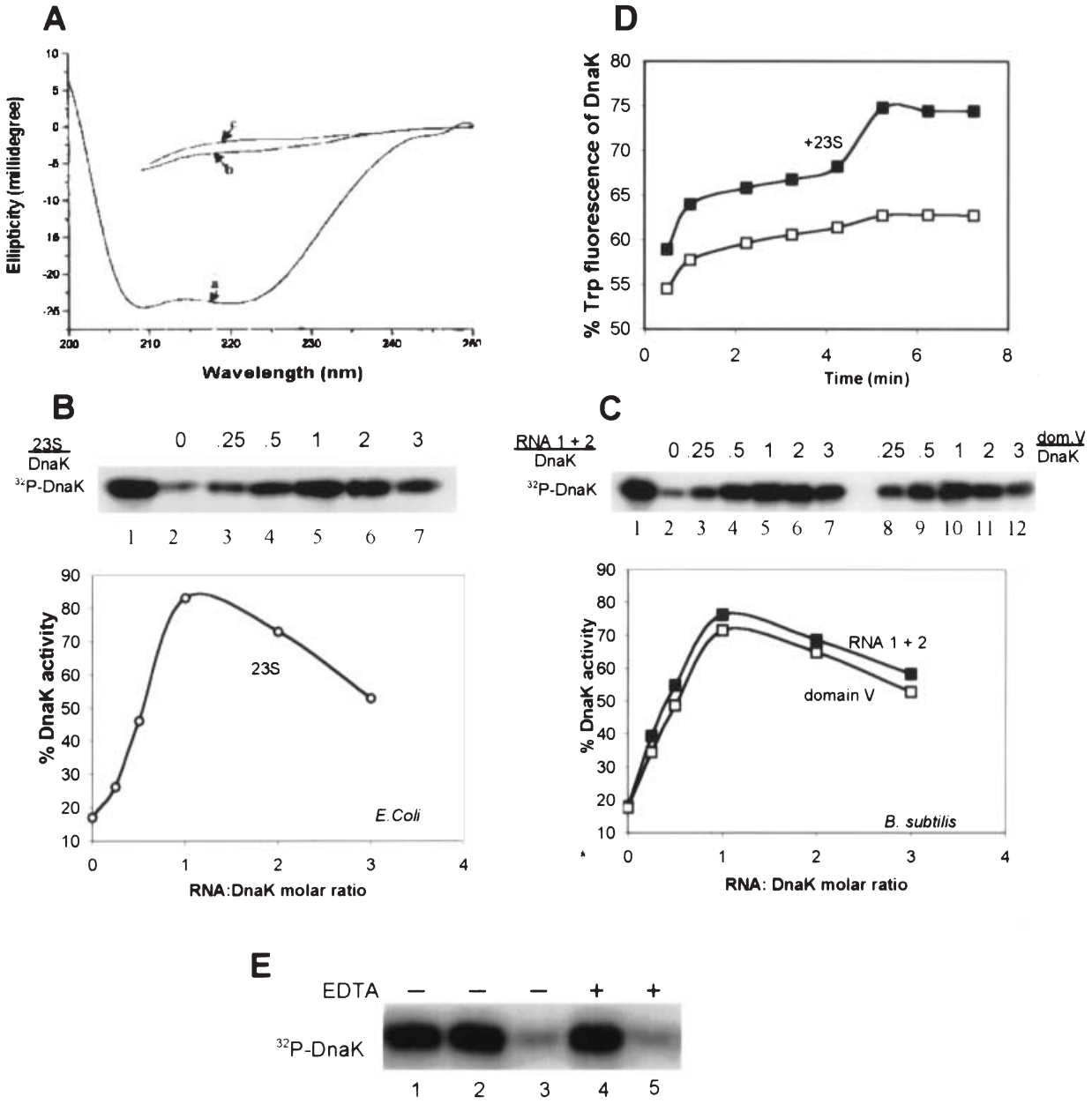

Fig. 5. A. Far UV CD spectra of native (a) and unfolded (b and c) DnaK (300 nM). Curves b and c represent DnaK denatured for 1 h and 2 h respectively.
B and C. RNA-mediated recovery of autophosphorylation activity of denatured DnaK. Unfolded DnaK (270 nM) was allowed to refold in the presence of increasing concentrations of folding modulators *E. coli* 23S rRNA (B), *B. subtilis* 23S rRNA domain V, and an equimolar mixture of *B. subtilis* RNA1 and RNA2 (C). The refolded DnaK samples were autophosphorylated with [γ-^{32}P]-ATP, precipitated and analysed by SDS-PAGE followed by autoradiography of the gel. Lanes 1 and 2 show the bands of the same amount of DnaK before denaturation and after self-folding, respectively. The intensity in lane 1 was taken as 100% in calculating the relative intensities of the other bands.
D. Recovery of intrinsic tryptophan fluorescence of denatured DnaK (600 nM) with time, when allowed to self-fold (empty squares) or fold in the presence of equimolar 23S rRNA of *E. coli* (filled squares).
E. Effect of EDTA on folding by 23S rRNA. DnaK (300 nM) was allowed to refold in presence of equimolar 23S rRNA (lane 1) or its absence (lane 3). Lane 5 is same as lane 1 except that 23S rRNA was preincubated with 1 mM EDTA. Lanes 2 and 4 are native DnaK without and with preincubation with 1 mM EDTA respectively. In lanes 4 and 5, [Mg^{2+}] was increased from 5 to 6 mM before assay.

