

Complementary Role of Two Fragments of Domain V of 23 S Ribosomal RNA in Protein Folding*

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We have shown that the domain V of bacterial 23 S rRNA could fold denatured proteins to their active state. This segment of 23 S rRNA could further be split into two parts. One part containing mainly the central loop of domain V could bind denatured human carbonic anhydrase I stably. This association could be reversed by adding the other part of domain V. The released enzyme was directed in such a way by the central loop of domain V that it could now fold by itself to active form. This agrees with our earlier observation that proteins fold within the cell posttranslationally, a process that is completed after release of the newly synthesized polypeptide from the ribosome (Chattopadhyay, S., Pal, S., Chandra, S., Sarkar, D., and DasGupta, C. (1999) *Biochim. Biophys. Acta* 1429, 293–298).

We have identified the ribosome as a general protein folding modulator on the basis of its ability to successfully fold all the denatured proteins that we have tried so far (e.g. lactate dehydrogenase, glucose-6-phosphate dehydrogenase, horseradish peroxidase, restriction endonucleases, alkaline phosphatase, malate dehydrogenase, β -lactamase, carbonic anhydrase, β -galactosidase, etc.) (1–6). This *in vitro* protein folding activity has been found to reside in the domain V of the 23 S rRNA in 50 S particle of the ribosome. This activity of ribosome has also been identified *in vivo* by showing slow posttranslational activation of the enzyme β -galactosidase in *Escherichia coli* that was synthesized just prior to the addition of the 30 S specific protein synthesis inhibitors kasugamycin and streptomycin. This posttranslational activation, however, was immediately arrested by adding antibiotics that bind to domain V of 23 S rRNA of 50 S ribosomal particle (1). The important question then is whether biological entities like molecular chaperons (7, 8) and ribosomes fold proteins to their active states following a pathway basically similar to spontaneous folding or whether there will be a paradigm shift in our understanding of protein folding in the cell when we know how ribosome (2–6, 9–11), which synthesizes the polypeptide, also folds it to active form.

We identified the protein folding activity in the large loop of domain V of 23 S rRNA of bacterial ribosome (4, 9, 10). We have reported in a number of publications (1–6, 10) that the 70 S

bacterial ribosomes, the 80 S wheat germ and rat liver ribosomes, the 50 S bacterial ribosomal subunit, its 23 S rRNA, as well as the 660-nt¹ domain V of 23 S rRNA could all fold denatured proteins, and at the end of the reaction they were found to dissociate completely from the proteins without the assistance of any co-factor. This implied that there were at least two steps in these reactions: (a) interaction with unfolded proteins to fold them and (b) dissociation from the folded proteins. We took the 660-nt-long domain V RNA from *Bacillus subtilis* and further split it into two smaller pieces that acted in a particular sequence on the unfolded protein to fold it. Here we present the role of these two parts of domain V in refolding denatured proteins.

EXPERIMENTAL PROCEDURES

Enzymes and Reagents—Human carbonic anhydrase I (EC 4.2.1.1), pig muscle lactate dehydrogenase (EC 1.1.1.27), and porcine heart cytoplasmic malate dehydrogenase (EC 1.1.1.37) were purchased from Sigma. The enzymes gave single bands of monomeric molecular weights 29,800, 31,000, and 35,000, respectively, in SDS-polyacrylamide gel electrophoresis. These enzymes are referred to as carbonic anhydrase, lactate dehydrogenase, and malate dehydrogenase in the text. All the laboratory reagents used were of analytical grade.

Preparation of *E. coli* 50 S Ribosomal Particle and Its 23 S rRNA—Purification of 70 S ribosome, its 50 S subunit, and 23 S rRNA from *E. coli* MRE 600 have been described previously (6). The 23 S rRNA was separated from 5 S RNA by gel filtration. The purity of 23 S rRNA was checked by (a) electrophoresis in a composite gel 0.5% agarose and 3% of a 19:1 (by mass) mixture of acrylamide/*N,N'*-methylene bisacrylamide, (b) an A_{260}/A_{280} greater than 2.0, and (c) 40% hyperchromicity on RNase digestion at 37 °C.

Preparation of Domain V of 23 S rRNA and Its Segments from *B. subtilis*—The 23 S rRNAs from many bacterial species including *E. coli* and *B. subtilis* have identical secondary structures where the nucleotides in the single-stranded region, especially the central loop of domain V, are invariant. The nucleotides can vary in the double-stranded regions from one bacterial species to another, but the conformations remain the same. Therefore, we took the cloned domain V of *B. subtilis* 23 S rDNA because it has convenient restriction sites that are lacking in the corresponding region of the *E. coli* 23 S rDNA. The 660-nt-long domain V RNA of *B. subtilis*, its 337-nt-long segment containing mainly the central loop of domain V, and another 425-nt-long segment from the 5' end were transcribed from plasmids pDK105 linearized with *Sma*I, pDK106 linearized with *Eco*RI, and pDK105 linearized with *Sma*I, respectively. All the transcriptions were done from SP6 promoter by SP6 RNA polymerase (Roche Molecular Biochemicals). The plasmids pDK105 and pDK106 were kind gifts from B. Weisblum, Madison, WI. The DNA templates were digested with RNase-free DNase I, and RNA was precipitated with ethanol after phenol extraction. The amount of RNA synthesized was estimated by adding a trace amount of [α -³²P]UTP with the ribonucleotides and measuring its incorporation in

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¹ The abbreviations used are: nt, nucleotide(s); FITC, fluorescein isothiocyanate.

