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In vitro protein folding by ribosomes from *Escherichia coli*, wheat germ and rat liver

The role of the 50S particle and its 23S rRNA

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Ribosomes from a number of prokaryotic and eukaryotic sources (e.g., *Escherichia coli*, wheat germ and rat liver) can refold a number of enzymes which are denatured with guanidine/HCl prior to incubation with ribosomes. In this report, we present our observations on the refolding of denatured lactate dehydrogenase from rabbit muscle and glucose-6-phosphate dehydrogenase from baker's yeast by ribosomes from *E. coli*, wheat germ and rat liver. The protein-folding acivity of *E. coli* ribosomes was found to be present in 50S particles and in 23S rRNA. The 30S particle or 16S rRNA did not show any protein-folding activity. The protein-folding activity of 23S rRNA may depend on its tertiary conformation. Loss of tertiary structure, by incubation with low concentrations of EDTA, inhibited the protein-folding activity of 23S rRNA. This low concentration of EDTA had no effect on folding of the denatured enzymes by themselves.

Keywords: protein folding; ribosomes; 23S rRNA; lactate dehydrogenase; glucose-6-phosphate dehydrogenase.

The process by which a genetic message is converted from a linear array of nucleotides to a linear polypeptide chain has been worked out in great detail in bacterial and eukaryotic cells. However, the mechanism of folding of linear polypeptides into three-dimensional structures has not been explained. Whereas some proteins can fold spontaneously from their denatured states (Anfinsen, 1973), the folding of many proteins into active conformations, which occurs rapidly during and after synthesis of the polypeptide, has been shown by means of genetic and biochemical experiments to be assisted by molecular chaperones (Gething and Sambrook, 1992; Hartle and Martin, 1992).

A number of examples of *in vitro* protein synthesis by cell extracts have been reported (Zubay et al., 1970; deCrombrugghe et al., 1971; Zubay, 1973) in which the synthesized polypeptide could fold into biologically active forms. We asked whether ribosomes, the site of protein synthesis, could also fold the newly synthesized polypeptide chain into its active form. Although the structure and function of some ribosomal proteins and rRNA species are known in great detail, new findings are emerging on the role of other proteins and rRNA in protein synthesis and in a number of other cellular processes. Resistance mutations against a number of antibiotics have been located in rRNA (reviewed by Noller, 1991), and 23S rRNA has been reported to possess peptidyl transferase activity (Noller et al., 1992). Recent reports on the initiation of folding of the β-subunit of tryptophan synthase during its translation on ribosomes (Fedorov et al., 1992) and on the *in vitro* folding of dihydrofolate reductase and rhodanese in a chaperone-free coupled transcription-translation system (Kudlicki et al., 1994) strongly support our assumption that ribosomes might possess protein-folding activity.

Ribosomes from bacterial sources can refold a number of denatured enzymes, e.g., alkaline phosphatase from *Escherichia coli*, horseradish peroxidase and a number of restriction endonucleases (Das et al., 1992; Bera et al., 1994; Chattopadhyay et al., 1994).

In this report, we show that the protein-folding activity is a general activity since ribosomes from *E. coli* (prokaryote), wheat germ and rat liver (eukaryote) could efficiently refold denatured lactate dehydrogenase (LDH) from rabbit muscle (a tetrameric enzyme of 140 kDa/tetramer) and glucose-6-phosphate dehydrogenase (Glc6*P*-DH) from baker's yeast (a dimeric enzyme of 102 kDa/dimer) to their catalytic forms. The finding that the protein-folding activity of ribosomes is found in ribosomes of different species supports the hypothesis that ribosomes possess a general protein-folding activity. The activity did not require ATP or GTP and was found to be present in 50S particles and in 23S rRNA of *E. coli* ribosomes. The 30S particles and 16S rRNA were found not to play any role in this process.

EXPERIMENTAL PROCEDURES

Preparation of *E. coli* 70S and wheat germ and rat liver 80S ribosomes. *E. coli* MRE 600 cells were grown in Luria-Bertani medium supplemented with 0.1% glucose until the A_{600} was approximately 0.8. Cells were then slowly cooled to 4 °C to produce run-off ribosomes (Ghose and Moore, 1979) and harvested in 20 mM Tris/HCl, pH 7.5, 10 mM magnesium acetate, 100 mM NH_4Cl and 5 mM 2-mercaptoethanol. Frozen cells were ground with alumina (two times by mass) in the same buffer containing 2 μg/ml DNAase I. Alumina and cell debris were removed by centrifuging the suspension twice at 12000 *g* for

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Abbreviations. Hsp, heat-shock protein; LDH, lactate dehydrogenase; Glc6*P*-DH, glucose-6-phosphate dehydrogenase.

Enzymes. L-lactate dehydrogenase from rabbit muscle (EC 1.1.1.27); glucose-6-phosphate 1-dehydrogenase from baker's yeast (EC 1.1.1.49).

10 min in a Hitachi RPR 20-2 rotor. The supernatant was centrifuged at 154000 *g* for 2 h in a Beckman Ti50 rotor and the pellet containing ribosomes was resuspended in TMA-10 buffer [20 mM Tris/HCl, pH 7.5, 10 mM magnesium acetate, 30 mM NH_4Cl, 5 mM 2-mercaptoethanol (Traub and Nomura, 1968)]. 1 M ammonium chloride was added and the suspension was kept at 0°C for 1 h. The ribosomal preparation was clarified by centrifugation at 12000 *g* for 20 min in a Hitachi RPR 20-2 rotor. The supernatant was centrifuged at 154000 *g* for 2 h in a Beckman Ti50 rotor and the pellet was resuspended in TMA-10. About 75 A_{260} units (one A_{260} unit is the amount of material that, when contained in 1 ml of solution, gives an absorbance at 260 nm of 1 in a 1-cm path-length cell) of this ribosomal preparation was loaded on the top of 5%-to-20% linear sucrose (RNAase-free) gradient in the same buffer and centrifuged at 35000 rpm for 90 min in a Beckman SW40.1 rotor at 4°C. The gradient was monitored at 260 nm and appropriate fractions containing 70S particles were pooled and centrifuged at 35000 rpm for 2 h at 4°C. The pellet of purified 70S particle was resuspended in TMA-10, and dialyzed against the same buffer at 4°C to remove traces of sucrose and stored at −70°C until use. The A_{260}/A_{280} ratio of this preparation was found to be greater than 1.8. One A_{260} unit of this preparation was assumed to contain 23 pmol of 70S particle when the absorbance was measured in 20 mM Tris/HCl, pH 7.5, 10 mM magnesium acetate, and 30 mM NH_4Cl (Spedding, 1990). The purity of 70S ribosomes was determined either by electrophoresis of the particle in a 0.5% agarose/3% acrylamide composite gel by a modified version of the procedure of Dahlberg (1979) or by loading 2−5 A_{260} units on a small analytical sucrose gradient in TMA-10 and centrifuging at 190000 *g* for 90 min at 4°C. The gradient was then analyzed at 260 nm.

