

Reactivation of denatured proteins by 23S ribosomal RNA: Role of domain V

(protein folding/chloramphenicol/erythromycin)

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ABSTRACT *Escherichia coli* ribosome, its 50S subunit, or simply the 23S rRNA can reactivate denatured proteins *in vitro*. Here we show that protein synthesis inhibitors chloramphenicol and erythromycin, which bind to domain V of 23S rRNA of *E. coli*, can inhibit reactivation of denatured pig muscle lactate dehydrogenase and fungal glucose-6-phosphate dehydrogenase by 23S rRNA completely. Oligodeoxynucleotides complementary to two regions within domain V (which cover sites of chloramphenicol resistant mutations and the putative A site of the incoming aminoacyl tRNA), but not to a region outside of domain V, also can inhibit the activity. Domain V of 23S rRNA, therefore, appears to play a crucial role in reactivation of denatured proteins.

Escherichia coli ribosomes can reactivate several denatured proteins and, therefore, may possess a general protein folding activity (1, 2). Such an activity of the ribosome has been traced first to the 50S particle and finally to its 23S rRNA component (2). Because the polypeptide chain forms on the 50S particle, one can reasonably expect it to play some role in the folding of the chain (3). The 23S rRNA appears to play a more active role than was appreciated earlier. The direct participation of 23S rRNA in peptidyl transferase reaction during polypeptide chain elongation has been demonstrated recently (4). The sites of the association of ribosome with aminoacyl tRNA and antibiotics, which inhibit protein synthesis, have also been located on the 23S rRNA (4). Because the sites for binding-charged tRNA and several antibiotics are located mainly on the central loop of domain V of 23S rRNA (4, 5), we tested the role of domain V of 23S rRNA in protein folding. In this paper, we show the effect of two different antibiotics that bind to domain V and oligodeoxynucleotides complementary to regions of domain V on the folding reaction.

MATERIALS AND METHODS

Denaturation and Refolding of Lactate Dehydrogenase (LDH) and Glucose-6-Phosphate Dehydrogenase (G6PD). LDH from pig skeletal muscle was denatured at a concentration of 3.2 μ M with respect to monomer with 1.0 M guanidium chloride at 20°C for 1 hr. G6PD from bakers' yeast was denatured at a concentration of 2.0 μ M with respect to monomer in 6 M guanidium chloride at 25°C for 45 min. For refolding, denatured LDH and G6PD were diluted 100- and 80-fold, respectively, and were incubated at 20°C and at 25°C, respectively, for 30 min in one of the following buffers: For refolding without antibiotic, the buffer contained 20 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, and 25 mM KCl (buffer A). For LDH, buffer A also contained 5 mM 2-mercaptoethanol. For refolding in the presence of chloramphenicol, the buffer contained 50 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, and 200 mM ammonium chloride. The 23S

rRNA was incubated with different concentrations of the antibiotic in the buffer at 4°C for 10 min (6) before adding the denatured enzyme. For refolding in the presence of erythromycin, the buffer contained 40 mM Tris-HCl (pH 7.5), 13 mM magnesium acetate, 270 mM KCl, and 33 mM NH₄Cl. The 23S rRNA was incubated with different concentrations of the antibiotic in this buffer at 25°C for 50 min (7) and then the denatured LDH was added. Each antibiotic needs its exclusive high-salt buffer to bind to 23S rRNA (6, 7). In folding experiments with antibiotics, the controls (without antibiotics) were also done in the same high-salt buffers. The activities of the refolded enzymes were measured after 10-fold dilution into assay buffers. For LDH, the rate of decrease in A_{340} was measured at 37°C in buffer containing 100 mM Tris-HCl (pH 7.5), 5 mM sodium pyruvate, and 250 μ M NADH (2). For G6PD, the rate of increase in A_{340} was measured at 37°C in buffer having 50 mM Tris-HCl (pH 7.8), 3 mM glucose 6 phosphate, 1 mM NADP, and 10 mM MgCl₂ (8).

For measurement of CD spectra, 3.2 μ M of LDH and 31.36 μ M of G6PD were denatured. The concentrations of guanidium chloride and other conditions of denaturation were the same as before. For refolding, the denatured LDH and G6PD were diluted 10- and 80-fold in buffer A (without 2-mercaptoethanol) containing 100 nM and 150 nM 23S rRNA, respectively, and incubated as before. The concentrations of native, denatured, and refolded proteins shown in CD spectra were 0.32 μ M for LDH and 0.39 μ M for G6PD. Such high concentrations of proteins were necessary to get sufficient CD signal. The denaturation and recovery of enzyme activities were not altered at higher protein concentrations.

