

Reactivation of denatured proteins by domain V of bacterial 23S rRNA

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ABSTRACT

***In vitro* transcripts containing domain V of the 23S rRNA of *Escherichia coli* and *Bacillus subtilis* can reactivate denatured proteins almost as efficiently as the total 23S rRNA. Here we show that almost the full length of domain V is required for reactivation of denatured pig muscle lactate dehydrogenase and pig heart cytoplasmic malate dehydrogenase: the central loop of this domain alone is not enough for this purpose. The antibiotic chloramphenicol, which binds to domain V of 23S rRNA, can inhibit reactivation of these proteins completely. Activity is eliminated by EDTA at a concentration of <1 mM, even in the presence of 4 mM MgCl₂, suggesting that the three-dimensional conformation of the RNA should be maintained for this activity.**

INTRODUCTION

Ribosomes from both prokaryotic and eukaryotic sources can reactivate several denatured proteins and, therefore, may possess a general protein folding activity (1–3). In *Escherichia coli* ribosomes such an activity has also been traced first to the 50S subunit and finally to its 23S rRNA component (2,4). Strong evidence has been obtained in favour of a similar role of 50S subunits/23S rRNA *in vivo* (manuscript submitted). Since the polypeptide chain forms on the 50S particle, one can reasonably expect it to play some role in folding of the chain (5–9). The 23S rRNA appears to play a more active role in protein synthesis than was appreciated earlier. The direct participation of 23S rRNA in the peptidyl transferase reaction during polypeptide chain elongation was demonstrated a few years ago (10). The sites of association of the ribosome with aminoacyl tRNA and antibiotics which inhibit protein synthesis have also been located on 23S rRNA (10,11). Domain V of 23S rRNA has been assigned as playing crucial roles in these processes. We have shown complete elimination of the protein folding activity of 23S rRNA by antibiotics specifically binding to domain V as well as oligodeoxynucleotides annealing with the single-stranded central loop of domain V (12). However, in all experiments reported so far on the different activities of 23S

rRNA it was extracted from the 50S ribosomal subunit by the usual phenol extraction procedure (10,12). The activities have not been shown in 23S rRNA transcribed *in vitro* from its gene. We have transcribed only domain V of 23S rRNA and its different parts *in vitro* from appropriate clones and have shown that the *in vitro* transcript containing almost the entire domain V has protein folding activity. If the central elaborate stem-loop region is deleted from domain V then the shorter transcripts fail to reactivate denatured protein. This protein folding activity of domain V is sensitive to the 23S rRNA binding antibiotic chloramphenicol, which binds to the central loop of domain V. This activity is also sensitive to EDTA, which breaks the tertiary organization (causing hyperchromicity) of this RNA.

MATERIALS AND METHODS

Denaturation and refolding of pig muscle lactate dehydrogenase (henceforth called lactate dehydrogenase) and pig heart cytoplasmic malate dehydrogenase (henceforth called malate dehydrogenase)

Lactate dehydrogenase at a concentration of 3.2 μM with respect to monomer was denatured with 1 M guanidium chloride at 20°C for 1 h (12). For refolding the enzyme was diluted 100-fold in 20 mM Tris-HCl, pH 7.5, 200 mM NaCl and 4 mM magnesium acetate and incubated at 20°C for 30 min with or without RNA unless otherwise mentioned. The enzyme concentration was 32 nM with respect to monomer during refolding. Malate dehydrogenase at a concentration of 1.15 μM with respect to monomer was denatured with 6 M guanidium chloride at 20°C for 40 min. For refolding the denatured enzyme was diluted 80-fold in 25 mM sodium phosphate, pH 7.6, 200 mM NaCl, 5 mM 2-mercaptoethanol and 4 mM magnesium acetate and incubated at 20°C for 15 min in the presence or absence of RNA unless otherwise mentioned. The enzyme concentration during refolding was 14 nM with respect to monomer. For refolding in the presence of chloramphenicol both the denatured enzymes were diluted in 50 mM Tris-HCl, pH 7.5, 200 mM NH₄Cl and 10 mM magnesium acetate. The antibiotic was incubated in this buffer for 15 min at 4°C prior to addition of denatured enzyme. For refolding in the presence of lincomycin both the denatured