Wheat germ 80S ribosomes were prepared by slightly modifying the procedures described by Fehling and Weidner (1988). Wheat germ (Sigma) was ground with an equal mass of neutral sand in 10 mM Tris/HCl, pH 7.8, 10 mM magnesium acetate, 90 mM KCl, 2 mM $CaCl_2$, 6 mM $KHCO_3$ and 5 mM 2-mercaptoethanol. Cell debris and sand were removed by centrifuging the suspension twice at 12000 *g* for 10 min in a Hitachi RPR 20-2 rotor. Crude 80S ribosomes were obtained by centrifuging the supernatant at 156000 *g* for 2 h in a Beckman Ti50 rotor. The pellet was resuspended in 10 mM Tris/HCl, pH 7.8, 10 mM magnesium acetate, 80 mM KCl and 5 mM 2-mercaptoethanol. About 75 A_{260} units of this preparation were loaded on the top of a 5%-to-20% linear sucrose gradient in the same buffer. The gradient was centrifuged at 35000 rpm for 90 min in a Beckman SW40.1 rotor at 4°C and was then analyzed at 260 nm. Appropriate fractions containing 80S ribosomes were pooled and centrifuged at 35000 rpm for 2 h at 4°C. The pellet of the purified 80S particle was resuspended in 10 mM Tris/HCl, pH 7.8, 10 mM magnesium acetate, 80 mM KCl and 5 mM 2-mercaptoethanol and dialysed at 4°C against the same buffer to remove traces of sucrose and then kept at −70°C until use. The dialysate had A_{260}/A_{280} and A_{260}/A_{235} values of 1.68 and 1.2, respectively. One A_{260} unit was considered to be equivalent to 30 pmol of 80S ribosomes (Spedding, 1990). The homogenity of the wheat germ 80S preparations was checked by sucrose-gradient sedimentation as described for *E. coli* 70S preparations.

80S ribosomes from rat liver were prepared following the procedures described by O'gata and Terao (1979). Rats of Wistar strain (100−150 g body mass, either sex) were starved for 15 h and killed by decapitation. Livers were immediately removed and homogenized in 50 mM Tris/HCl, pH 7.6, 25 mM KCl, 10 mM $MgCl_2$, 0.25 M sucrose (medium A) at 0°C with six strokes at 1000 rpm. The suspension was centrifuged at 10000 *g* for 10 min in a Hitachi RPR 20-2 rotor. The supernatant was removed and the turbid zone and the pellet were resuspended in an equal volume of the same medium and homogenized and centrifuged as before. The supernatant fractions were combined and centrifuged at 158000 *g* for 2 h in a Beckman Ti50 rotor. The pellet was homogenized in 1 vol. of of original tissue mass of 35 mM Tris/HCl, pH 7.8, 25 mM KCl, 10 mM $MgCl_2$ and 0.15 M sucrose in a Dounce homogenizer. The homogenized microsomal solution was incubated in 1% sodium deoxycholate for 1 h at 0°C, which released the membrane-bound ribosomes from the endoplasmic reticulum. The suspension was layered over an equal volume of 35 mM Tris/HCl, pH 7.8, 0.6 M KCl, 10 mM $MgCl_2$, 0.3 M sucrose and centrifuged at 156000 *g* for 2 h in a Beckman Ti50 rotor. The pellet was rinsed with a small amount of 50 mM Tris/HCl, pH 7.8, 50 mM KCl, 10 mM $MgCl_2$ and 0.25 M sucrose and resuspended in the same medium. About 75 A_{260} units of this crude 80S rat liver ribosome preparation were loaded onto the top of a linear sucrose gradient prepared by mixing additional 5% and 20% sucrose in medium A. The gradient was centrifuged at 35000 rpm for 90 min at 4°C in a Beckman SW40.1 rotor and was analysed at 260 nm. Appropriate fractions containing 80S rat-liver ribosomes were pooled and centrifuged at 35000 rpm in a Beckman SW40.1 rotor. The pellet containing purified rat liver ribosomes was resuspended in medium A and dialyzed against the same medium at 4°C to remove traces of sucrose and stored at −70°C until use. The A_{260}/A_{280} and A_{260}/A_{235} ratios of the dialysate were 1.87 and 1.62, respectively. One A_{260} unit is considered to be equivalent to 18 pmol of 80S ribosomes (Spedding, 1990). The purity and homogenity of the 80S rat-liver ribosomes was checked either by gel elctrophoresis (Dahlberg, 1979) or by means of an analytical sucrose gradient as described for *E. coli* 70S ribosomes.

Preparation of 50S and 30S particles. 70S particles in TMA-10 were dialyzed twice against 300 vol. of 20 mM Tris/HCl, pH 7.5, 0.1 mM magnesium acetate, 30 mM NH_4Cl, and 5 mM 2-mercaptoethanol for 24 h at 4°C. The dialyzed sample was layered upon a 5%-to-20% linear sucrose gradient made up in the same buffer and centrifuged at 35000 rpm for 6 h at 4°C, in a Beckman SW40.1 rotor. Gradient fractions were collected and their absorbance monitored at 260 nm to allow detection of the separate peaks due to 50S and 30S particles. To avoid cross-contamination, fractions from the heavier side of the 50S peak and the lighter side of the 30S peak were pooled separately and precipitated with chilled ethanol. The precipitate was resuspended in TMA-10, dialyzed against the same buffer to remove ethanol and sucrose and kept at −70°C until use. The purity of each subunit was tested either by electrophoresis through a 0.5% agarose/3% acrylamide gel (Dahlberg, 1979) or by loading 2−5 A_{260} units on an analytical sucrose gradient in 20 mM Tris/HCl, pH 7.5, 5 mM magnesium acetate, 30 mM NH_4Cl and 5 mM 2-mercaptoethanol and centrifuging them at 160000 *g* for 2 h 15 min in a Beckman SW50.1 rotor. The gradients were analysed at 260 nm. The concentration of each subunit was measured spectrophotometrically assuming one A_{260} unit is equivalent to 69 pmol and 34.5 pmol of 30S and 50S subunits, respectively (Spedding, 1990).

