CHAPTER - III

Complementary Role of Two Fragments of 23S rRNA in Protein Folding
3.1. INTRODUCTION:

We have identified the ribosome as a general protein folding modulator on the basis of its ability to successfully fold all the unfolded proteins which we tried so far (e.g., lactate dehydrogenase, glucose 6-phosphate dehydrogenase, horse radish peroxidase, restriction endonucleases, alkaline phosphatase, malate dehydrogenase, β-lactamase, carbonic anhydrase, β galactosidase etc.) (Chattopadhyay et al., 1999; Das and DasGupta, 1992; Bera et al., 1994; Das et al., 1996; Chattopadhyay et al., 1994; Bera et al., 1994). This in vitro protein folding activity has been found to reside in the domain V of the 23S rRNA in 50S particle of the ribosome. This activity of ribosome has also been identified in vivo by showing slow post translational activation of the enzyme β-galactosidase in E.coli that was synthesized just prior to the addition of the 30S specific protein synthesis inhibitors kasugamycin & streptomycin. This post translational activation, however, was immediately arrested by adding antibiotics like chloramphenicol and lincomycin which bind to domain V of 23S rRNA of 50S ribosomal subunit (Chattopadhyay et al., 1999). The important question then is whether biological entities like molecular chaperons (Hartl and Martin, 1992; Gething et al., 1995) and ribosomes fold proteins to their active states following basically similar pathway as spontaneous folding or will there be a paradigm shift in our understanding of protein folding in the cell when we know how ribosome (Das and DasGupta, 1992; Bera et al., 1994; Das et al., 1996; Chattopadhyay et al., 1994; Bera et al., 1994, Chattopadhyay et al., 1996; Pal 1997; Kudlicki et al., 1997), which synthesizes the polypeptide also folds it to active form.

If we observe the protein folding activity of different ribosomal particles as well as that of 23S rRNA and its domain V region, we find that at the end of the folding reaction they
dissociate completely from the folded proteins and no co-factor is required for this process. This implies that there are at least two steps in this reactions (a) interaction with unfolded proteins to fold them and (b) dissociation from the folded proteins. To understand the mechanism of this ribosome mediated folding process we started with the simplest folding modulator, the domain V region of 23S rRNA. We looked at the secondary structure of the 660 nt long domain V region of *B. subtilis* 23S rRNA (fig. 2.5.B). Since the central loop is strongly implicated in protein synthesis we thought that we could divide it into two structural parts (1) the central loop region and (2) the bushy stem-loop region and see if they complement in the protein folding reaction. The central loop region is most conserved throughout the process of evolution from mitochondria, the chloroplast, the smallest prokaryotic cells to higher eukaryotes (Douthwate, 1992) where as the structure of the stem loop region varies from prokaryotes to eukaryotes, among different species of the eukaryotes and also in mitochondrial large ribosomal RNA. We took 660 nt long domain V of *B. subtilis* 23S rRNA and split it into the central loop region (337 nt long) and the 425 nt long bushy stem loop part (fig. 3.1). They had a small overlap. We called these two fragments RNA1 and RNA2.

We observed the protein folding activity of these two RNA fragments. For this purpose we used human carbonic anhydrase I and lactate dehydrogenase as test enzymes. We found that RNA1 and RNA2 complemented in the protein folding reaction which went through the following steps. (1) Binding of refolding enzyme with RNA1. This RNA1 bound enzyme was not active. (2) Release of the enzyme from RNA1 by RNA2 in a folding competent state and (3) slow attainment of active form by this folding competent protein, free from both RNA1 and RNA2.
3.2. EXPERIMENTAL PROCEDURES:

3.2.1. Enzymes and reagents:
Human carbonic anhydrase I (EC 4.2.1.1), pig muscle lactate dehydrogenase (EC 1.1.1.27) were purchased from SIGMA (USA). Fluorescent probe FITC (fluorescein 5-isothiocyanate) were purchased from Molecular Probes, Inc. (USA). Other reagents were of Analytical Grade.