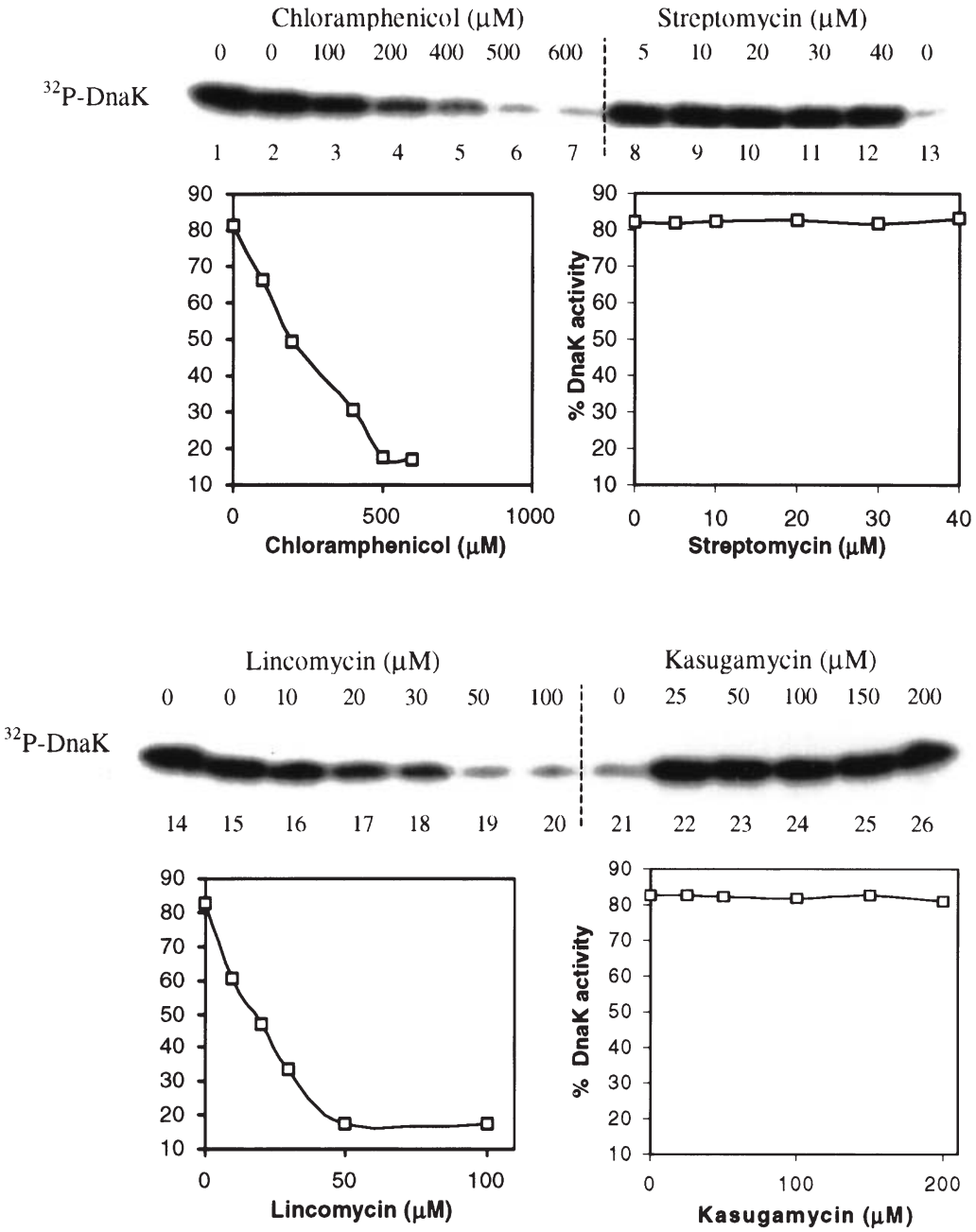

Fig. 6. Effect of antibiotics on 23S rRNA mediated reactivation of unfolded of DnaK. Unfolded DnaK (270 nM) was allowed to refold in the presence of equimolar 23S rRNA from *E. coli*. The buffer also contained different antibiotics. Autophosphorylation activity of DnaK was measured from autoradiograms (as in Fig. 5B) and plotted against different concentrations of the antibiotics. Lanes 1 and 14, and 2 and 15 have identical amounts of native and 23S rRNA-reactivated DnaK, respectively, in the absence of antibiotics. Self-folded DnaK bands are shown in lanes 13 and 21.

suggest that domain V normally participates in the folding of DnaK *in vitro*, and therefore refolding of DnaK is not special compared to the other proteins we have tested earlier.

Involvement of domain V in folding of DnaK in vivo

To determine the role of domain V in the folding of DnaK *in vivo*, the effect of antibiotics on the level of phosphorylation of DnaK was determined in *E. coli* (Zylicz *et al.*, 1983; Seeger *et al.*, 1996). DnaK synthesis was induced in the presence of $H_3{}^{32}PO_4$ and phosphorylation of DnaK was monitored by autoradiography. The amount of phosphorylated DnaK increased in the absence of antibiotics, and also to some extent in the presence of streptomycin (Fig. 7A) and kasugamycin (Fig. 7B). This residual