The ribosomes and subunit preparations obtained were checked for possible contaminating GroEL [heat-shock protein (Hsp) 60] and DnaK (Hsp 70) proteins by competitive ELISA with antibodies raised against GroEL and DnaK (provided by Dr D. K. Chattoraj, NIH, USA).

Competitive ELISA. Detection of the antigens GroEL or DnaK in our ribosome preparation was performed by means of competitive ELISA. For detection of GroEL, five series of reaction tubes were set up, each containing a fixed concentration of anti-GroEL Ig [15 ng/ml in NaCl/P_i (0.36 g/l KH_2PO_4, 5.47 g/l $Na_2HPO_4 \cdot 12\ H_2O$, 0.36 g/l KCl, 10.04 g/l NaCl, pH 7.4)].

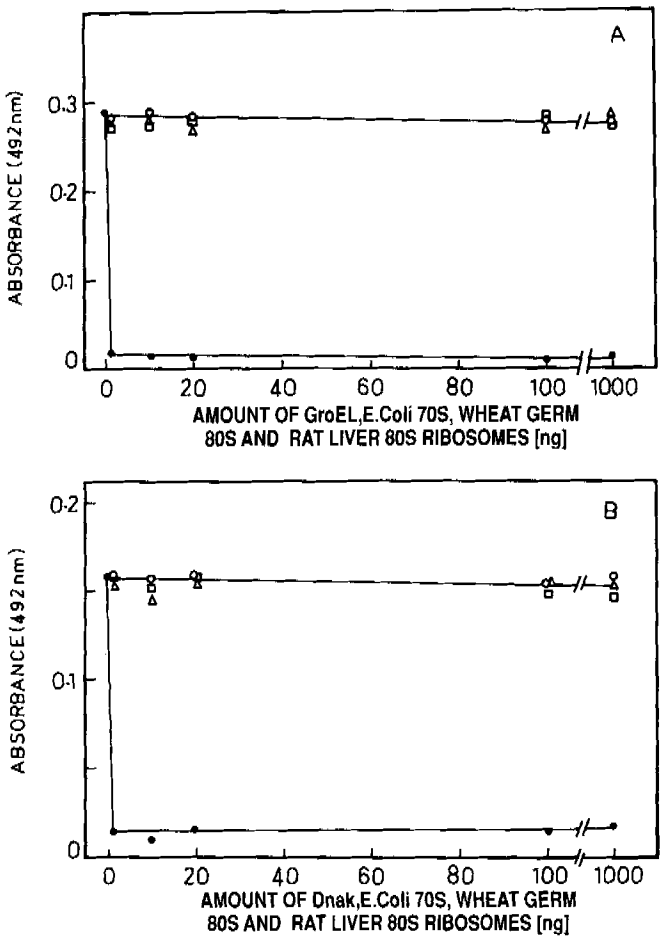

Fig. 1. Competitive ELISA for Hsp-like proteins. (A) *E. coli* GroEL and ribosomal preparations from different sources detected with anti-GroEL antibody. Absorbance at 492 nm with increasing concentrations of GroEL (●), and *E. coli* 70S (○), wheat germ 80S (□) and rat liver (△) ribosomes. (B), *E. coli* DnaK and ribosomal preparations from different sources detected with anti-DnaK antibody. Absorbance at 492 nm with increasing concentrations of DnaK (●), and *E. coli* 70S (○), wheat germ 80S (□) and rat liver 80S (△) ribosomes.

Increasing amounts of GroEL, *E. coli* 70S ribosomes, wheat germ 80S ribosomes and rat liver 80S ribosomes in the range 0–1 μg, were added to four series of tubes. The other series was used as a control. After incubation for 60 min at 37 °C the five series of the samples were added to five series of wells of the microtitre plate. All of the wells were previously coated with 1 ng GroEL protein in NaCl/P_i for 60 min then unsaturated sites on the wells were blocked with 1% BSA (Sigma) in NaCl/P_i containing 0.05% Tween 20 for 1 h at 37 °C. The microtitre plate was then incubated for 60 min at 37 °C and the immunoprecipitation reaction was monitored by adding anti-rabbit secondary Ig (horseradish peroxidase-conjugated) to the respective wells and incubated for 1 h at 37 °C. The bound horseradish peroxidase conjugate was detected by adding the substrate and 1,2-phenylenediamine (Diagnostics Pasteur) to the wells. The absorbance was measured in an ELISA reader, and plotted as a function of the amount of antigens GroEL, 70S and 80S ribosomes as shown in Fig. 1 A.

A similar set of reactions were performed to detect the presence of Hsp70 with anti-Hsp70 antibody and the antigens DnaK, *E. coli* 70S ribosomes, wheat germ 80S ribosomes and rat liver 80S ribosomes. Fig. 1 B shows the competitive ELISA for the detection of Hsp70.

Preparation of rRNA. Total rRNA and 23S and 16S rRNA were isolated from *E. coli* 70S, 50S and 30S ribosomal particles, respectively, by slightly modifying the procedure of Zimmerman (1970). Ribosomal particles were extracted 10 times with phenol and the final aqueous phase was precipitated by addition of 2 vol. chilled ethanol and 0.1 vol. 20% potassium acetate. 23S rRNA was separated from 5S rRNA by gel-filtration chromatography. The homogenities of rRNA were verified by electrophoresis in a composite gel containing 0.5% agarose and 3% of a 19:1 (by mass) mixture of acrylamide/*N*,*N*′-methylenebisacrylamide. After electrophoresis, the gel was stained with ethidium bromide. Only one band was visible in samples of 23S and 16S rRNA.

rRNA concentration was estimated spectrophotometrically assuming an $A_{260}^{1\%}$ value of 24. The extracted RNA samples had a A_{260}/A_{280} ratio of greater than 2. The 16S and 23S RNA samples showed 40% hyperchromicities after RNAase digestion at 37 °C and they exhibited no detectable fluorescence emissions when excited at 285 nm and 290 nm at a concentration of 300 μg/ml with excitation and emission band-pass set at 10 nm. We found this analysis to be a convenient way to detect even a trace quantity of protein contamination.