3.2.2. Buffers:
Buffers used for the dilution and denaturation of the enzymes were same as discussed in section 2.2.IV. For RNA1 and RNA2 mediated protein folding experiment, the refolding buffer used was similar to that used in case of intact domain V mediated folding (section 2.2.IV).

3.2.3. Preparation of segments of domain V of 23SrRNA from B. subtilis:
The 23S 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. So, we took the cloned domain V of *B. subtilis* 23S rDNA because it has convenient restriction sites which are lacking in the corresponding region of the *E. coli* 23S rDNA. RNA1, containing mainly the central loop of domain V, and RNA2 were transcribed from
plasmids pDK 106 linearized with EcoR1, and pDK105 linearized with SmaI respectively. Transcription produced one 377nt long fragment (RNA1), containing 337 nt long central loop region of B. subtilis domain V and one 425 nt long RNA2 (fig. 3.2). All the transcriptions were done from SP6 promoter by SP6 RNA polymerase (Roche Molecular Biochemicals). The plasmids pDK105 and pDK 106 (constructed by deleting part of domain V DNA outside the central loop) were kind gifts from B. Weisblum, Madison, USA. The DNA templates were digested with RNase free DNase 1 and RNA was precipitated with ethanol after phenol extraction. The amount of RNA synthesized was estimated by adding trace amount of α³²-P UTP with the ribonucleotides and measuring its incorporation in RNA.

3.2.IV. Labelling Carbonic anhydrase with fluorescent probe FITC (Fluorescein-5-isothiocyanate):

100 mM of the enzyme was mixed with 50 fold molar excess of FITC (M.W. 389.38) at pH 8.7 and kept in ice for one hour. The labelled enzyme was separated from unincorporated FITC by Sephadex G-25 gel filtration column. The enzyme activity did not change due to FITC labelling and it did not interfere with denaturation and refolding of the enzyme. Fluorescence emission from FITC labelled protein was obtained by exciting at 495nm and measuring emission at 520nm using a Hitachi F 3010 fluorescence spectrophotometer (fig. 3.3). Fluorescence labelling of the enzyme was necessary for quantitation in experiments where the amount of enzyme was so small that we could not use A₂₈₀ or intrinsic fluorescence of tryptophan residues for this purpose.
3.2.V. Unfolding and refolding of enzymes:

Carbonic anhydrase was unfolded at a concentration of 10μM with 6 M Gdn-Cl for two hours at 25°C. For refolding, the unfolded protein was diluted 100 fold (final concentration 100nM) in a buffer containing 50 mM Tris-HCl (pH -7.6), 5mM Mg -Acetate and 200 mM NaCl and incubated at 25°C for 30 mins. with or without folding modulators. The activity of refolded enzyme was assayed by adding 500μM para nitrophenyl acetate (pNPA) 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 μM with respect to monomer with 1 M guanidine- hydrochloride at 20°C for 1 hour. For refolding, the enzyme was diluted 100 fold in 20mM Tris-HCl, pH 7.5, 200 mM NaCl and 4mM Mg-acetate and incubated at 20°C for 30 mins with or without RNA (Chattopadhyay et al., 1996). The enzyme concentration was 32 nM with respect to monomer during refolding. 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.

3.2.VI. Gel retardation assay for RNA bound to the enzyme:

Linearized plasmid pDK 106 was transcribed in presence of $\alpha^{32}$P UTP to prepare $^{32}$P labelled 337 nt long RNA1, the DNA template was destroyed, and then the RNA was
purified by phenol extraction and ethanol precipitation. Unfolded carbonic anhydrase was added in refolding buffer containing this radiolabelled RNA1 at 25°C. The concentrations of RNA1 and the enzyme were 25 nM 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 minutes and loaded on the same gel. The gel was run in TBE buffer and exposed to X-ray film for autoradiography.