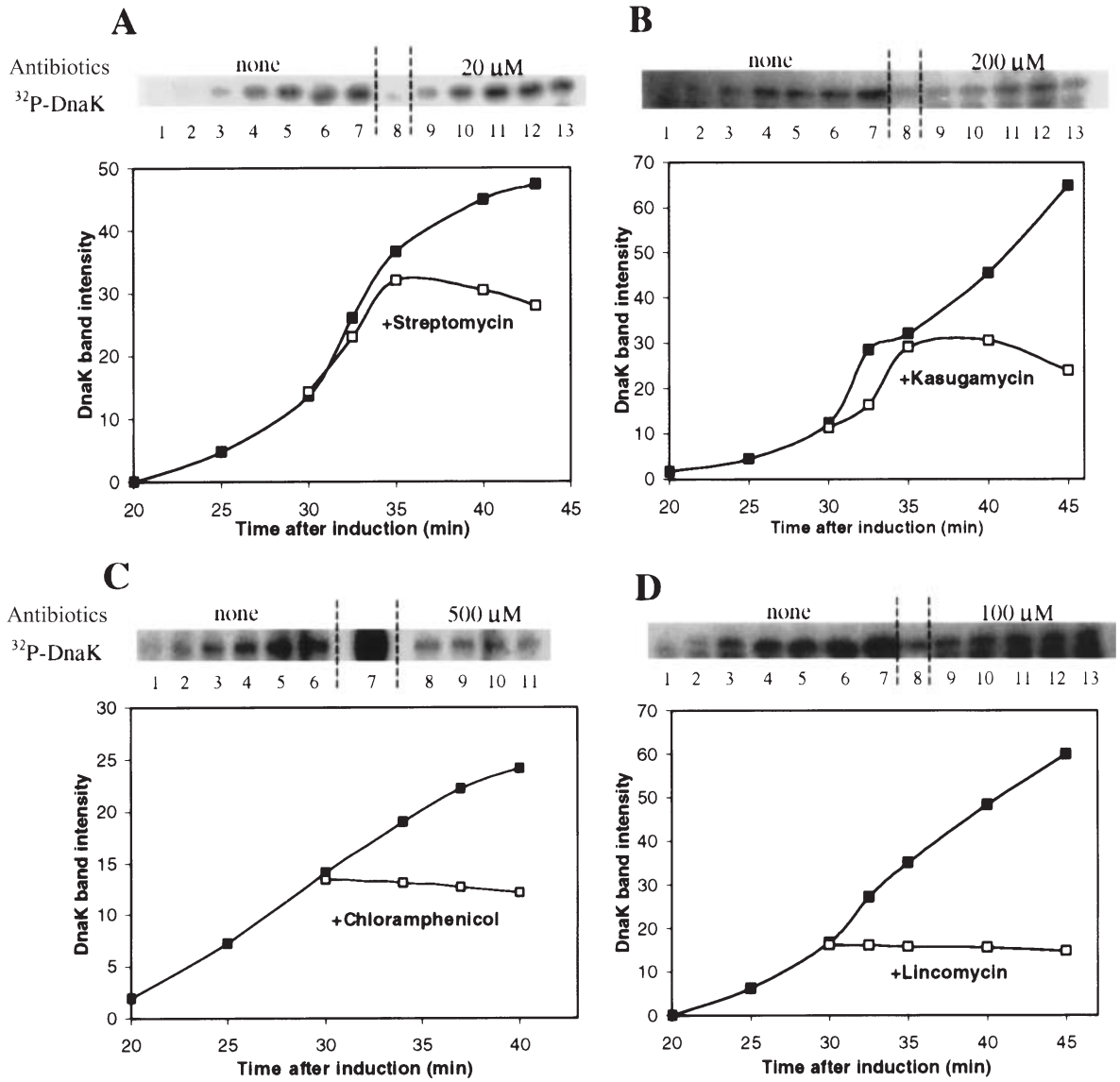

Fig. 7. Effect of antibiotics on phosphorylation of DnaK *in vivo*. Expression of DnaK was induced in *E. coli* and its phosphorylation was monitored by labelling with ^{32}P. After 30 min of DnaK induction, the culture was divided into two equal parts. One was allowed to grow as such and to the other an antibiotic of choice was added at a level that completely shuts off protein synthesis. At different time points after DnaK induction aliquots were withdrawn and an equal OD of cells were used in all cases before cell lysis. The lysate of each fraction, after removal of nucleic acids, was precipitated and then subjected to SDS-PAGE followed by autoradiography. DnaK band intensity was measured by densitometric scanning (in arbitrary units) and plotted against time after induction of DnaK expression. Lane 8 in A, B and D, and lane 7 in (C) show bands of *in vitro* autophosphorylated DnaK, used to locate the position of DnaK phosphorylated *in vivo*.