Denaturation of the enzymes. Rabbit muscle LDH was obtained from Sigma. 10 μg of this preparation gave a single band of about 35 kDa upon SDS/PAGE after silver staining. The concentration of this enzyme was measured spectrophotometrically assuming an $A_{280}^{1\%}$ value of 14.5. The stock solution was diluted 10-fold in 20 mM Tris/HCl, pH 7.5 to a final a concentration of 1 mg/ml LDH. The enzyme was denatured with 1 M guanidine/HCl, pH 7.5 in the presence of 5 mM 2-mercaptoethanol for 60 min at 25 °C, unless otherwise mentioned. The concentration of the protein in the denaturation reaction was 0.47 μM (with respect to the enzyme tetramer). The activity of the enzyme was completely abolished when the enzyme was diluted into the assay mixture directly from the denaturation reaction. The intrinsic tryptophan fluorescence of the enzyme, measured in a 1-ml cuvette of 1-cm path length in a Hitachi F 3010 spectrofluoremeter, showed that the peak height at 340 nm diminished with time of denaturation to a minimum intensity after 30 min at 25 °C (data not shown).

Glc6*P*-DH from baker's yeast was purchased from Sigma as lyophilized crystal which was reconstituted by addition of 20 mM Tris/HC, pH 7.5 to give a final Glc6*P*-DH concentration of 1 mg/ml. The concentration of the enzyme was measured spectrophotometrically assuming an $A_{278}^{1\%}$ value of 9.65 which agreed well with the concentration determined by the method of Lowry et al. (1951). 10 μg of this enzyme gave a single band at about 50 kDa upon SDS/PAGE after silver staining. The enzyme was further diluted in the same buffer to a final concentration of 0.4 mg/ml and incubated with 6 M guanidine/HCl, pH 7.5, at 25 °C for 45 min. The enzyme concentration during denaturation was 0.98 μM (with respect to the dimer). The tryptophan-fluorescence intensity at 340 nm measured as described for LDH diminished to a minimum value within 5 min and the peak shifted to 352 nm for the denatured enzyme (data not shown). The enzyme activity was totally lost when the enyzme was diluted into assay buffer directly from the denaturation mixture.

Every time the enzymes were diluted from their stock solutions, their denaturation profiles were monitored to ensure the reproducibility of the process. All the experiments described below were repeated at least five times to make sure that the patterns were reproducible. The data given are, however, from single experiments and not averages of all the experiments, since the standard deviations were too small to be shown by bars.

Refolding of the denatured enzyme. Refolding of LDH was initiated by diluting the denatured enzyme 80-fold in

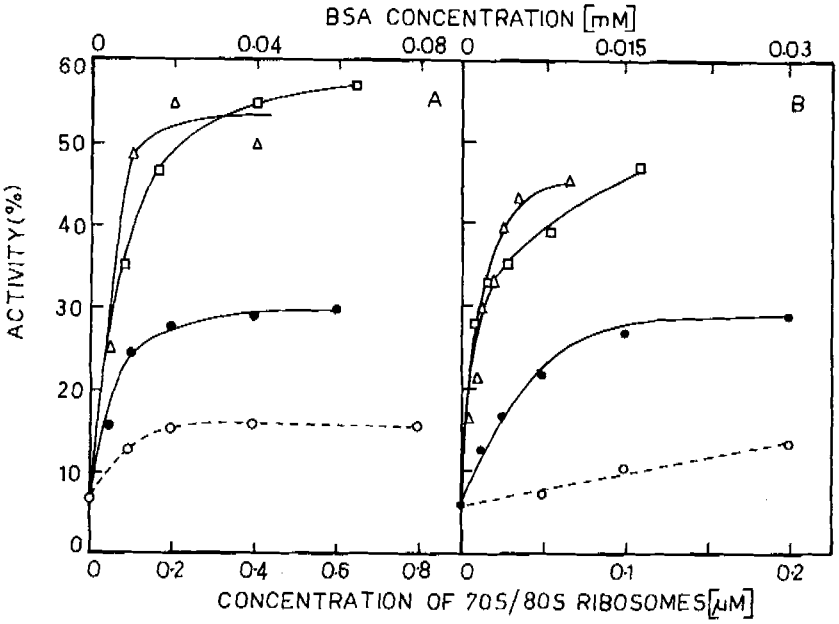

Fig. 2. Refolding of denatured LDH and denatured Glc6*P*-DH by ribosomes from different sources. Refolding of LDH (A) and Glc6*P*-DH (B) is shown in presence of different concentrations if *E. coli* 70S (●), wheat germ 80S (□), and rat liver 80S (△) ribosomes and BSA (○). The concentrations of LDH and Glc6*P*-DH during refolding were 6 nM and 12 nM, respectively. The activities of the refolded enzymes were measured after 30 min refolding and are expressed as percentages of the activity of same amount of native enzymes.

20 mM Tris/HCl, pH 7.5, 10 mM magnesium acetate, 5 mM 2-mercaptoethanol and ribosomal particles or rRNA. The mixture was incubated for 30 min at 25 °C unless otherwise mentioned. The concentration of the enzyme tetramer during refolding was 6 nM.

The refolding of Glc6*P*-DH was initiated by diluting the denatured enzyme 80-fold in 20 mM Tris/HCl, pH 7.5, 10 mM magnesium acetate, 25 mM KCl and ribosomal particles or rRNA. The mixture was incubated at 25 °C for 30 min unless otherwise mentioned. The concentration of the enzyme in all refolding reactions was 12 nM. The residual guanidine/HCl concentration in refolding reactions was very small and this amount of guanidine/HCl had no effect on the activity of the native enzyme. During refolding of the enzymes, the volume of the additions such as ribosomal particles or rRNA never exceeded 10 µl in a total reaction volume of 200 µl. An equivalent amount of buffer was added in the refolding reactions, without ribosomes/rRNA.