RNA. We call the 337- and 425-nt-long RNA molecules RNA1 and RNA2, respectively (see Fig. 2).

Labeling Carbonic Anhydrase with Fluorescent Probe FITC—The enzyme (100 nM) was mixed with a 50-fold molar excess of FITC at pH 8.7 and kept in ice for 1 h. The labeled enzyme was separated from unincorporated FITC by Sephadex G-25 gel filtration column. The enzyme activity did not change due to FITC labeling, and it did not interfere with denaturation and refolding of the enzyme. Fluorescence emission from FITC-labeled protein was obtained by exciting at 495 nm and measuring emission at 520 nm using a Hitachi F3010 fluorescence spectrophotometer. Fluorescence labeling of the enzyme was necessary for its quantitation in experiments where the amount of enzyme was so small that we could not use its activity, A_{280} , or intrinsic fluorescence of tryptophan residues for quantitation.

Denaturation and Refolding of Enzymes—Carbonic anhydrase was denatured at a concentration of 10 μ M with 6 M guanidine-hydrochloride for 2 h at 25 °C. The protein lost its secondary structure as revealed by its CD spectrum. For refolding, the denatured protein was diluted 100-fold (final concentration: 100 nM) in a buffer containing 50 mM Tris-HCl (pH 7.6), 5 mM magnesium acetate, and 200 mM NaCl and incubated at 25 °C for 30 min with or without folding modulators. The activity of refolded enzyme was assayed by adding 500 μ M *para*-nitrophenyl acetate to the refolding mixture and measuring the increase in A_{400} with time when incubated at 25 °C. The concentrations of enzyme and ribosomal RNA, etc., varied in different experiments and are mentioned in appropriate places. Lactate dehydrogenase, a homotetrameric enzyme, was denatured at a concentration of 3.2 μ M with respect to monomer with 1 M guanidine hydrochloride at 20 °C for 1 h (9). 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 (9). The enzyme concentration was 32 nM with respect to monomer during refolding. Malate dehydrogenase, a homodimeric enzyme, was denatured at a concentration of 1.15 μ M with respect to monomer with 6 M guanidine hydrochloride 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. The enzyme concentration during refolding was 14 nM with respect to monomer. For all the enzymes, refolding was over by the time of incubation mentioned above. The extent of refolding was calculated by taking the ratio of the activity of the refolded enzyme to the activity of the same amount of native enzyme.

Gel Retardation Assay for RNA Bound to the Enzyme—Linearized plasmid pDK106 was transcribed in presence of [α - 32 P]UTP to prepare 32 P-labeled 337-nt-long RNA1, the DNA template was destroyed, and then the RNA was purified by phenol extraction and ethanol precipitation. Denatured carbonic anhydrase was added in refolding buffer containing this radiolabeled RNA1 at 25 °C. The concentrations of RNA1 and the enzyme were 25 and 50 nM, respectively. All RNA1 molecules would not be trapped by the enzyme at this concentration. The enzyme-RNA1 complex was divided in two equal parts. One part was loaded on a 5% native polyacrylamide gel, and the other part was treated with 1% SDS at 50 °C for 15 min and loaded on the same gel. The gel was run in TBE buffer and exposed to x-ray film for autoradiography.

Acrylamide Quenching Studies on Native, Spontaneously Folded, RNA1-bound and RNA1/RNA2-mediated Folded Enzyme—Steady state fluorescence was measured using a Hitachi F 3010 spectrofluorimeter. Quenching of fluorescence emission from tryptophan residues was obtained by recording the intensities (excitation: 290 nm; emission: 330 nm) after successive addition of small aliquots of quencher (acrylamide) stock solutions. A nominal bandpass of 5 nm for the excitation and 5 nm for the emission was used. The fraction of tryptophan residues that are accessible to the quencher molecule could be estimated from the equation $F_0/(F_0 - F) = 1/(K_{sv}f_e(Q) + 1/f_e)$ (12), where f_e is the fraction of total number of tryptophan residues in carbonic anhydrase accessible to the quencher, F_0 is the fluorescence intensity in absence of the quencher, F is the fluorescence intensity at the quencher concentration Q , and K_{sv} is Stern-Volmer quenching constant assuming purely dynamic quenching.