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enzymes were diluted in 60 mM Tris-HCl, pH 7.5, 400 mM KCl and 4 mM magnesium acetate. The antibiotic was incubated in this buffer for 20 min at 0°C before adding denatured enzymes. Each antibiotic needs an exclusive high salt buffer to bind 23S rRNA (36,37). The activities of the refolded enzymes were measured after dilution in the assay buffer. For lactate dehydrogenase 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 malate dehydrogenase the rate of decrease in A_{340} was measured at 25°C in buffer containing 150 mM sodium phosphate, pH 7.6, 2 mM 2-mercaptoethanol, 500 μ M oxaloacetate and 200 μ M NADH.

Cloning domain V of *E.coli* 23S rDNA on the T7 promoter in the plasmid vector pGEM4Z and its transcription by T7 polymerase

A part of the 23S rDNA gene covering most of domain V was PCR amplified from plasmid pKK 3535, which contains the entire 23S rDNA, using oligonucleotides (A) 5'-GAGAAGCTTGTACCCG-CGGCAAGACG GAA-3' and (B) 5'-GAGGGATCCTCTCCAGCGCCACGGCAGA-3' for the 5'- and 3'-ends of the amplified DNA. The bold sequences of the oligonucleotides constitute the two ends of the domain V segment amplified by PCR, while the rest of the nucleotides constitute restriction sites for the enzymes *Bam*HI and *Hind*III. The PCR product was digested with restriction enzymes *Hind*III and *Bam*HI at the 5'- and 3'-end respectively and ligated to vector pGEM4Z (38) digested with the same enzymes between the T7 and SP6 promoters in the polyclonal site. The cloned fragment contained 595 nt of 23S rDNA. This plasmid, designated pDP1, was then linearized with *Bam*HI and transcribed with T7 RNA polymerase to give a run-off RNA containing 595 nt from 2040 to 2634 of 23S rRNA. Transcription with SP6 RNA polymerase of the plasmid linearized with *Hind*III gave the RNA strand complementary to 23S rRNA. The RNA strand transcribed with SP6 polymerase annealed with 23S rRNA, whereas the RNA strand transcribed with T7 polymerase failed to do so. Therefore, T7 polymerase synthesized the same strand as domain V of 23S rRNA.

The T7 and SP6 RNA polymerases, ribonucleotides and RNase inhibitors required for *in vitro* transcription were obtained from Ambion (Maxiscript kit no. 1322). Taq polymerase was obtained from GENEI (Bangalore, India).

RESULTS

Transcription of different parts of domain V of *B.subtilis* 23S rDNA cloned in transcription vectors by SP6 and T7 RNA polymerases

The putative secondary structure (39) of the 595 nt transcript from plasmid pDP1 containing domain V of *E.coli* 23S rRNA is shown in Figure 1A. Domain V of 23S rRNA of *Bacillus subtilis* cloned in plasmid pDK105 (34) was transcribed with SP6 RNA polymerase after linearization of the plasmid with *Eco*RI. The secondary structure of this 660 nt RNA is shown in Figure 1B. The 337 nt RNA fragment containing the central loop of domain V of *B.subtilis* 23S rRNA was transcribed with SP6 RNA polymerase from plasmid pDK106 (34) linearized with *Eco*RI. The structure of this RNA is shown in Figure 1C. The 113 nt RNA containing the upper half of the central loop of domain V of *B.subtilis* 23S rRNA was transcribed by T7 RNA polymerase