3.2. VII. Filter binding assay:

Nitrocellulose filter binding assay was used to observe the interaction of refolding protein with RNA1 and RNA 2. A constant concentration of RNA (30 nM) was incubated with various concentration of unfolded carbonic anhydrase in buffer containing 50 mM Tris-HCl (pH-7.6), 5mM Mg Acetate and 200 mM NaCl. ³²-P labelled RNA1 and RNA 2 were prepared from linearised pDK 106 and pDK 105. Denatured carbonic anhydrase at different concentration was added in the above buffer containing the radiolabelled RNA1 or RNA2. After incubation at 25°C for 15 minutes samples were filtered through pre-soaked nitrocellulose filters (0.45 μm, Sartorius). The filters were dried and the retained count was measured by liquid scintillation counter (Beckman LS 6000 SE). Release of RNA1 bound carbonic anhydrase by RNA2 and ethanol was also monitored by filter binding assay. Unlabelled RNA2 was added to radiolabelled RNA1-protein complex and samples were filtered at different time interval and the amount of retained radiolabelled RNA1 on the filter membrane was measured as earlier.
3.2. VIII. 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 (Ex: 290nm; Em: 330nm) after successive addition of small aliquots of quencher (Acrylamide) stock solutions. A nominal bandpass of 5nm for the excitation and 5nm for the emission was used. The fraction of tryptophan residues that are accessible to the quencher molecule could be estimated from the equation

$$\frac{F_0}{F_0-F} = \frac{1}{K_{sv} f_e(Q)} + \frac{1}{f_e}$$

(Lehrer, 1971) 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.

3.3. RESULTS:

3.3.I. Two fragments of the domain V of 23SrRNA complement to fold unfolded proteins:

We took 660 nucleotide long domain V of \(B.\text{subtilis}\) 23S rRNA for this purpose. As mentioned in materials and methods, it can be easily divided into two parts with some
overlap. One of them is a 377 nucleotide long in vitro transcript, obtained using EcoRI cleaved pDK 106 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, Md, USA). We call this RNA1. The other RNA is transcribed from plasmid pDK 105 after cutting it with restriction endonuclease Smal. This gives a 425 nt long RNA having mainly the elaborate stem loop part towards the 5' end of domain V, but it lacks most of the large circle. We call this RNA2. The putative secondary structures of 660 nt, 337 nt and 425 nt RNA are shown in fig. 3.1 (Kovalic et al., 1995). Refolding of unfolded carbonic anhydrase (100 nM), and lactate dehydrogenase (32 nM) with different concentration of the two RNA moieties mixed in 1:1 ratio are shown in fig. 3.4. The extent of refolding was the same as that of refolding with 660 nt long RNA (compare with fig. 2.16). 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 unfolded protein.

3.3.11. 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 unfolded carbonic anhydrase, waited for 15 minutes and then added the other. Following further incubation of 30 minutes, 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 sufficiently long time, even more than one hour, 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. 3.5 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 the figure, 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 (Das et al., 1996; Chattopadhyay et al., 1996; Pal, et al., 1997). Fig.3.6 shows the time course of recovery of enzyme activity in an experiment where RNA2 concentration was varied. Here the RNA1 was added in equimolar ratio with the unfolded enzyme (132 nM) and RNA2 was added after 15 minutes in different molar ratios of RNA1 : RNA2, from 1:1 to 1:1/8. After a subsequent incubation of 30 minute, the enzyme activity was measured. As is apparent from fig. 3.6A, the RNA1 bound enzyme was not active. About 20% enzyme activity which appeared in presence of 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 on 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 as an "on pathway" folding intermediate. RNA2 therefore seems to have acted like an enzyme, recycling after the refolding reaction a number of times when present in sub-stoichiometric concentration. 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. 3.6B). On the other hand, the first step of the refolding reaction - the binding of refolding 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 more than five fold excess of unfolded enzyme molecules to bind all of them (chapter IV).