Assays of the enzymes. The activities of the enzymes were monitored spectrophotometrically. LDH was incubated in 100 mM Tris/HCl, pH 7.5, 5 mM sodium pyruvate, 250 µM NADH (Badcoe et al., 1991) for 3 min at 37 °C. The decrease in absorbance at 340 nm was measured for 3 min during which the rate of decrease was linear. The refolded enzyme was diluted 10-fold in this reaction mixture for assay. The activity of the refolded enzyme was expressed as a percentage of the activity of the same amount of native enzyme. Incubation of the native enzyme with ribosomes or rRNA did not change its activity.

Glc6*P*-DH was assayed in 33 mM Tris/HCl, pH 7.8, 10 mM $MgCl_2$, 3 mM glucose 6-phosphate, 1 mM NADP following a slightly modified procedure of Noltmann et al. (1962). The reaction was incubated for 5 min at 37 °C. The linear increase in absorbance at 340 nm due to reduction of NADP was recorded during this time. The activity of the refolded enzyme was expressed as a percentage of the activity of the same amount of native enzyme. Incubation of the native enzyme with ribosomes and rRNA did not change its activity.

RESULTS

Analysis of the presence of Hsp in ribosome preparations. The 70S ribosomes and 50S particles from *E. coli* and the 80S ribosomes from wheat germ and rat liver were analyzed for the presence of Hsp such as GroEL (Hsp60) and DnaK (Hsp70). Competitive ELISA for the presence of GroEL-like proteins in ribosome preparations are shown in Fig. 1A. All the ribosomal preparations were found to be free from such contamination. Competitive ELISA with an antibody raised against DnaK protein of *E. coli* are shown in Fig. 1B. From these competitive ELISA experiments we concluded that our ribosome preparations were free from contamination GroEL or DnaK. We also found protein-folding activity in the preparation of 23S rRNA of *E. coli* ribosomes which were thoroughly deproteinized.

Recovery of activity of denatured LDH and Glc6*P*-DH in the presence of ribosomes from different sources. Denatured LDH was incubated with different concentrations of ribosomes from wheat germ, rat liver and *E. coli* and assayed for enzyme activity. The recovery of activity increases with increasing concentrations of ribosome (Fig. 2A). In the absence of ribosomes the denatured enzyme itself could recover only about 6% of its activity in 30 min. The maximal recovery of activity was 50–60% in different experiments with ribosomes from rat liver and wheat germ. *E. coli* 70S ribosomes were found to be less efficient, yielding a maximum recovery of 30%. While ribosomes from different sources assisted the folding of the denatured enzyme considerably, very poor recovery of activity (only 7%) was observed when BSA was added as a possible non-specific protector of labile folding intermediates.

Similar recovery of activity was also observed for denatured Glc6*P*-DH (Fig. 2B). Folding of the enzyme by itself and in the presence of BSA are also shown (Fig. 2B). The 80S ribosomes from wheat germ and rat liver were more efficient in supporting the refolding of the denatured enzyme than 70S ribosomes from *E. coli*. In the absence of ribosomes the denatured enzyme by itself recovered about 6–8% of its activity, whereas the recov-

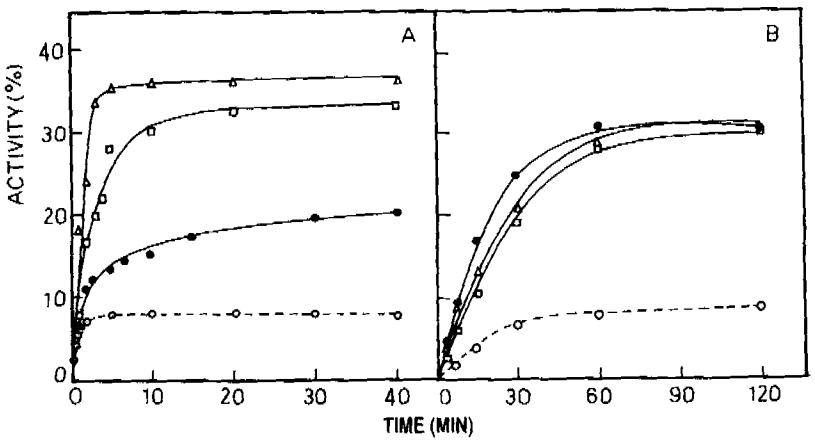

Fig. 3. Time-courses of recovery of LDH and Glc6*P*-DH with ribosomes from different sources. (A) Denatured LDH (6 nM) was incubated with 0.1 μM *E. coli* 70S (●), 0.1 μM wheat germ 80S (□), 0.05 μM rat liver 80S (△) ribosomes or in the absence of ribosomes (○) at 25°C. At different times samples were withdrawn and assayed. Activities are expressed as percentages of the activity of the equivalent amount of native enzyme. (B) Denatured Glc6*P*-DH (12 nM) was incubated with 0.12 μM *E. coli* 70S (●), 0.02 μM wheat germ 80S (□) and 0.02 μM rat liver 80S (△) ribosomes or in the absence of ribosomes (○) at 25°C. At different times samples were withdrawn and assayed. Activities are expressed as percentages of the activity of the equivalent amount of native enzyme.