increase was totally inhibited as soon as chloramphenicol (Fig. 7C) and lincomycin (Fig. 7D) were added to the culture. In all four cases, the concentrations of the antibiotics were such that they stopped protein synthesis in less than 30 s as measured by blockage of ^{35}S-methionine incorporation (Noller, 1991; Chattopadhyay *et al.*, 1999). The increase of phosphorylated DnaK in the presence of streptomycin and kasugamycin indicate activation of protein molecules synthesized before antibiotic addition. The increase continued for about 5 min after the drug addition. This indicates that the post-translational activation of DnaK can take up to 5 min that possibly represents the time to achieve the correct tertiary organization of this 70 kDa protein. This post-translational activation was not possible in the presence of chloramphenicol and lincomycin that bind and inactivate domain V. Because streptomycin and kasugamycin do not interact with domain V, this rRNA region apparently remains active to modulate the post-translational folding of DnaK. We conclude the domain V is specifically involved in assisting post-

translational folding of DnaK at least as it pertains to its phosphorylation activity.

Role of DnaK in post-translational activation of β-galactosidase

As DnaK increases the rate of protein folding *in vitro* (Fig. 2), we asked whether DnaK could be playing a similar role *in vivo*. β-galactosidase synthesis from the wild-type *lac*-operon was induced with IPTG (final concentration 1 mM), and after 30 min, a 30S specific translation inhibitor, tetracycline, was added (final concentration 20 μg ml^{-1}). Although new protein synthesis stopped instantly as measured by ^{35}S-methionine uptake (Chattopadhyay *et al*., 1999), the activity of β-galactosidase continued to increase for several minutes (Fig. 8). The time to reach maximal increase was longer in Δ(*dnaK-dnaJ*) cells compared to Δ*dnaJ* or wild-type cells or when the Δ(*dnaK-dnaJ*) cells were complemented with a plasmid carrying the *dnaK-dnaJ* genes. These results may suggest a facilitatory role of DnaK in post-translational activation of β-galactosidase *in vivo* in a DnaJ independent manner.

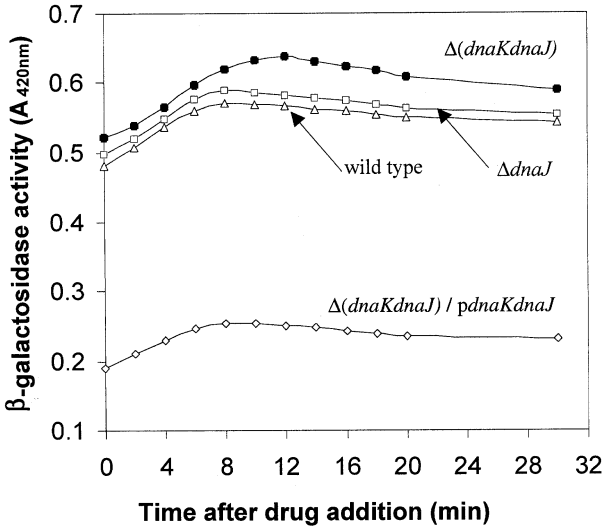

Fig. 8. Post-translational activation of β-galactosidase in *E. coli* cells. The enzyme synthesis was induced with IPTG, and after 30 min, tetracycline was added at 20 μg ml^{-1} to block protein synthesis. The enzyme activity in crude cell extract was then measured at different times. The peak activity reached in about 12 min after the antibiotic addition in Δ(*dnaK-dnaJ*) (= PK101) (Kang and Craig, 1990) cells as opposed to about 8 min for the wild type (= C600), Δ*dnaJ* (= PK102) and Δ(*dnaK-dnaJ*) cells complemented with a plasmid (pBN15) carrying the wild-type *dnaK and dnaJ* genes under the control of *ptac* promoter. No IPTG was used in the plasmid carrying cells as induction caused severe reduction of β-galactosidase activity. All cells were used at 30°C.

Discussion

The molecular chaperones perform a large number of functions inside the cell. All these activities are linked to the ability of the chaperones to influence the conformation of protein molecules. Not many experiments have been done to understand the folding of the chaperone proteins themselves. It is imperative to know the answer for full understanding of the biological protein-folding problem.