Acrylamide Quenching of the Refolded LDH. Quenching of fluorescence emission from tryptophan residues (Ex: 290 nm, Em: 345 nm; band pass – Ex: 5 nm, Em: 5 nm) was recorded after successive addition of small aliquots of acrylamide stock solution. The concentration of LDH was 0.16 μ M and of 23S rRNA, when present, was 40 nM in buffer A. The fraction of Trp residues that were accessible to the acrylamide could be estimated from the modified Stern–Volmer equation,

$$F_0/(F_0 - F) = 1/(K_{sv}f_e(Q)) + 1/f_e,$$

where f_e = fraction of the total number of Trp residues in LDH that were accessible to the quencher, F_0 is the fluorescence intensity in the absence of the quencher, F is the intensity at the quencher concentration Q and K_{sv} ; the Stern–Volmer quenching constant $K_{sv} = (F_0/F - 1)/(Q)$.

Oligodeoxynucleotide Probing of 23S rRNA. Three oligonucleotides complementary to the 23S rRNA, namely, oligo A, 5_{OH}AGCCTGTTATC_{OH}3; oligo B, 5_{OH}TAAACCCAGC-T_{OH}3; and oligo C, 5_{OH}CCCCTATTCGGTT_{OH}3 were synthesized (Bangalore Genei, India). For annealing, 20 pmol of 23S

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Abbreviations: LDH, lactate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase.

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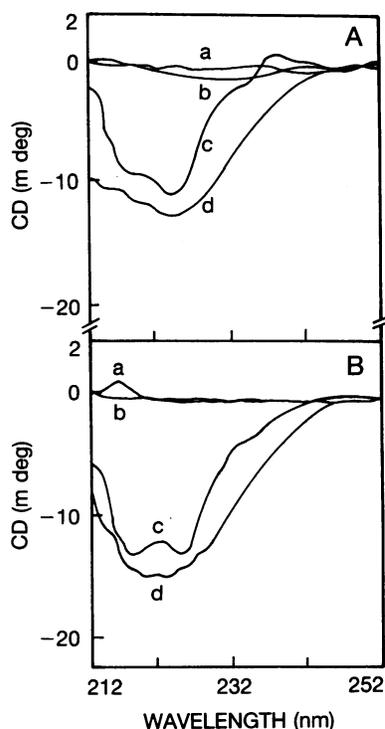


FIG. 1. CD spectra of (A) LDH and (B) G6PD. (a) Base line with buffer. (b) Protein denatured with guanidium chloride. (c) Protein refolded with 23S rRNA from which the contribution of RNA was deducted. (d) Native protein. Helicities calculated from Θ_{222} were: native 29%, denatured 2%, and refolded 24.2% for LDH and native 18%, denatured 0%, and refolded 13.2% for G6PD.

rRNA and 195 pmol of an oligonucleotide were incubated in 10 mM Tris-HCl (pH, 7.5), 150 mM NaCl, and 1 mM EDTA for 70 min at 50°C and then left on ice overnight. To monitor the extent of annealing, the oligonucleotides were end-labeled with [γ - 32 P]ATP and after annealing with 23S rRNA, the mixture was run on a 20-cm long gel, of which the top 7 cm contained 4% and the remaining part 12% acrylamide. The free oligonucleotides separated widely from the annealed ones, which remained close to the well. The extent of annealing was calculated from the ratio of counts in these two fractions. Under the above condition, about 70–80% of 23S rRNA could be annealed with the oligos. Free oligos were separated from the RNA by gel filtration through Sephadex G-200 column.

RESULTS

Refolding and Reactivation of Denatured Protein by 23S rRNA. Fig. 1 shows the CD spectra of native, denatured, and

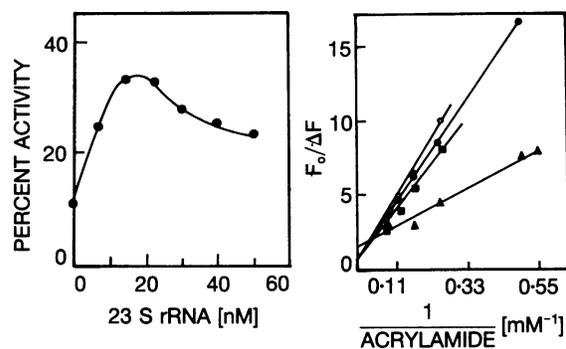


FIG. 2. (Left) Refolding of denatured LDH with 23S rRNA and (Right) modified Stern–Volmer plots for acrylamide quenching of native protein with 23S rRNA (●), native protein only (○), protein refolded by 23S rRNA (■), and self-folded protein (▲).