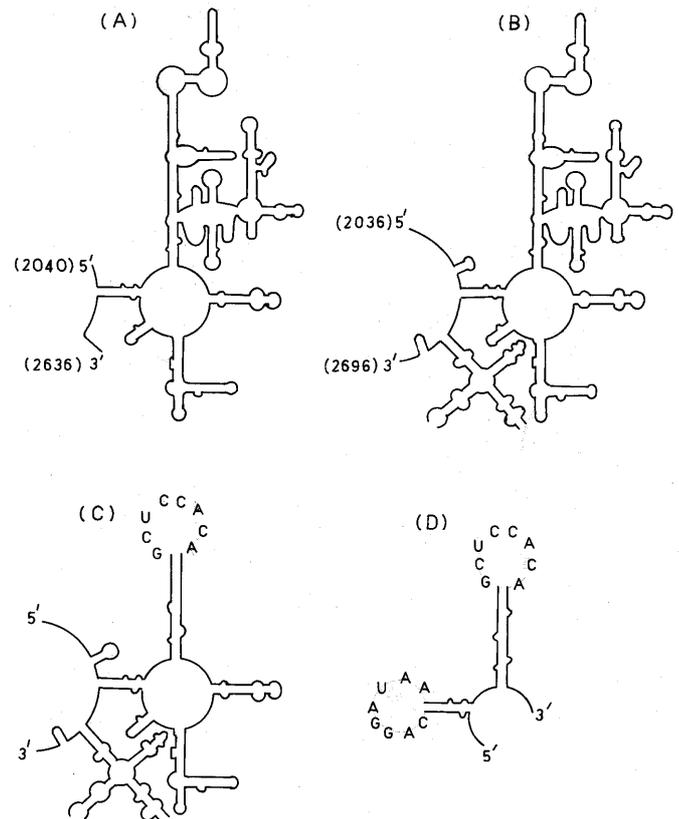


Figure 1. Secondary structures of different segments of domain V of bacterial 23S rRNA. (A) 595 nt from *E.coli*, (B) 660, (C) 337 and (D) 113 nt respectively from *B.subtilis*. The bases shown in the loops in (C) and (D) were introduced to join the stems by Kovalic *et al.* (34), they do not belong to the authentic domain V of 23S rRNA.

from plasmid pDK107 (35) linearized with *Eco*RI. The structure of this RNA is shown in Figure 1D. Plasmids pDK105, pDK106 and pDK107 were kind gifts from Prof. Bernard Weisblum (University of Wisconsin, Madison). The secondary structure of the four RNA species (Fig. 1A–D) are drawn according to Gutell and Fox (39). A few bases shown in the loops in Figure 1C and D were introduced by Kovalic *et al.* (34) to join the stems. These are not present in authentic domain V of 23S rRNA.

After transcription the RNA molecules were purified by phenol extraction, ethanol precipitation and subsequent gel filtration to get rid of the DNA. A small amount of [α - 32 P]UTP was added to the transcription reaction to calculate the concentration of RNA molecules from incorporation of [α - 32 P]UTP in acid-precipitable material.

The length of all the transcripts above were found to be as expected when run on 5% acrylamide, 8 M urea gels, as shown in the autoradiogram in Figure 2.

Reactivation of denatured proteins by 23S rRNA and its fragments

Figure 3 shows reactivation of denatured malate dehydrogenase and lactate dehydrogenase with *E.coli* 23S rRNA and domain V

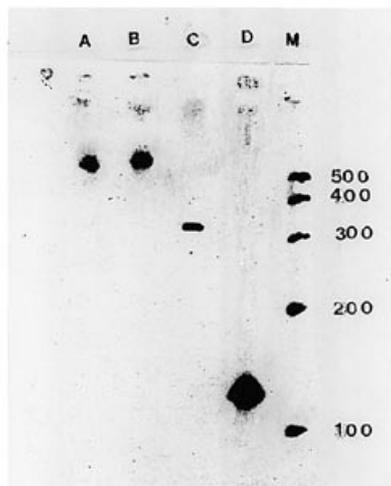


Figure 2. Autoradiogram of a 5% acrylamide, 8 M urea gel showing transcripts of different parts of domain V of 23S rDNA. The length of the transcripts are (A) 595 nt transcribed from pDP1, (B) 660 nt transcribed from pDK105, (C) 337 nt transcribed from pDK106 and (D) 117 nt transcribed from pDK107. The Century marker RNA fragments are shown in lane M. These markers were synthesized on templates provided by Ambion (catalogue no. 7780) by T7 RNA polymerase.