Our discovery of a general protein folding activity in the peptidyl transferase loop of the large rRNA adds a new paradigm to the problem. The finding that a nucleic acid molecule can help in protein folding helps us to understand how chaperones that help fold other proteins, can fold themselves, assuming that they do not fold spontaneously. Having assured ourselves that a dozen or more proteins could be folded by rRNA, we went on to check if this general protein folding activity applies to chaperones. We selected DnaK not only because it is well conserved and extensively studied but also because it is a monomer and the assay for its autophosphorylation activity is quick and quantitative (Tilly *et al*., 1983; Georgopoulos *et al*., 1990; McCarty and Walker, 1991). The physiological significance of this activity of DnaK is indicated by the fact that heat shock promotes phosphorylation of DnaK within *E. coli,* and this phosphorylated chaperone shows a higher affinity towards unfolded proteins (Sherman and Goldberg, 1993).

Our experiments indicate that DnaK cannot fold by itself to active form after synthesis *in vivo* (Fig. 7) or from unfolded state *in vitro* (Fig. 5). Other chaperones such as DnaJ involved with many of the activities of DnaK could not help it to fold either, because in our *in vivo* experiments with antibiotics both DnaJ and DnaK were induced from the plasmid pBN15 (Fig. 7). It appears that the domain V of 23S rRNA could cause a significant increase in the autophosphorylation activity of DnaK post-translationally *in vivo* (Fig. 7) and of the unfolded protein *in vitro* (Fig. 6). The unfolded DnaK was as good a substrate for folding by rRNA as any other protein we have tested.

We find that native DnaK binds to the 50S ribosomal subunit of *E. coli* with a 1:1 stoichiometry (Fig. 1). Our results thus confirm and extend recent works that suggested that DnaK could be a ribosome-associated protein. The binding does not interfere with the subunit's protein folding activity (Fig. 2). Even DnaK can be cross-linked to 50S without compromising the folding activity. We also could not detect fluorescence energy transfer between 50S bound DnaK and denatured BCA by labelling BCA with FITC (donor) and DnaK with RITC (acceptor) in the case of 50S–DnaK complex mediated folding of BCA. Because there was energy transfer between free DnaK and denatured BCA in the absence of 50S (data not shown), DnaK and denatured BCA apparently bind to

non-overlapping sites on 50S. The candidate sites, 5S and the central loop of domain V RNAs, respectively, are also well separated in the crystal structure of ribosome (Nissen *et al*., 2000). The binding of DnaK, however, makes the nucleotides of 23S rRNA more accessible to antibiotics indicating a conformational change of 50S. This change may account for the modest increase in the rate of protein folding found *in vitro* when 50S-mediated folding of a denatured protein was measured in the presence of DnaK (Fig. 2), or increased rate of folding of β-galactosidase post-translationally *in vivo* (Fig. 8). The binding of the denatured protein to 50S apparently also changes the subunit's conformation that results in the release of DnaK (Fig. 3). Thus both DnaK and a denatured protein may influence the conformation of 50S. Whether interactions with 50S influence the chaperone activity of DnaK remains to be studied.

Is DnaK normally ribosome associated? The two cohorts of the DnaK chaperone system, DnaJ and GrpE, are much less abundant. The concentrations as percent of total cell protein are <0.02:0.14:1.4 for DnaJ:GrpE:DnaK (Neidhardt and VanBogelen, 1987). On the other hand, the abundance of DnaK and ribosomes seems to be comparable on a molar basis. Depending upon the growth rate ribosomes can constitute from about 9–22% of total cell protein, about 10-fold higher than DnaK (Bremer and Dennis, 1996). As the molecular weight of ribosomal proteins is also about 10-fold higher compared to DnaK (850 versus 69 kDa), the bulk of DnaK could therefore be available for association with ribosomes. As DnaK is released upon binding of denatured proteins to 50S, heat shock or other stresses may release DnaK so that it can perform its conventional chaperone function. The ribosome association may be a mechanism to utilize an abundant protein like DnaK when cells are not stressed. DnaK-bound ribosomes also remain translationally active *in vivo* (Vysokanov, 1995).