refolded LDH and G6PD. The secondary structures were completely lost due to denaturation; little residual helicity was observed as judged by the CD data on mean residue ellipticity at 222 nm using the criteria provided by Chen and Yang (9). The secondary structures recovered considerably when refolded with 23S rRNA. The recovery of the biochemical activity and structural conformations of LDH in the presence of 23S rRNA are shown in Fig. 2. The biochemical activity was restored to about 35%. The Trp accessibility of the neutral quencher acrylamide for native, self-folded, and enzyme-folded in presence of 23S rRNA were 100%, 67%, and 100%, respectively, when calculated from the quenching of their intrinsic fluorescence. These results indicate the close similarity between the conformations of the native and the refolded LDH in the presence of 23S rRNA. On the other hand, the self-folded protein had different conformation since the accessibility of acrylamide for its tryptophans was very different. The native and the refolded enzymes also eluted in exactly the same volume in the Sephacryl S-300 column showing that their Stokes radii were identical. The refolded enzyme was separated from the 23S rRNA by the column, and was active. Therefore, continuous association with the RNA was not necessary (data not shown).

Inhibition of 23S rRNA-Mediated Reactivation of Denatured Enzymes by Antibiotics. The denatured LDH and G6PD were incubated in the reactivation buffer, in the presence and absence of 23S rRNA and varying concentrations of chloramphenicol. In the absence of the antibiotic, reactivation of LDH with and without 23S rRNA, as measured by the enzyme activity, were 30% and 12%, respectively. The corresponding figures for G6PD were similar (29% and 16%, respectively). With increasing concentrations of chloramphenicol, the rRNA-mediated reactivations were increasingly inhibited, and reached the level of self-folding and not lower for both the

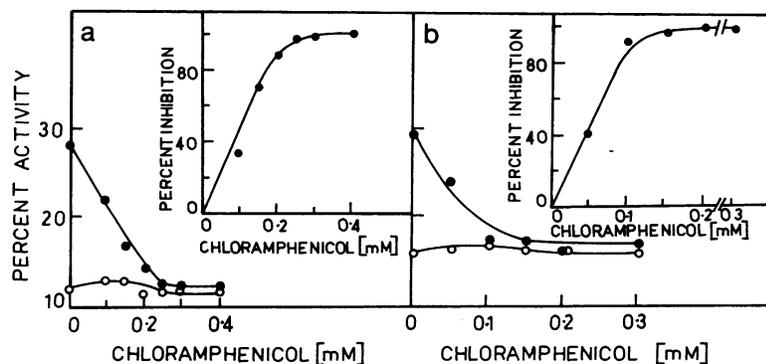


FIG. 3. Effect of chloramphenicol on the refolding of denatured (a) LDH and (b) G6PD with (●) and without (○) 23S rRNA. Insets show percent inhibition of 23S rRNA-mediated refolding at different concentrations of chloramphenicol.

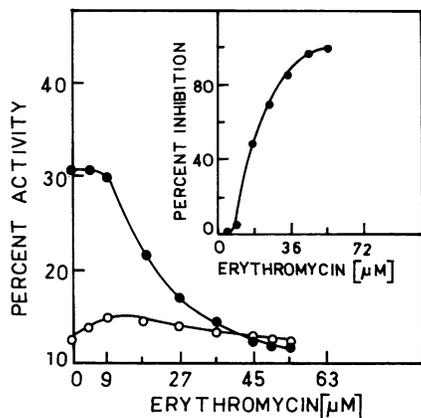


FIG. 4. Effect of erythromycin on the refolding of denatured LDH with (●) and without (○) 23S rRNA. Inset shows percent inhibition of 23S rRNA-mediated refolding at different concentrations of erythromycin.

enzymes (Fig. 3 *a* and *b*). Erythromycin had similar inhibitory effects on the reactivation of LDH. In the absence of the antibiotic, the enzyme activities were 30% and 12% in the presence and absence of 23S rRNA. With increasing concentrations of erythromycin, the reactivation in the presence of 23S rRNA reached a plateau at 12% (Fig. 4). This experiment could not be done with G6PD because the antibiotic drastically interfered with the activity of the native enzyme.