of 23S rRNAs from *B.subtilis* (660 nt) and *E.coli* (595 nt). The shorter fragments from domain V of *B.subtilis* 23S rRNA of lengths 337 and 113 nt failed to reactivate the denatured proteins. Therefore, the elaborate stem-loop regions protruding from the upper part of the central loop of domain V (Fig. 1A and B) appear to be indispensable for the protein folding reaction. The central loop alone is not enough for this activity. The extents of reactivation of the proteins were similar in the presence of complete 23S rRNA and the 660 nt RNA containing the entire domain V. The 595 nt transcript, which was missing ~40 nt from the 3'-OH end of domain V of *E.coli* 23S rRNA, reactivated the proteins to a little lesser extent. Like 23S rRNA the requirements of domain V RNA molecules for optimum reactivation of the enzymes were stoichiometric. The ratios of RNA to protein molecules required for optimum reactivation were identical for intact 23S rRNA and domain V RNA. Also, as observed earlier in the case of 23S rRNA (2,3), at higher concentration of domain V RNA the extent of reactivation decreased. The protein folding activity of 23S rRNA is therefore present in domain V only. Since the 595 nt RNA obtained from plasmid pDP1 is so far the smallest length of domain V having protein folding activity, we used this RNA to characterize this reaction.

Time course of reactivation of proteins by 23S rRNA and its domain V

The time course of reactivation of malate dehydrogenase and lactate dehydrogenase with 23S rRNA and its 595 nt fragment are shown in Figure 4A and B. The concentrations of enzymes were as described in Materials and Methods. The concentrations of both the RNAs were 14 and 28 nM respectively for malate and lactate dehydrogenase. The 595 nt RNA reactivated denatured malate dehydrogenase and lactate dehydrogenase at a rate considerably faster than 23S rRNA. With this small RNA

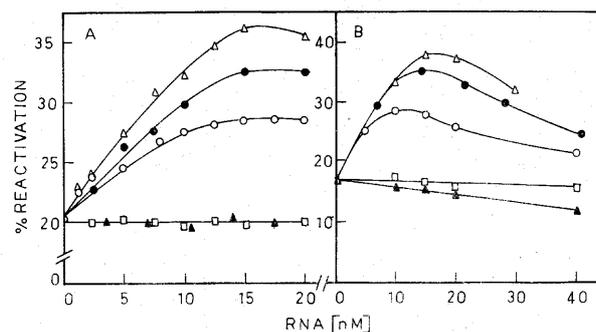


Figure 3. Reactivation of malate dehydrogenase (A) and lactate dehydrogenase (B) with 23S rRNA of *E.coli* (open triangles), 660 nt RNA (filled circles), 595 nt RNA (open circles), 337 nt RNA (open squares) and 113 nt RNA (closed triangles).

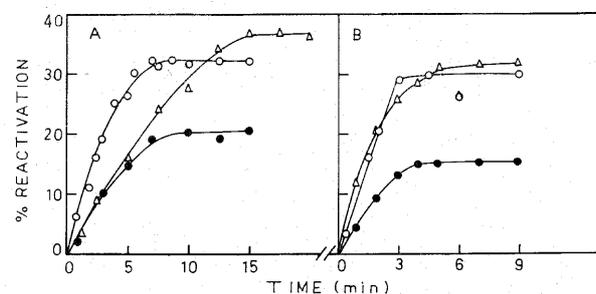


Figure 4. Time course of reactivation of malate dehydrogenase (A) and lactate dehydrogenase (B) with 23S rRNA of *E.coli* (open triangles), 595 nt RNA (open circles) and without RNA (closed circles).

fragment maximum reactivations were obtained at the same time as spontaneous folding for both proteins. This is in contrast to our earlier observations on 70S ribosomes, the 50S subunit and 23S rRNA-mediated protein folding, all of which took appreciably longer than spontaneous folding of the proteins. Obviously, these modulators controlled the rate limiting steps of protein folding.