Another protein known to interact with 50S is the trigger factor (TF), a *tig* gene product (Deuerling *et al*., 1999; Kramer *et al*., 2002). Both DnaK (Hesterkamp and Bukau, 1998) and TF (Deuerling *et al*., 1999) are known to interact with a wide variety of nascent polypeptides. Whereas single null mutants of *dnaK* and *tig* are viable, the double mutant is not and it accumulates aggregated proteins in the cytosol (Teter *et al*., 1999; Kramer *et al*., 2002). Thus ribosome association of DnaK may be required to share TF function.

DnaK consists of two domains that act in a coupled manner. The N-terminal domain (44 kDa) binds ATP (Flaherty *et al*., 1990) and the C-terminal domain (18 kDa) interacts with substrate polypeptides (Zhu *et al*., 1996), which are linear polypeptide sequences rich in hydrophobic amino acid residues (Flynn *et al*., 1991; Rudiger *et al*., 1997). Binding and release of the substrate depends on modulation of the intrinsic peptide affinity of DnaK by cycles of ATP binding and hydrolysis by the N-terminal domain (Hartl, 1996; Bukau and Horwich, 1998). Our study indicates that binding of DnaK to 50S and its release upon binding of unfolded proteins can happen in the absence of ATP. The mechanism of DnaK interactions with ribosomes therefore appears to be different from those with polypeptide substrates. The basis of this difference remains to be studied.

Experimental procedures

Preparation of E. coli *ribosomal particles, 23S rRNA and* B. subtilis *rRNA fragments*

The 70S ribosome and its subunits, 50S and 30S, and the 23S rRNA are from *E. coli* MRE600 and were prepared as described (Chattopadhyay *et al*., 1994). Ammonium chloride was used during the preparation of 70S ribosomes. The domain V of 23S rRNA, and its segments RNA1 and RNA2, are from *B. subtilis*, and were prepared as described (Pal *et al*., 1999; Chowdhury *et al*., 2002). RNA1 covers nucleotide (nt) positions 2036–2696 excluding positions 2111–2252 and 2271–2470 (total deletion of 340 nt). To this 16 nt were added to maintain the stem 74 + 75. RNA2 is from 2036 to 2461. The RNA1 and RNA2 regions of *B. subtilis* have identical secondary structure to those of *E. coli* (Kovalic *et al*., 1995). The bases known to play crucial roles in translation (Lieberman and Dahlberg, 1994) and protein folding (Chowdhury *et al*., 2002; S. Pal *et al*. in preparation) are invariant in *E. coli* and *B. subtilis*, and in bacteria in general.

Unfolding and refolding of DnaK

DnaK was purified through Mono Q HR 5/5 column using FPLC (Hwang *et al*., 1990). The purified protein eluted as a monomeric species when applied to Sephadex G-200 gel filtration column and eluted using buffer A [100 mM Tris.Cl (pH 7.5), 100 mM NaCl, 5 mM 2-mercaptoethanol, 5 mM $MgCl_2$ and 10% (v/v) glycerol] (Fig. 1C). The protein was unfolded with 2 M guanidium hydrochloride in 20 mM Tris.Cl, pH 7.5, 20 mM NaCl at 25°C for 2 h. Complete loss of the native secondary structure of the protein was achieved in this condition of denaturation, as seen in the far UV CD spectrum. In fact, loss of secondary structure was almost complete even in 1 M guanidium hydrochloride (data not shown). For refolding, the unfolded protein was diluted 27.2-fold (final DnaK concentration of 270 nM) in the presence or absence of folding modulators. The residual concentration of guanidium hydrochloride (73.4 nM) had no effect on the folding or the activity of the native protein (data not shown). The buffer A was also used as the refolding buffer. After incubation for 15 min at 25°C, the autophosphorylation activity of the protein was measured.