3.3.III. Release of the folding intermediate from RNA1 by detergent/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-X100 (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. 3.7. 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 unfolded 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 23S 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 refolding enzyme was a true intermediate in this process of folding.

3.3.IV. Stable association of the folding intermediate with the RNA1:
During refolding, the refolding 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 labelled with α³²P 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. 3.8a, 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. 3.8a-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 RNA1 bound enzyme, RNA2 was added at
a ratio of protein : RNA as 1:1/8. After incubation for an hour, the enzyme activity was assayed and the reaction mixture was loaded on the same column. As shown in the elution profile (fig. 3.8 a-d), the reactivated enzyme was not associated with 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, that is, 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.

3.3.V. Gel retardation assay on refolding carbonic anhydrase bound RNA 1:

We have also seen the stable binding of the refolding carbonic anhydrase to RNA1 in 5% polyacrylamide gel. As shown in fig 3.9 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 five times that of RNA1 (see chapter-IV).
3.3.VI. Filter binding assay on refolding carbonic anhydrase bound RNA 1 and RNA 2:

The stable binding of RNA 1 with refolding carbonic anhydrase and release of the protein by RNA 2 could be observed by the nitrocellulose filter binding assay, where the RNA 1 gets trapped on the filter only when it is bound by the refolding protein. This assay is very helpful to quantitate the extent the RNA-protein complex. It was found that with increasing concentration of protein the extent of RNA-protein complex retained on the nitro-cellulose filter increased and reached to saturation level when protein concentration is 6 fold higher than the RNA concentration. At this concentration nearly 40% of the total RNA was retained on the filter as RNA-protein complex (fig. 3.10A). There could be many reasons for a maximum retention of about 40% RNA 1. For example, unlike its structural homogeneity in the intact ribosome, the RNA 1 molecules in test tube could be a dynamic population of structural ensembles and not all of them are capable of folding proteins. Similarly, not all refolding intermediates of protein molecule could engage in productive binding with RNA 1. We are not in a position to make any concrete statement in this regard until many more experiment are done. A similar filter binding experiment with RNA 2 showed only 6% retention. When RNA 2, at equimolar concentration to RNA 1, and ethanol, at 3% final concentration, were added to the radiolabelled RNA 1-carbonic anhydrase complex, the refolding carbonic anhydrase released from RNA 1 within 30 seconds (fig. 3.10B).
3.3.VII. **Fluorescence studies on the tertiary organization of native and refolding carbonic anhydrase:**

Carbonic anhydrase has six tryptophan residues (Barlow, 1987) 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. 3.11A, the tryptophan fluorescence quenched dramatically when the unfolded 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. 3.11A. The released enzyme was not active but took some time—presumably for fine tuning of the active site—to show its activity (fig. 3.11B). 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. 3.8A) we thus see that most of the refolding 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 post translationally (Chattopadhyay et al., 1999; Netzer and Hartl, 1997; Netzer and Hartl, 1996). 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. 3.12, the tryptophan accessibility of the quencher was 100% for the native, self folded and RNA
mediated folded proteins. But the accessibility was lower for the RNA1 bound unfolded 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 get higher fluorescence signal. In case of RNA bound/released enzyme, we had to work with lower concentrations to avoid inner filter problems which could arise at high RNA concentration.