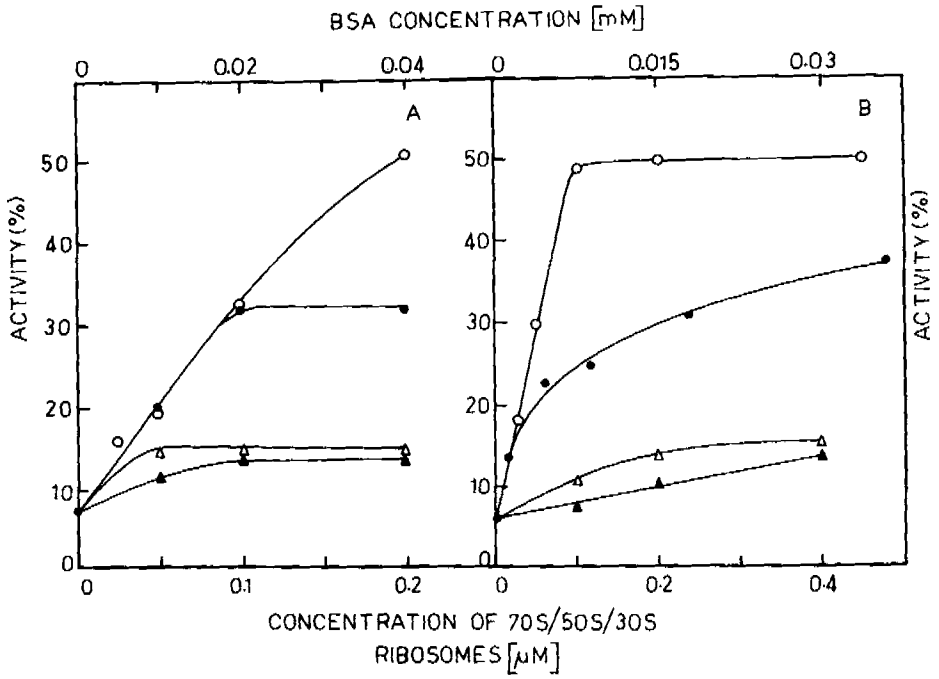

Fig. 4. *E. coli* 50S subunits support the refolding of denatured LDH and Glc6*P*-DH. The effect of different concentrations of *E. coli* 70S (●), 50S (○) and 30S (△) particles and BSA (▲) on the recovery of activity of 6 nM LDH (A) or 12 nM Glc6*P*-DH (B). For both enzymes, the refolding reactions were carried out for 30 min at 25°C and the recovered activities were expressed as percentages of the activities of the equivalent amount of native enzymes.

ery of activity in the presence of ribosomes from wheat germ and rat liver was 45–50%. With *E. coli* ribosomes the recovery of enzyme activity was 25–30%.

Addition of ATP, GTP or UTP had no effect on the recovery of the enzyme activities. The refolded enzymes could be separated from ribosomal particles by ultracentrifugation and gel filtration (data not shown).

Time-course of refolding of denatured LDH and Glc6*P*-DH by ribosomes from different sources. Fig. 3 shows the time-courses of recovery of activity of denatured LDH and Glc6*P*-DH by ribosomes from *E. coli*, wheat germ and rat liver. The concentration of denatured LDH was 6 nM, whereas the concentrations of *E. coli* 70S ribosomes and wheat germ 80S ribosomes were 0.1 μM and that of rat liver 80S ribosome was 0.05 μM (Fig. 3A). The time-course of recovery of enzyme activity in the absence of ribosomes is also shown (Fig. 3A). The time taken for ribosome-mediated recovery of enzyme activity was longer than the time taken for spontaneous recovery. The time-course of recovery of activity of Glc6*P*-DH ist shown in Fig, 3B. Unassisted folding of this enzyme led to the recovery of only 6% of its activity. The concentration of wheat germ ribosomes and rat liver ribosomes in this experiment was 0.02 μM and that of *E. coli* ribosomes was 0.12 μM, the enzyme concentration being 12 nM. The time taken by denatured Glc6*P*-DH to recover in the presence of ribosomes from any of the sources was longer

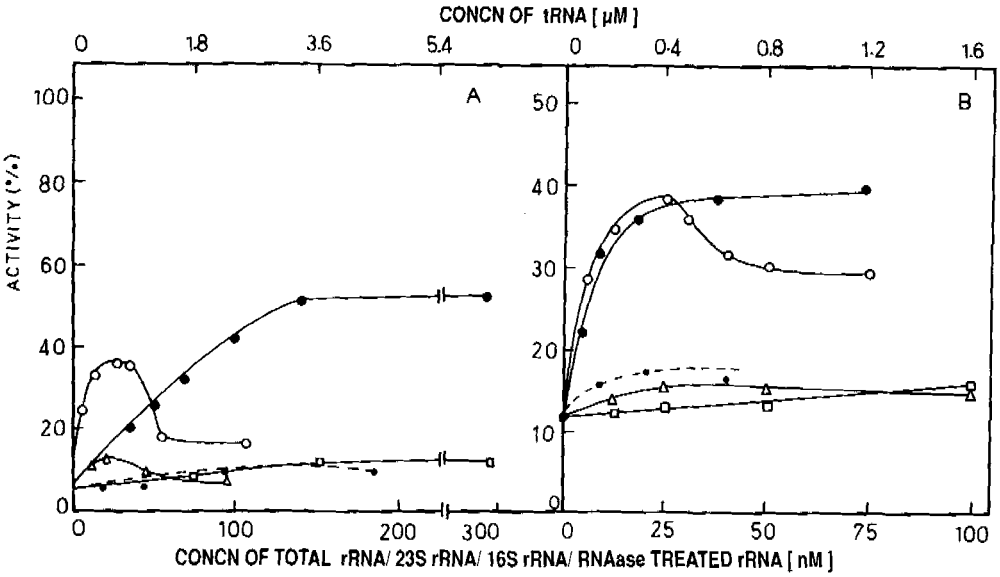

Fig. 5. Reactivation of denatured LDH and denatured Glc6*P*-DH by rRNA. Effect of increasing concentrations of total rRNA (●), 23S rRNA (○), 16S rRNA (△) and transfer RNA (□) of *E. coli* and total rRNA treated with ribonuclease A (100 μg/ml) at 37°C for 45 min then 30 min at 65°C (●) on the reactivation of denatured 6 nM LDH (A) and 12 nM Glc6*P*-DG (B). Refolding reactions were carried out at 25°C for 30 min and the recovered activities are expressed as percentages of the equivalent amount of native enzymes.

than that taken for unassisted folding. The activity increased linearly with time up to about 30% recovery with ribosomes from all the sources. In these experiments, the amounts of ribosomes used were less than those needed to obtain maximum recovery of denatured enzymes.

Refolding of denatured LDH and Glc6*P*-DH by 70S and 50S ribosomal particles. Having detected protein-folding activity in the ribosomes from a number of sources, we wanted to identify, which subunits of the ribosome, if any, could possess the activity. Fig. 4 shows the recovery of activity of denatured LDH and Glc6*P*-DH by the 30S and 50S particles of *E. coli* ribosomes. One might expect the 50S particles to provide the active site(s) for protein folding since the polypeptide chain is synthesized on this subunit. In the presence of 30S particles the activity recovered was only 5–7% higher than that recovered by unassisted folding for both enzymes. This could be due to a non-specific effect, as demonstrated with BSA.

50S particles recovered more LDH and Glc6*P*-DH acitvity than 70S particles at the same molar concentrations. The active component taking part in protein folding could be partially inaccessible in the 70S conformation, and more exposed in 50S particles.