The protein folding activity of 23S rRNA is therefore present in domain V only. This is not surprising. In fact, chemical and other probes revealed that >20 nt in this domain only are accessible, even when it is covered by ribosomal proteins as part of 23S rRNA. No other region of 23S rRNA is as exposed (13-19).

Effect of the antibiotics chloramphenicol and lincomycin on reactivation of lactate dehydrogenase and malate dehydrogenase by domain V of 23S rRNA

We have shown that protein folding by 23S rRNA was inhibited by chloramphenicol and erythromycin (12), antibiotics which bind to this RNA. Since the antibiotics bind mainly to domain V of 23S rRNA, we checked the sensitivity of protein folding by the 595 nt RNA to such antibiotics. The antibiotics used in these experiments were chloramphenicol and lincomycin. Figure 5A shows inhibition of reactivation of denatured malate dehydrogenase and lactate dehydrogenase by chloramphenicol. At 0.2 mM chloramphenicol

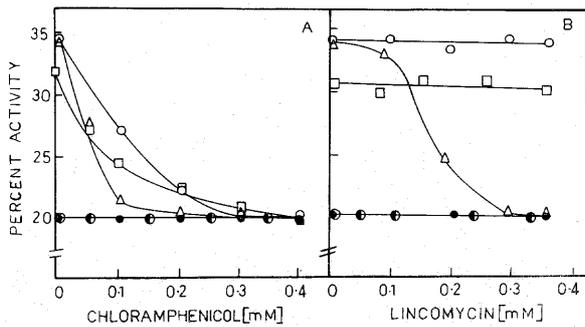


Figure 5. Effect of chloramphenicol (A) and lincomycin (B) on reactivation of the enzymes in the presence and absence of rRNA. Reactivation of lactate dehydrogenase (open circles) and malate dehydrogenase (open squares) with 595 nt RNA and reactivation of lactate dehydrogenase with 23S rRNA from *E. coli* (open triangles). Reactivation of lactate dehydrogenase (filled circles) and malate dehydrogenase (half filled circles) without RNA.

recovery of enzyme activity decreased to the level of spontaneous folding. Whereas lincomycin had an inhibitory effect on reactivation of denatured lactate dehydrogenase and malate dehydrogenase by full-length 23S rRNA, it did not inhibit reactivation of these enzymes by the 595 nt fragment (Fig. 5B). This could be due to the absence of nt 2032, which is implicated in lincomycin binding (11), in this RNA fragment. On the other hand, most of the nucleotides involved in chloramphenicol binding occur throughout the length of the central loop of domain V, so this antibiotic could easily bind to the RNA fragment.

Effect of EDTA on reactivation of proteins by domain V of 23S rRNA

We have shown that even in the presence of 4 mM MgCl₂ very low concentrations of EDTA (<1 mM) could completely inhibit reactivation of proteins by 23S rRNA (3). This small amount of EDTA caused hyperchromicity of 23S rRNA (3), probably by destroying its tertiary level organization and hence protein folding activity. The 595 nt RNA-mediated reactivation of malate dehydrogenase and lactate dehydrogenase was also completely inhibited by ~0.75 mM EDTA, as shown in Figure 6.