RESULTS

The Folding of Denatured Proteins by 50 S Ribosomal Particle and Domain V of Its 23 S rRNA—Fig. 1 shows the refolding of three denatured enzymes, carbonic anhydrase (monomer), malate dehydrogenase (homodimer), and lactate dehydrogenase (homotetramer) with 50 S ribosomal subunit, its 23 S rRNA, and the domain V of 23 S rRNA. The concen-

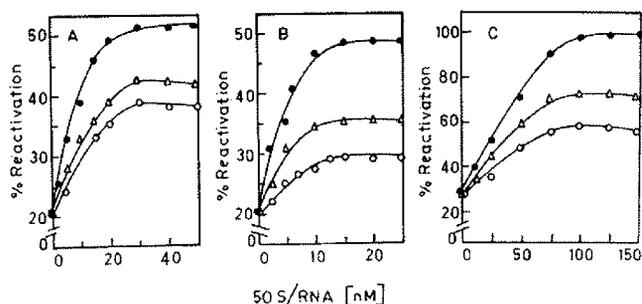


FIG. 1. Reactivation of denatured lactate dehydrogenase (A), malate dehydrogenase (B), and human carbonic anhydrase I (C) with 50 S ribosomal subunit (●), 23 S ribosomal RNA (Δ), and 660-nt domain V of 23 S rRNA (○).

tration of denatured enzymes were 100, 14, and 32 nM, respectively, with respect to monomer. Although all of them could refold the denatured enzyme, there was a gradual decrease in the extent of refolding as we went from 50 S subunit to the domain V of its 23 S rRNA. This protein folding activity was specific for domain V RNA. The 16 S rRNA, tRNA, and regions of 23 S rRNA other than domain V (e.g. the domain II of 23 S rRNA) did not show this activity (3, 4, 9). The activity could also be destroyed by RNase (3, 4). As mentioned under "Discussion," although the domain V provides the crucial site for refolding (9, 10) other regions of 23 S rRNA may also provide additional sites to interact with denatured proteins to increase the yield of refolded enzymes. However, the domain V appears to provide the minimum length of RNA that shows peptidyl transferase activity (13, 14) and protein folding activity, which is sensitive to antibiotics that prevent protein synthesis and folding *in vitro* and *in vivo* (1, 10). Therefore, we turned our attention to the domain V and went on to see if the active site(s) could be further narrowed down to any part of it.

The increased activity of the refolded enzymes was not due to some fortuitous effect of the ribosomes/the subunits and RNAs. The native enzymes did not show any increase in activity in presence of any of the modulators.

The reduced ability of domain V RNA to fold protein compared with 50 S particle could also be due to partial loss of its structure in absence of ribosomal proteins. The 23 S RNA has been shown to lose its peptidyl transferase activity due to deproteinization (13). It may also be necessary to vary reaction conditions to optimize the protein folding activity of domain V RNA.

It should also be mentioned here that ribosomes/ribosomal RNA refolds some intermediate(s) of spontaneous folding of the enzyme since the secondary and some tertiary structures are formed in the dead time of dilution of denaturant.

Two Fragments of the Domain V of 23 S rRNA Complementary to Fold Denatured Proteins—We took the 660-nucleotide-long domain V of *B. subtilis* 23 S rRNA for this purpose. As mentioned under "Experimental Procedures," it can be easily divided into two parts with some overlap. One of them is a 337-nucleotide-long *in vitro* transcript, obtained using *Eco*RI-cleaved pDK106 template, which possesses mainly the single stranded large circle of domain V but lacks the elaborate stem loop part, which is deleted in the cloned gene (kindly provided by Prof. B. Weisblum, University of Wisconsin, Madison, WI). We call this RNA1. The other is transcribed from plasmid pDK105 after cutting it with restriction endonuclease *Sma*I. This gives a 425-nt-long RNA having mainly the elaborate stem loop part toward the 5' end of domain V, but it lacks most of the large circle. We call this RNA2. The putative secondary structures of 660-, 337-, and 425-nt RNA are shown in Fig. 2 (15). Refolding of denatured carbonic anhydrase (100 nM), malate

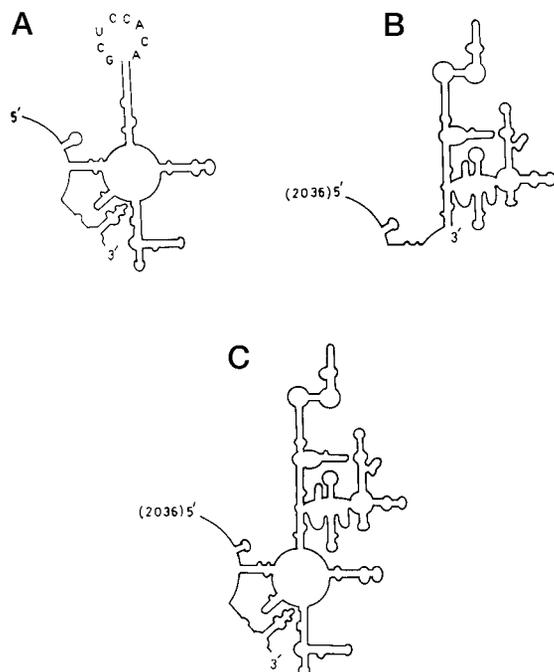


FIG. 2. Secondary structures of different segments of domain V of bacterial 23 S ribosomal RNA, 337-nt RNA1 (A), 425-nt RNA2 (B), and 660-nt total domain V region (C). The bases shown in the loop in A were introduced to join the stem by Kovalic *et al.* (15). They do not belong to the authentic domain V of 23 S rRNA.