Refolding of denatured LDH and Glc6*P*-DH by total rRNAs and 23S rRNA of *E. coli*. The protein-folding activity of the 70S particles did not disappear when the particles were washed with 0.8 M LiCl to remove many of surface proteins from the particles (data not shown). This finding prompted us to look for the activity in the -rRNA. The discovery of peptidyl transferase activity in 23S rRNA (Noller et al., 1992) supported our idea that it would be worthwhile to look for the refolding activity in rRNA. The 23S RNA was of particular interest since the 50S particle was found to be active in protein folding. We found that denatured LDH and Glc6*P*-DH could be refolded by total rRNA and 23S rRNA (Fig. 5). That RNA was responsible for this activity could be demonstrated by the finding that the total rRNA lost its protein-folding activity when it was digested with RNAase A. The specificity of the 23S rRNA in this process was implicated by the finding that neither 16S rRNA from 30S

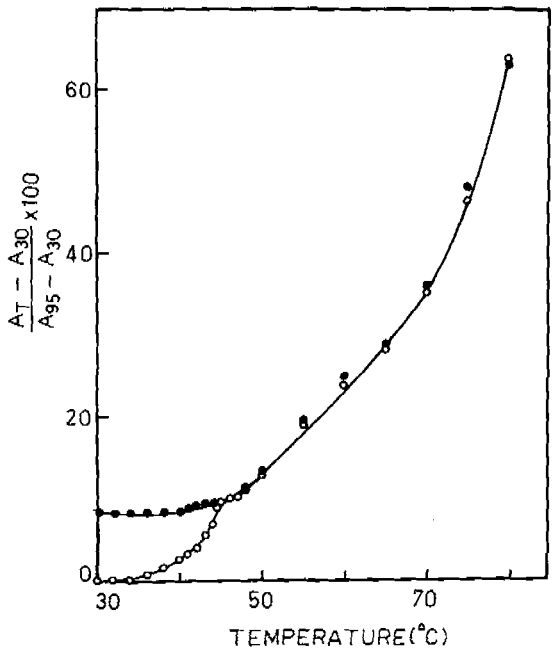

Fig. 6. Thermal-melting profile of 23S rRNA in the presence (●) and absence of 1 mM EDTA (○). 23S rRNA was diluted in 20 mM Tris/HCl, pH 7.6, 4 mM magnesium acetate containing either 0 or 1 mM EDTA and the absorbance at 260 nm of these samples at different temperatures were recovered and plotted as $(A_T - A_{30}/A_{95} - A_{30})X100$ as a function of temperature, where A_T is the absorbance at any given temperature, A_{30} is the absorbance at 30°C (absorbance of 23S rRNA remained unaltered up to 35°C), A_{95} is the absorbance at 95°C (complete denaturation of 23S rRNA giving maximum hyperchromicity was observed above 90°C. The temperature of the samples in spectrophotometer was maintained by constant circulation of water in the sample and reference cell holder from an LKB constant-temperature water circulator.

particles nor *E. coli* tRNA (which has an elaborate stem-loop structure) could support the refolding of the denatured proteins. The reactivation of enzymes with increasing concentrations of 23S rRNA and total rRNA did not follow similar patterns

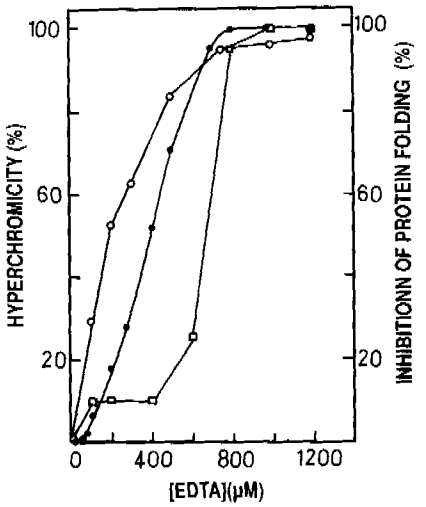

Fig. 7. Effect of increasing concentrations of EDTA on the hyperchromicity (●) of 23S rRNA and on protein folding. 23S rRNA was incubated at 25°C in a sample cell holder of Hitachi U2000 spectrophotometer and increasing amounts of EDTA were added. The difference in absorbance in the absence of EDTA and in the presence of 1 mM EDTA was taken as 100%. The percentage inhibitions of refolding activity of 23S rRNA towards denatured LDH (□) and Glc6*P*-DH (○) by increasing concentrations of EDTA are also shown. The differences between the yield of refolding of the denatured LDH and Glc6*P*-DH with and withouth 23S rRNA in the absence of EDTA were taken as 100%.

(Fig. 5). The reactivation of both proteins gradually increased with increasing concentrations of 23S rRNA to a maximum (at 25 nM RNA for both proteins) and then decreased to a saturating value. These patterns were seen repeatedly in a number of experiments and we do not have any explanation for this.

Tertiary conformation of 23S rRNA could be required for protein folding. The tertiary folds in rRNA and tRNA have been studied by following their sensitivity to a number of reagents, including EDTA (Latham and Cech, 1989; Celander and Cech, 1990, 1991; Wang and Cech, 1992). It has also been demonstrated that *in vitro* peptidyl transferase activity of *E. coli* and *Thermus aquaticus* 50S ribosomal particles and 50S particles depleted of protein by SDS/proteinase K treatment from the same sources were inhibited by 5 mM EDTA (Noller et al., 1992). We found that even in the presence of 4 mM magnesium acetate, small concentrations of EDTA could largely destroy the tertiary folds in 23S rRNA. Melting of 23S rRNA (Fig. 6) showed an initial rise in absorbance at 260 nm with increased temperature up to 48°C. This rise in absorbance was indicative of nearly complete destabilization of the tertiary fold. When the temperature was increased further, melting ensued in a second phase causing opening up of the stem-loop structure of the molecule. However, addition of 1 mM EDTA to this RNA, even at 25°C, destroyed the tertiary fold causing an increase in absorbance comparable to that obtained at high temperature (45–50°C). Increasing the temperature did not increase the absorbance at 260 nm in the presence of EDTA until about 50°C above which the melting curve followed the same path as in the absence of EDTA. Above about 50°C, the melting profiles in the presence or absence of 1 mM EDTA were identical. Therefore EDTA did not reduce the melting temperature noticeably. We take the difference in absorbance at 260 nm of 23S rRNA at 30°C in the presence and absence of 1 mM EDTA as the total contribution of the tertiary folds to absorbance. Fig. 7 shows the change in absorbance at 260 nm of 23S rRNA due to the addition of increasing concentrations of EDTA as a percentage of the total contribution of the tertiary folds to the absorbance. Inhibition of 23S-rRNA-mediated folding of denatured LDH and Glc6*P*-DH by increasing concentrations of EDTA was also observed (Fig. 7). Although the refolding of denatured LDH by 23S rRNA was slightly more resistant to EDTA than that of Glc6*P*-DH, 23S-rRNA-mediated refolding of both enzymes was completely suppressed at less than 1 mM EDTA. The recovery of enzyme activity was comparable to that obtained by self-folding, which was not influenced by this concentration of EDTA.