DISCUSSION

We have shown that the protein folding activity of 23S rRNA of the bacterial ribosome (1-4,12) actually resides in domain V. The 660 and 595 nt *in vitro* transcripts, containing domain V or most of it of *B. subtilis* and *E. coli* 23S rRNA respectively, could reactivate denatured lactate dehydrogenase and malate dehydrogenase almost as efficiently as 23S rRNA. This observation completely eliminates the possibility of any contaminating ribosomal protein factor in our rRNA preparations which could be responsible for protein folding. Also, these transcripts take up their tertiary conformations required for protein folding spontaneously, without the assistance of any ribosomal proteins. This is a much less stringent requirement compared with the domain V conformation required for peptidyl transferase activity. For the peptidyl transferase reaction *E. coli* 23S rRNA was isolated by proteinase K and SDS treatment, but phenol extraction destroyed such activity (10). This reaction also involves tRNA, nucleotides, etc. Therefore, we can assume that

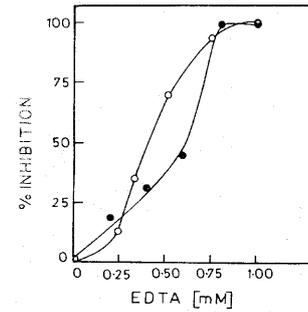


Figure 6. Inhibition of reactivation of lactate dehydrogenase (filled circles) and malate dehydrogenase (open circles) with 595 nt RNA in the presence of EDTA.

the protein folding activity in the largest rRNA, which involves a very simple reaction without requiring any cofactors, etc. (12,4), evolved earlier than peptidyl transferase activity. These arguments are valid if we assume that the original ribosome was made up solely of RNA, as suggested by Crick (20) and asserted more strongly by Woese (21). As mentioned before, even in the 50S subunit itself a large number of nucleotides, almost exclusively in domain V of 23S rRNA, are accessible by chemical agents, amino acyl tRNA, the 30S subunit, etc. (13-19). Therefore, it is not surprising that this domain having the peptidyl transferase centre could act within or without the ribosomal protein coat to form the peptide bond and fold the newly synthesized protein. The presence of an activity in RNA *in vitro* which is normally seen in the ribonucleoprotein complex *in vivo* is not surprising: RNase P, snRNP, etc. are wonderful examples (22-30). The tertiary conformation of this RNA domain was required for this activity, as we discuss later. This points to a number of interesting possibilities. The protein folding activity in domain V of 23S rRNA could evolve before the complete ribosome structure was determined with further addition of nucleotides and proteins. In a prebiotic soup only a small RNA could assist some spontaneously synthesized polypeptides to fold to meaningful (active) conformers. The 'RNA world' (31,32) envisages a versatile role of RNA in biochemical and biological evolution. In the central dogma of molecular biology the RNA then has overwhelming control over the genetic information. It transmits information from DNA to the ribosome active centre and eventually translates the information into a three-dimensional 'active' protein. RNA directs the protein to attain the active three-dimensional state (2-4,10,12), a role which is perhaps the most deterministic in the central dogma of molecular biology. The requisite conformation of 23S rRNA for protein folding is destroyed by EDTA and presumably blocked by the antibiotics chloramphenicol, erythromycin, lincomycin, etc. (3,12), which bind to the 50S subunit of the *E. coli* ribosome.

The protein folding activity of the 595 nt transcript is resistant to lincomycin. If this is true *in vivo*, then we could use this information with advantage in follow-up experiments. We could overproduce this transcript in the cell in the presence of lincomycin, which inhibits the protein folding activity of all full-length 23S rRNA molecules, and see if this transcript plays any role in protein folding *in vivo*.

Finally, a comparison of nucleotide sequences of the 660 and 595 nt RNA species (33,34) brings home what is already known about domain V of bacterial 23S rRNA. The central loop is totally conserved and a few single-stranded regions are also conserved. Changes in sequence within stems are always compensated by changes in complementary sequences to restore base pairing. Our task now is to change individual bases within the central loop and destabilize the stems one at a time to determine their effects on protein folding.

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