dehydrogenase (14 nM), and lactate dehydrogenase (32 nM) with different concentration of the two RNA moieties mixed in 1:1 ratio are shown in Fig. 3. The extent of refolding was the same as that of refolding with 660-nt-long RNA (compare with Fig. 1). Therefore, these two RNA molecules complement each other in the refolding reaction. They either act independently on the unfolded proteins (see below) or form a composite RNA through tertiary interactions between themselves and then refold the denatured protein.

The Refolding Process Goes through Independent Steps—This and the subsequent experiments were carried out with carbonic anhydrase only because with a relatively small monomeric protein the results would be easier to interpret. To check whether these two RNA moieties reacted independently or not, we added one of them to denatured carbonic anhydrase, waited for 15 min, and then added the other. Following an additional incubation of 30 min, the enzyme activity was assayed. We found recovery of enzyme activity only when the order of addition was RNA1 followed by RNA2. We could wait for a sufficiently long time, even more than 1 h, after adding RNA1 and then add RNA2. The final recovery of activity was the same as the recovery with the two RNA molecules added together in unimolecular ratio. Fig. 4 shows that the enzyme activity recovered when RNA1 was added first, but did not do so when RNA2 was added first. The total concentration of RNA varied in different sets, but the ratio of the RNA moieties was always maintained as 1:1. As shown in Fig. 4, maximum recovery of enzyme activity was obtained when the ratio of enzyme (33 nM):RNA (unimolecular mixture of RNA1 and RNA2) was 1:1. There was slight inhibition in the recovery at higher RNA concentration (6, 9, 10). Fig. 5 shows the time course of recovery of enzyme activity in an experiment where RNA2 concentration varied. Here the RNA1 was added in equimolar ratio with the denatured enzyme (132 nM), and RNA2 was added after 15 min in different molar ratios of RNA1:RNA2, from 1:1 to 1:1/8. As is apparent from Fig. 5A, the RNA1-bound enzyme was not active. About 20% enzyme activity, which appears in presence of

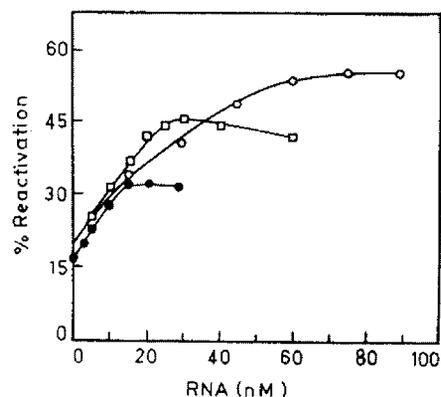


FIG. 3. Reactivation of denatured lactate dehydrogenase (□), malate dehydrogenase (●), and human carbonic anhydrase I (○) with different amounts of 1:1 mixture of RNA1 and RNA2.

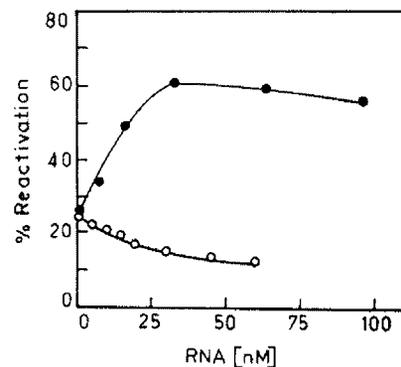


FIG. 4. Activity of denatured carbonic anhydrase after sequential incubation with equal concentration of RNA1 and RNA2. Recovery of enzyme activity was seen when RNA2 was added 15 min after addition of RNA1 (●), but there was no recovery of activity when RNA1 was added 15 min after addition of RNA2 (○). An additional incubation of 30 min was done before measuring enzyme activity after adding RNA2 and RNA1, respectively, in the above cases.