DnaK autophosphorylation activity was measured by slightly modifying the conditions as described (McCarty and Walker, 1991). The reaction mixture for autophosphorylation contained 100 mM Tris.Cl (pH 7.5), 100 mM NaCl, 5 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 10% (v/v) glycerol, 1 μM ATP

and 10 μCi of [γ-^{32}P]-ATP. The reaction was carried out at 37°C for 15 min. The ^{32}P-labelled protein was precipitated with cold TCA, centrifuged and washed with chilled acetone. The precipitated protein following resuspension was subjected to10% SDS-PAGE, and visualized by autoradiography of the gel.

FITC and RITC labelling of proteins

The protein bovine carbonic anhydrase (BCA) and DnaK were labelled with the fluorescent probe FITC or RITC as described (Pal *et al*., 1999; Sanyal *et al*., 2002). The fluorescent labelling of DnaK did not alter its autophosphorylation activity significantly. FITC-labelled proteins were measured by fluorescence emission at 520 nm when excited at 495 nm using a Hitachi F-3010 fluorimeter. The wavelengths were 575 nm and 520 nm, respectively, for RITC-labelled proteins. The BCA activity was measured as described (Pal *et al*., 1999).

Synthesis and phosphorylation of DnaK in E. coli

For *in vivo* overexpression of DnaK, plasmid pBN15 was used that contained the genes for DnaK and DnaJ under P_{tac} promoter (Blum *et al*., 1992). The plasmid was isolated from *E. coli* PBL328 (Blum *et al*., 1992) and was used to transform *E. coli* XL1Blue. XL1Blue/pBN15 cells were grown in low phosphate media till OD_{600} of the culture reached about 0.1. DnaK synthesis was induced with 60 μM IPTG (Blum *et al*., 1992). After 20 min of induction, 5 μCi ml^{-1} of $H_3{}^{32}PO_4$ was added to the culture. After another 10 min (i.e. 30 min after induction) the culture was divided into two equal parts. One was allowed to grow as control. To the other, one of the four antibiotics, chloramphenicol, lincomycin, streptomycin and kasugamycin, was added at concentrations that stop cellular protein synthesis instantly as measured by stoppage of ^{35}S-methionine incorporation (Chattopadhyay *et al*., 1999). The arrest of DnaK protein synthesis was confirmed by immunoblotting using anti-DnaK antibody. At different times aliquots were withdrawn from the two flasks. Because the number of cells would increase with time in the absence of antibiotics, the aliquots withdrawn from the control were adjusted to keep the OD units identical to that at the time of antibiotic addition. The aliquots were centrifuged for 1 min at 10 000 r.p.m. as soon as they were collected, suspended in buffer A (refolding buffer) and lysed with toluene. The lysis prevents further autophosphorylation of DnaK in the extract (data not shown). The aqueous phase was collected, and the cellular debris removed by centrifugation at 10 000 r.p.m. To remove nucleic acid from the supernatant, it was mixed with DEAE-cellulose equilibrated with buffer A. After gentle swirling for several min at room temperature the mixture was centrifuged. DnaK was eluted from the pellet by washing with 400 mM NaCl in 100 mM Tris.Cl, pH 7.8, and centrifugation at 10 000 r.p.m. for 5 min at room temperature. This led to complete removal of ^{32}P-labelled nucleic acids that were retained by the DEAE-cellulose in the pellet. The supernatant was mixed with chilled TCA to a final concentration of 5%. The mixture was incubated in ice for 30 min and the protein recovered by centrifugation at 14 000 r.p.m. for 30 min at 4°C. The pellet was washed with chilled acetone and, after resuspension, run in a 10% SDS-PAGE. After autoradiography, the band corresponding to DnaK was scanned to determine its intensity in arbitrary units. The DnaK band was identified by using as marker purified DnaK labelled *in vitro* with ^{32}P. The identity was further confirmed by immunoprecipitation of the cell extract with anti-DnaK polyclonal antibody that removed the band from the supernatant, as monitored by SDS-PAGE (data not shown).

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