Inhibition of Reactivation by Deoxyoligonucleotides Complementary to the Large Loop of Domain V of 23S rRNA. We have shown that 23S rRNA-mediated reactivation could be completely inhibited by chloramphenicol and erythromycin. These antibiotics also inhibit protein synthesis and mutations that confer resistance to these antibiotics are located within the central loop of domain V of 23S rRNA (ref. 4; Fig. 5). The

antibiotics also bind to these sites of the RNA (4). Because this binding may also block the 23S rRNA-mediated protein reactivation, we tested this hypothesis independently. We used complementary deoxyoligonucleotides annealed to domain V and other regions of 23S rRNA and followed their effect on protein folding. Three different oligonucleotides were used. Oligo A is an 11-mer complementary to nucleotides 2447–2457 of 23S rRNA. The region covers three of the seven sites in domain V that can be mutated to confer chloramphenicol resistance. Oligo B is also an 11-mer, but complementary to nucleotides 2577–2587, which cover the putative A site for incoming aminoacyl tRNA. Oligo C is a 13-mer complementary to nucleotides 608–620, which cover a part of domain II, which is outside the central loop of domain V. Since the single-stranded loop (Fig. 5), which binds erythromycin consists of only five bases that are A and U only, we could not decide on a complementary oligo that would stably base pair with it. The loop is also flanked by stable stems rich in G+C base pairs. Therefore, annealing with longer oligos would have required disruption of the stem. So the site of action of erythromycin was not tested. Oligos A and B both inhibited 23S rRNA-mediated reactivation of LDH and G6PD. Complete inhibition was not obtained most likely because the efficiency of annealing the oligos to the 23S rRNA was at most 80% (Fig. 6 *a–d*). Increasing the concentration of RNA reduced the level of folding even in controls without the oligos as we observed earlier (2). Unlike oligos A and B no inhibition was seen with oligo C, although the degree of annealing to 23S rRNA was identical in all three cases (Fig. 6 *e* and *f*). Control experiments were also done only with the oligo probes to ensure that they had no effect on the folding of LDH and G6PD. These results therefore suggest that the availability of the central loop of domain V of 23S rRNA is particularly important for protein folding.

DISCUSSION

The central loop of domain V of 23S rRNA spanning nucleotides 2000–2624 has been marked as an active core where