RNA1 only, was about the same as the recovery of activity without ribosomal assistance. This might be due to the enzyme molecules, which failed to bind to RNA1 but went straight to the true pathway of spontaneous folding. RNA-assisted recovery of activity was seen after RNA2 was added. The maximum recovery of activity was the same irrespective of the concentration of RNA2, the only difference being the rate of the recovery of enzyme activity, which was slower with lower concentration of RNA2. A quick gel filtration assay with the sample where RNA1:RNA2 ratio was 1:1 showed that the enzyme dissociated from RNA before its activity reached its peak. This showed that the dissociated folding intermediates could be protected by RNA and then released on the true folding pathway. All these time courses of recovery of enzyme activity with different concentrations of RNA2 could be plotted in a linear logarithmic plot, showing that they represented first order reaction (Fig. 5B). On the other hand, the first step of the refolding reaction (the binding of denatured enzyme to RNA1, which gave a stable RNA-bound folding intermediate) was obviously a first order reaction with respect to enzyme concentration, but it was too fast to be considered as the rate-limiting step in the overall refolding reaction. It should be mentioned here that we cannot vary the concentration of RNA1 below the level of enzyme concentration. In fact, we need it in 5-fold excess of denatured enzyme molecules to bind all of them.²

Release of the Folding Intermediate from RNA1 by Deter-

² S. Chowdhury, S. Pal, S. Chandra, D. Sarkar, A. N. Ghosh, and C. Das Gupta, unpublished observation.

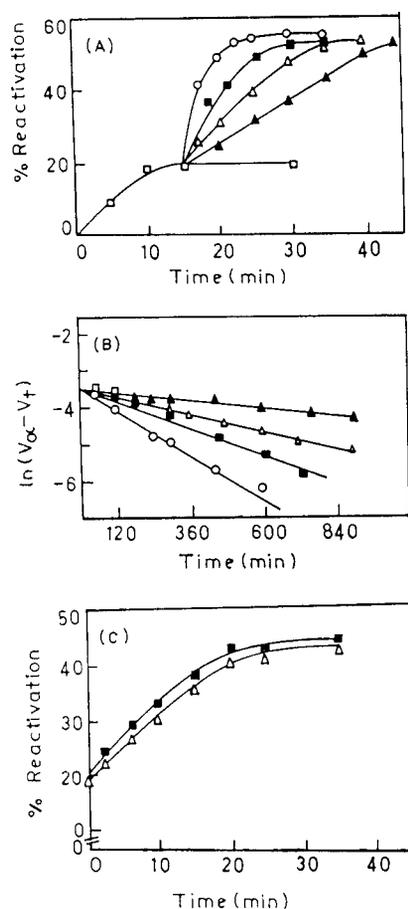


FIG. 5. A, recovery of activity of denatured carbonic anhydrase when RNA1 was added to it (\square), followed by RNA2 at mole to mole ratio of RNA1: RNA2 of 1:1 (\circ), 1:1/2 (\blacksquare), 1:1/4 (\triangle), and 1:1/8 (\blacktriangle). B, first order plot with the same symbols as in the time course of recovery shown in A. C, time course of recovery of activity of RNA1-bound denatured carbonic anhydrase when released by 0.2% Triton X-100 (\blacksquare) and 3% ethanol (\triangle).

gent/Ethanol—The RNA2 could be just competing with the protein folding intermediate to bind to RNA1 so that the latter was displaced, or RNA2 could play an active role in the process of folding the RNA1-bound denatured protein and then release it. To distinguish between these two possibilities, we added small quantities of non ionic detergent Triton X-100 (final concentration 0.2%) or ethanol (final concentration 3%) to the RNA1-bound carbonic anhydrase (100 nM). In both the cases, the enzyme dissociated from RNA1 and, like the RNA2-mediated released enzyme, folded slowly to active form although the recovery of activity was slightly less than the RNA2-mediated process, as shown in Fig. 5C. The small amount of ethanol and Triton X-100 had no effect on the activity of enzyme. Therefore, the RNA2 did not play any active role in folding denatured carbonic anhydrase. Its function was to release the protein so that it could fold to active form by itself. This puts the RNA1, which is basically the large central loop of the domain V of 23 S rRNA, at the center stage of protein folding. This 337-nt-long RNA could possibly trap the protein folding intermediates at a stage where misfolding due to non-native interactions of its different segments would lead to loss of enzyme activity. A simple reduction in the non-native interactions of different protein segments might ensure the formation of on-pathway folding intermediates. The experiments below show that the RNA1-bound denatured enzyme was a true intermediate in this process of folding.

Stable Association of the Denatured Enzyme with the

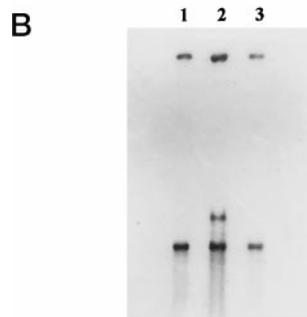
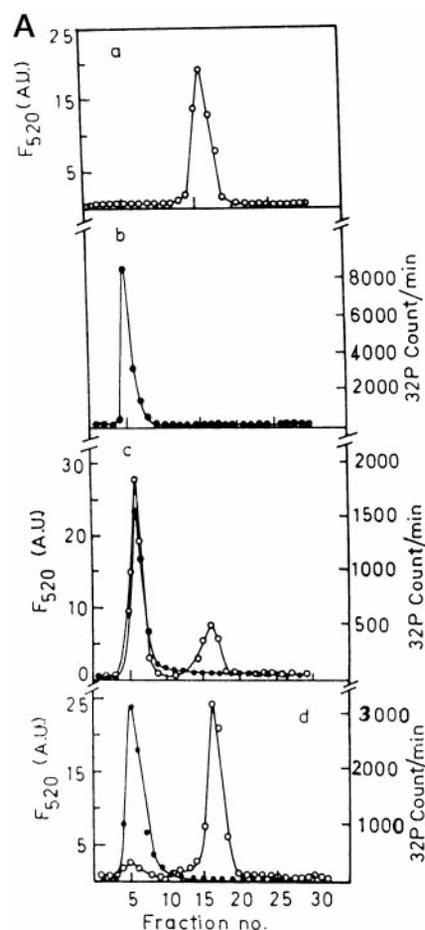