DISCUSSION

We have demonstrated a protein-folding activity *in vitro* in ribosomes from bacterial, plant and animal sources. We have shown previously that a number of enzymes, denatured with guanidine/HCl or by heating, could be refolded to their active forms by prokaryotic ribosomes including ribosomes from a methanogenic bacterium (Das et al., 1992; Bera et al., 1994; Chattopadhyay et al., 1994). All the proteins that we have tested could be refolded from their denatured states by ribosomes. The only criterion for selection of the proteins was that they should be amenable to simple, fast and precise assays. The proteins included bacterial alkaline phosphatase, Glc6*P*-DH from baker's yeast (Das et al., 1992), horseradish peroxidase, LDH from rabbit muscle and pig muscle (Chattopadhyay et al., 1994) and several restriction endonucleases (Bera et al., 1994). Our observations strongly suggested a second role of ribosomes, that of folding all the polypeptide chains after synthesizing them.

Although protein-folding-deficient mutants of ribosome have not been isolated, we think that our observation of a protein-folding ability in the ribosomes from different species supports the argument that this activity could be biologically significant.

A few characteristics of this novel activity of ribosomes have been reported here and earlier (Das et al., 1992; Bera et al., 1994; Chattopadhyay et al., 1994). The ratio of denatured proteins to ribosomes or rRNA were stoichiometric, not catalytic. Very high concentrations of ribosomes and RNA were necessary to achieve optimum recovery of the denatured proteins. However, the exact stoichiometry varied from one enzyme to another. The stoichiometry was determined by measuring physical parameters of interaction between the denatured proteins and the ribosomes or 23S rRNA, and these were much lower than the ratios which were necessary for optimum folding (unpublished results). The requirement for excess ribosomes was mainly due to the ribosomes being prevented from binding to the substrate (denatured proteins) in the desired conformation throughout the process of folding, as occurs for an enzyme-substrate reaction. After folding the first denatured protein molecules, that it encountered, the ribosome did not bind another denatured protein molecule because the latter folded itself (either in the correct or an incorrect) as soon as the denaturant was diluted out. We have found that ribosomes, while refolding denatured Glc6*P*-DH, could be recycled like an enzyme if more freshly denatured Glc6*P*-DH was added at a later time (Bera, A. K., unpublished results).

The time-course of ribosome-mediated refolding was found to be different from that of the small amount of spontaneous refolding for all the proteins tested. There appears to be some discrepency between the rates of folding of denatured proteins (with or without ribosomes) in this report, compared with our earlier data (Chattopadhyay et al., 1994). This discrepancy arises because here we refolded the denatured enzyme in presence of ribosomes only, whereas in some of the experiments reported earlier (Chattopadhyay et al., 1994) the enzymes were refolded in the presence of ribosomes, cofactors and substrates.

We have not been able to localize the active site(s) for folding of denatured protein on 23S rRNA. We have seen that unfolding of the tertiary structure of the 23S rRNA inhibited its protein-folding activity. EDTA at low concentrations largely destroyed the tertiary fold of this rRNA but the stem-loop structures remained largely intact. At low concentrations of EDTA, less than 1 mM, even in the presence of 10 mM magnesium-acetate the protein-folding activity of 23S rRNA towards Glc*P*-DH and LDH was inhibited completely. The failure of 30S particles and 16S rRNA to support the recovery of enzyme activities suggested that the protein folding by ribosomes/23S rRNA was not due to passive stabilisaiton of labile folding intermediates to prevent misfolding.

Our ribosomal preparations contained no contaminating Hsp60-like proteins as seen by competitive ELISA using antibodies against GroEL and DnaK proteins. The possibility of chaperone contamination could also be eliminated by the findings that refolding reactions were independent of ATP hydrolysis, whereas chaperones, with the exception of HSP90 (Wiech et al., 1992), need ATP to refold denatured proteins, and the protein-folding activity was found to be located in the 23S rRNA which was completely free of protein as determined by the complete absence of fluorescence emission when excited at 285 nm and 290 nm at high concentration and the high ratio of absorbance values at 260 nm and 280 nm (> 2.0).

LDH and Glc6*P*-DH dissociated from ribosomes after folding and could be separated from the ribosomes by ultracentrifugation and gel filtration (data not shown).

Our finding of the role of 23S rRNA in protein folding suggests an evolutionary functional relationship between RNA and proteins. The ribosomal proteins, at least those of 50S particles, could be folded by 23S rRNA, leading to its morphogenesis.

The discovery of a general protein-folding activity on the 23S rRNA could provide further insights into the process of natural selection. We can argue that a protein only becomes a part of cellular process if it is folded to an active form by ribosome with the help of the other activities (e.g., prolyl isomerase, disulfide isomerase). The question of the survival of the cell under specific selection pressure comes next.

In addition to the proteins used in this study, we observed previously the folding of denatured horseradish peroxidase, alkaline phosphatase, glucose oxidase and restriction endonucleases with ribosomes from bacterial sources (Das et al., 1992; Bera et al., 1994; Chattopadhyay et al., 1994). These proteins include periplasmic proteins, heme-containing glycoprotein and proteins from organelles such as peroxisomes. Therefore ribosomes must be able to recognize the amino acid sequences of these proteins and fold them accordingly to active forms. The 50S particles and its 23S rRNA (known to posses the peptidyl transferase activity which joins the amino acids to form polypeptides) could also recognize the sequence of the amino acids in the polypeptides. We have to examine whether protein-free preparations of 23S rRNA, such as an *in vitro* transcript of 23S rRNA gene, can fold proteins and determine the location of the protein-folding activity on the 23S rRNA and its sites of action on polypeptide chains.

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