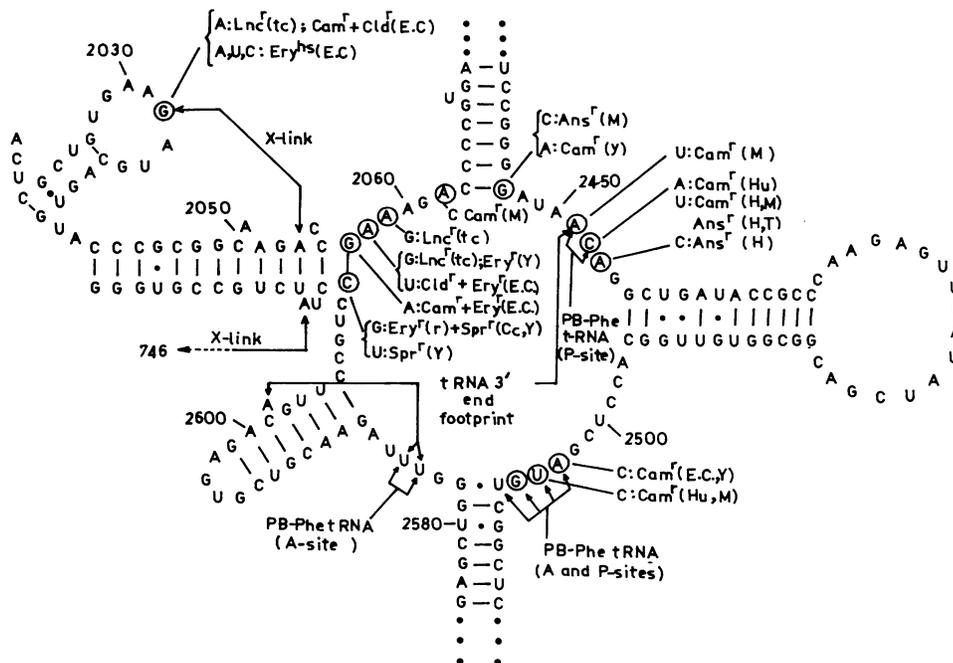


FIG. 5. Secondary structure of peptidyl transferase loop and adjacent regions of 23S rRNA. Arrows indicate bases that could be crosslinked (X-link) and the positions photoaffinity-labeled by benzophenone-derivatized Phe-tRNA (PB-Phe tRNA) and *p*-azidopuromycin. Sites within this region where interaction of the tRNA 3' end causes altered reactivities to the chemical reagents are shown (tRNA 3' end footprint). Positions where mutations confer drug resistance are shown. Ans, anisomycin; Cam, chloramphenicol; Cld, clindamycin; Ery, erythromycin; Lnc, lincomycin; Spr, spiramycin; r, resistant. Resistant mutations were detected in Ec, *E. coli*; H, halobacteria; Hu, human mitochondria; M, mouse mitochondria; T, tetrahymena cytoplasmic ribosomes; Tc, tobacco chloroplasts; Y, yeast mitochondria. [Reproduced with permission from ref. 5 (copyright American Society for Microbiology).]

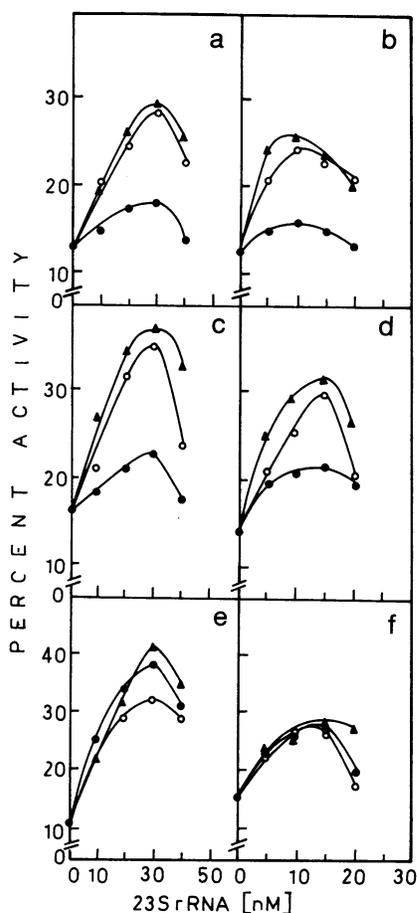


FIG. 6. Refolding of denatured LDH (left panels) and G6PD (right panels) with 23S rRNA pre-treated in various ways, namely, taken from stock (\blacktriangle), mock-treated for annealing with oligo probe without adding the oligo (\circ), and annealed with oligos (\bullet). Oligo A was used in a and b, oligo B in c and d, and oligo C in e and f.

aminoacyl tRNA and a number of antibiotics bind and the peptidyl transferase reaction takes place (4). Here we show by two different approaches that the same region of the rRNA play a crucial role in reactivation of denatured proteins. We used two antibiotics, chloramphenicol and erythromycin, and two oligonucleotides complementary to different regions of this loop. The folding of denatured LDH and G6PD were inhibited when 80 $\mu\text{g}/\text{ml}$ of chloramphenicol and 40 $\mu\text{g}/\text{ml}$ of

erythromycin were added to 23S rRNA. These concentrations are normally used to stop protein synthesis in growing cells (10). The binding of these antibiotics to their respective binding sites on 23S rRNA inhibited its protein-folding activity, suggesting the involvement of the region in protein folding. This inference was supported by the results of another set of experiments where oligodeoxynucleotides complementary to different regions of the rRNA were used. The oligonucleotides that were effective in blocking the activity of 23S rRNA spanned the region where mutations conferring resistance to chloramphenicol map. Thus, the integrity of domain V appears to be required for the refolding activity. As mentioned in the results, the antibiotics could not suppress the level of folding of denatured LDH and G6PD below the level of self-folding. Because the antibiotics had no effect on the self-folding of the denatured proteins in the absence of rRNA (Fig. 3), it appears that they could abrogate at best the stimulatory effect of rRNA by preventing their binding to denatured proteins.

The central loop of domain V could be directly acting as the folding modulator or it could be determining the tertiary conformation of 23S rRNA that is required for the folding activity. To help sort out these possibilities, we would synthesize different segments of 23S rRNA and possibly scan the entire molecule to determine the role of other segments of the RNA, if any, to refold denatured proteins.

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