FIG. 6. A, Sephadex G-100 gel filtration profile of native carbonic anhydrase (a), ^{32}P -labeled RNA1 (b), ^{32}P -labeled RNA1-bound denatured carbonic anhydrase (c), and carbonic anhydrase released from ^{32}P -labeled RNA1 after addition of RNA2 (d). Carbonic anhydrase was labeled with FITC and quantitated from fluorescence emission intensity at 520 nm when excited at 495 nm. FITC labeling was necessary to quantitate very small amount of enzyme used in these experiment. B, gel retardation assay on 5% polyacrylamide gel of denatured carbonic anhydrase-bound RNA1. Lane 1, RNA1 only; lane 2, RNA1 bound to denatured enzyme; lane 3, sample of lane 2 treated with 1% SDS at 50 °C.

RNA1—During refolding, the denatured carbonic anhydrase remained bound as a stable intermediate with RNA1 and this RNA-enzyme complex could be recovered by gel filtration through Sephadex G-100 column. The RNA and protein were labeled with $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ and FITC, respectively. The FITC helped to quantitate the enzyme even at low concentration which was used in these experiments irrespective of whether it was in native or in denatured state. A part of the RNA-protein complex was loaded on the column. As shown in Fig. 6A, the complex eluted out in the void volume whereas a small fraction of unbound enzyme was retained in the column and eluted later

in the same fraction as the native enzyme (Fig. 6A, c). The RNA-bound enzyme in void volume showed no activity. The small amount of unbound enzyme could have gone through the process of spontaneous folding and its activity was too small to measure (spontaneous folding was 20% in such experiments). To the remaining RNA-bound enzyme, RNA2 was added at a ratio of protein:RNA as 1:1/8. After incubation for 1 h, the enzyme activity was assayed and the reaction mixture was loaded on the same column. As shown in the elution profile (Fig. 6A, d), the reactivated enzyme dissociated from the RNA1 and eluted at the same position as the native enzyme. Here, the total count in the RNA in void volume was equal to the sum of counts in RNA1 and RNA2. More than 80% of the refolded enzyme activity was found in the protein peak. Very little enzyme remained associated with RNA in the void volume. Therefore, the RNA1 keeps the folding intermediates of the enzyme tightly bound, which can be dissociated from it by RNA2 before or after refolding. If it is dissociated before refolding, the intermediate must fold spontaneously, *i.e.* it must be an on-pathway intermediate for spontaneous folding. In such a case the role of RNA1 and RNA2 could be rather passive, that of protecting the folding intermediates against the forces of misfolding. In any event, the RNA-bound protein gave a stable intermediate in this refolding pathway that should be thoroughly characterized.

Gel Retardation Assay on Denatured Carbonic Anhydrase-bound RNA1—We have also seen the stable binding of the refolding carbonic anhydrase to RNA1 in 5% polyacrylamide gel. As shown in Fig. 6B, the enzyme-bound RNA1 (50 nM enzyme:25 nM RNA1) migrated slower than the control unbound RNA1. When the enzyme was released by treatment with 1% SDS, RNA1 migrated to the same position as its control. RNA1 was added at half the enzyme concentration to ensure that both the enzyme-bound as well as free RNA1 could be detected in the gel. To bind all RNA1 molecules, the enzyme concentration should be about 5 times that of RNA1 (data not shown).

Recently, we have developed a filter binding assay where the RNA1 gets trapped on the filter only when it is bound by the refolding protein. Therefore, the binding to and the release of the protein from RNA1 can be quantitated. This would help to select mutants of RNA1, made by site-directed mutagenesis, which are deficient in binding/refolding proteins. We have preliminary observation with one such mutant, which is defective in binding/refolding denatured carbonic anhydrase.² We are thus in the process of identifying nucleotides in RNA1 that directly interact with the protein or ensure its binding to RNA1.

Fluorescence Studies on the Tertiary Organization of Native and Refolding Carbonic Anhydrase—Carbonic anhydrase has six tryptophan residues (16), which fluoresce strongly in the native enzyme when excited at 290 nm. We used this strong fluorescence emission, which peaks at around 330 nm, to probe the tertiary organization of the protein during denaturation and refolding. As shown in Fig. 7A, the tryptophan fluorescence quenched dramatically when the denatured protein was bound to RNA1. The intensity of emission increased quickly as soon as RNA2 was added. This was due to the release of the bound enzyme, and its time course is shown in *inset* of Fig. 7A. The released enzyme was not active but took some time (presumably for fine tuning of the active site) to show its activity (Fig. 7B). A quick spin column gel filtration assay after adding RNA2 but before the appearance of enzyme activity also showed that the enzyme dissociated from RNA1 (data not shown). Combining this with results of gel filtration experiments (shown in Fig. 6A), we thus see that most of the dena-

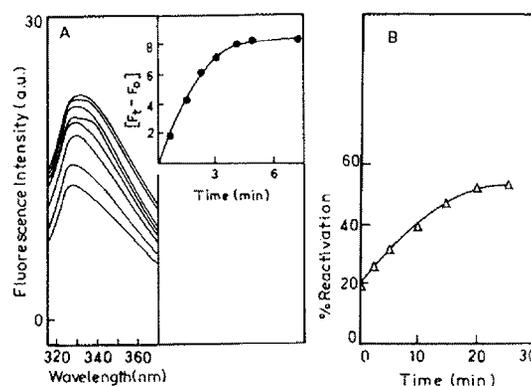


FIG. 7. Gradual increase in intensity of tryptophan fluorescence emission peak (A) and the activity of carbonic anhydrase (B) after addition of RNA2- to RNA1-bound denatured enzyme. The mole to mole ratio of RNA1: RNA2 was 1:1/4. The *inset* in A shows the increase in fluorescence emission with time from the initial value F_0 .

tured protein molecules bound to RNA1 were destined to go through on-pathway folding intermediates by themselves to be active once they were released by RNA2. The activity appeared after the release from RNA1 supported the studies, which suggested that bacterial proteins mostly fold posttranslationally (1, 17, 18). We then made a comparative study on the tertiary structures of the native, self-folded, RNA1-bound, and RNA-mediated folded enzyme, using the tryptophan accessibility of the quencher acrylamide in case of all the forms. As shown in Fig. 8, the tryptophan accessibility of the quencher was 100% for the native, self-folded, and RNA-mediated folded proteins. However, the accessibility was lower for the RNA1-bound denatured enzyme. Some of the tryptophans were thus inaccessible to the quencher, presumably because they were blocked by binding of the RNA1 with the folding intermediates. The difference in the slope of the Lehrer plot for native/self-folded and RNA-bound/released enzyme was due to the fact that greater amount of protein was taken in the first case. This was done to obtain higher fluorescence signal. In case of RNA-bound/released enzyme, we had to work with lower concentrations to avoid inner filter problems that could arise at high RNA concentration.

DISCUSSION

The studies on protein folding by modulators like *E. coli* ribosome, its 23 S rRNA, and the domain V of bacterial 23 S rRNA were all done with stoichiometric amount of these modulators with respect to protein concentration. This was due to the following compulsion. If all the denatured protein molecules were not sequestered by the folding modulators as soon as the denaturant was diluted out, the unbound protein molecules would go through spontaneous folding mode (both on and off pathways) and the yield of refolded protein would go down. Ideally, all the denatured protein molecules should have been sequestered by the modulator. We could achieve this sort of stable association of the refolding protein with RNA1 of domain V of 23 S rRNA. In fact, with a mole to mole ratio of 5:1 for RNA1: protein molecules, we could trap all the folding intermediates on RNA1 (data not shown). For ribosomal particle, 23 S rRNA, and 660-nt domain V RNA, this mole to mole ratio could not be increased much above 1:1 since that would reduce the extent of folding (6, 9, 10). This could be due to the RNA2 region competing with RNA1 for the denatured protein instead of allowing the RNA1 to bind the folding intermediate first. We think that since only one domain of 23 S rRNA molecule (the domain V) could interact with newly synthesized protein *in vivo*, the question of a stoichiometry lower or higher than 1

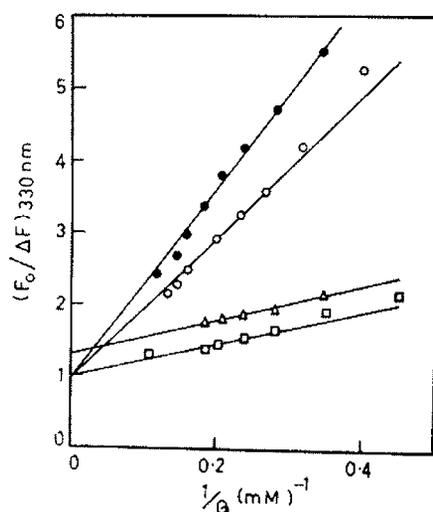


FIG. 8. Lehrer plot of acrylamide quenching of tryptophan fluorescence of carbonic anhydrase. Native enzyme (●), self-folded enzyme (○), RNA1-bound denatured enzyme (△), and refolded enzyme after release from RNA1 by RNA2 (□). The enzyme concentrations were higher in the first two cases and lower in the remaining two samples (see text).

does not arise. Therefore, the mechanism of protein folding by ribosome and ribosomal RNA should be considered strictly within the stoichiometric relationship and it would not make sense to use ribosome/ribosomal RNA in large excess or in catalytic amount; however, when we could break the reaction in two parts, the RNA2 could obviously be added in catalytic amount, and it did turn over to give higher yield of refolded protein with time.

Within a very short period after diluting out the denaturant, the protein reforms its secondary structure. We saw that a delay of 10–15 s in adding the modulator after diluting out the denaturant did not reduce reactivation of a number of proteins noticeably (data not shown). The secondary structures must have been completely formed within this time. Therefore, some spontaneously folding intermediates beyond the level of secondary structure formation should be the substrate for ribosome-assisted folding. This agrees with *in vivo* studies and studies on transcription-translation process in cell-free extract, where the polypeptides have been shown to take up secondary structure during synthesis on the ribosome (19–23). At this state, the polypeptide is trapped by RNA1. The binding is stronger at higher salt concentration and could be mainly due to hydrophobic nature of the interaction between denatured protein and RNA1. When released by RNA2, most of the folding intermediates by themselves turn into active protein and there is no more loss of activity due to off-pathway folding. This implies that the dissociated intermediates must be the on-pathway folding intermediates, which would all end up as active protein. This last, relatively slow step appears to be the rate-limiting step in this reaction. It is basically a first order reaction with respect to protein concentration. Therefore, the overall reaction is the result of the following independent events: (a) very fast binding of refolding intermediate to RNA1, (b) slow release of folding intermediates from RNA1 by RNA2, and (c) self-folding of the released on-pathway folding intermediates. This third step is the slowest one. The situation corresponds to posttranslational protein folding *in vivo*, believed to be mainly true in bacteria (17, 18). We have also observed such slow posttranslational formation of active β -galactosidase in *E. coli* several minutes after the stoppage of synthesis of the protein by translation inhibiting antibiotics (1). It should be emphasized, however, that this posttranslational folding could

be “guided” by the translational machinery, like ribosomal RNA (1, 3, 4, 6, 9, 10) and protein like EF_{Tu}. (24). Several studies on conformation of nascent proteins on ribosomes and the contribution of ribosome in their folding process point to similar possibilities (17, 20, 21, 23, 25–30). A number of studies were done in the laboratory of Brimacombe (31), where aminoacylated initiator tRNA and peptidyl tRNA of different lengths obtained by coupled transcription-translation of N-terminal part of different lengths of *E. coli* ompA protein, bacteriophage T4 gene 60 protein, and tetracycline resistance gene product were photo-cross-linked to the ribosome. After deproteinization, the amino acid and short polypeptides were found to be cross-linked with many bases in the large loop of domain V and with few other bases outside the domain V of 23 S rRNA. Thus, the association of nascent polypeptide mainly with domain V of 23 S rRNA is well established. The role of RNA2 in the process of protein folding is in releasing the refolding intermediates and this passive role can be mimicked by 0.2% Triton X-100 or 3% ethanol, which reduce the solvent polarity and weaken hydrophobic interaction.

Thus, the 337-nt-long domain V region of 23 S rRNA directs protein folding intermediates to take up the active tertiary structure by themselves. This region of ribosomal RNA turns out to be the most conserved one in terms of the base sequences and secondary structure in course of evolution from unicellular to multicellular organisms. This could be the primordial RNA, which helped spontaneously synthesized polypeptides to fold and be selected for biological activities in course of even pre-cellular evolution. Even the large rRNAs in mitochondria from various sources that are considerably smaller than their bacterial and eukaryotic counterpart possess this stretch of domain V, while other region of domain V (for example the RNA2 part) appears to be missing in them. Thus, this 337-nt RNA might represent the basic molecular fossil (32) that has been carrying out the process of trapping protein folding intermediates to shunt more newly synthesized protein molecules to the activation pathway. While the RNA1 and a small part of RNA2 are present in the mitochondrial ribosome, both the RNA1 and the RNA2 stretches are present in 23 S rRNAs, whereas even larger stretches of nucleotides are present in more evolved 26 and 28 S rRNAs.

An extension of this study with mitochondrial, bacterial, and eukaryotic large ribosomal RNA segments could throw more light on the interaction of these RNAs with proteins and the evolution of the protein folding activity.

As mentioned in the text, we are now collecting mutants in RNA1 by site-directed mutagenesis so that the nucleotides that directly interact with the proteins or ensure their binding to RNA1 can be identified to understand the role of domain V RNA in ribosome-assisted protein folding.

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Complementary Role of Two Fragments of Domain V of 23 S Ribosomal RNA in Protein Folding

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The role of the NH₂- and COOH-terminal domains of the inhibitory region of troponin I in the regulation of skeletal muscle contraction.

Danuta Szczesna, Ren Zhang, Jiaju Zhao, Michelle Jones, and James D. Potter

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Page 32776, Ref. 1: One of the authors was omitted. The correct reference is:

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