3.4. DISCUSSION:

The studies on protein folding by modulators like E.coli ribosome, its 23S rRNA and the domain V of bacterial 23S rRNA were all done with stoichiometric amount of these modulators with respect to protein concentration. The reason was discussed earlier in section 2.4. Ideally, all the unfolded 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 23S rRNA. In fact, with a mole to mole ratio of 5:1 for RNA1 : protein molecules, we could trap nearly all the folding intermediates on RNA1(see Fig. 4.3). For ribosomal particle, 23S 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 (Das et al., 1996; Chattopadhyay et al., 1996; Pal et al., 1997). This could be due to the RNA2 region, competing with RNA1 for the refolding protein instead of allowing the RNA1 to bind the folding intermediate first. We think that since only one domain of 23S rRNA molecule (the domainV) could interact with newly synthesized protein in vivo,
question of a stoichiometry lower or higher than one does not arise. Therefore, the mechanism of protein folding by ribosome and ribosomal RNA should be considered strictly within the stoichiometric relationship and would not make sense to use ribosome / ribosomal RNA in large excess or in catalytic amount, but when we could break the reaction in two parts, the RNA2 could obviously be added in catalytic amount and it did turnover 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 seconds in adding the modulator after diluting out the denaturant did not reduce reactivation of a number of proteins noticeably (fig 2.11A inset). 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 (Federov et al., 1992; Kudlicki et al., 1994a; Kudlicki 1994b; Federov and Baldwin, 1997; Komar et al., 1997). 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 refolding 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) slow self folding of the released on pathway 
folding intermediates. This third step is the slowest one. The situation corresponds to post 
translational protein folding in vivo, believed to be mainly true in bacteria (Netzer and 
Hartl, 1997; Netzer and Hartl, 1996). We have also observed such slow post translational 
formation of active β-galactosidase in E. coli several minutes after the stoppage of 
synthesis of the protein by translation inhibiting antibiotics (Chattopadhyay et al., 1999). It 
should be emphasized, however, that this post translational folding could be “guided” by 
the translational machinery, like ribosomal RNA and protein like EFTu (Kudlicki et al., 
1997). Several studies on conformation of nascent proteins on ribosomes and the 
contribution of ribosome in their folding process point to similar possibilities (Netzer and 
Hartl, 1997; Kudlicki et al., 1994a; Kudlicki et al., 1994b; Kudlicki et al., 1997a; Kudlicki 
et al., 1997b; Hendrik et al., 1993; Hansen et al., 1994; Hartl, 1994; Hardesty et al., 1995; 
Federov and Baldwin, 1995; Ellis and Hartl 1996). A number of studies were done in the 
laboratory of Brimacombe (Choi and Brimacombe, 1998) 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 small length 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 23S rRNA. Thus the association of nascent polypeptide mainly 
with domain V of 23S 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-X100 or 3% ethanol which reduce the solvent polarity and weaken hydrophobic interaction.

Thus the 337 nt long domain V region of 23S 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 which 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. So this 337 nt RNA might represent the basic molecular fossil (Maizels and Weiner, 1993) which has been carrying out the process of trapping protein folding intermediates to shunt more newly synthesized protein molecules to the activation pathway. While the length of the domain V RNA increases steadily from the smallest mitochondria to the largest eukaryotic ribosomes, the RNA1 region remains remarkably constant in them.

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.

One point must be raised here to account for a maximum retention of about 40% RNA1 when loaded with several fold excess of unfolded carbonic anhydrase, as it appears from all the experiments reported here, the RNA1 bound enzyme is folding intermediate
with most of its secondary and a significant extent of tertiary folds already formed. *In vitro*, this step is spontaneous and not guided by the ribosome. So only a small fraction of the total number of protein molecules could be competent to bind. *In vitro* the process of folding of the newly synthesized enzyme initiates on the ribosome and it could be an efficiently guided process.
Fig. 3.1. Secondary structures of different segments of domain V of *B. subtilis* 23S ribosomal RNA, (A) RNA1, (B) RNA2 and (C) total domain V region. The bases shown in the loop in (A) were introduced to join the stem by Kovalic et al. (1996).
Fig. 3.2. Autoradiogram of the 5% polyacrylamide, 8M urea gel, showing the 377 nt long RNA1 (lane 1) and 425 nt long RNA2 (lane 2). The century marker RNA fragments are shown in lane M.
Fig. 3.3. Fluorescence spectrum of different fractions of Sephadex G-25 gel filtration profile of FITC-labelled carbonic anhydrase. Excitation wavelength was 485 nm and both the excitation and emission band passes were 5 nm. Inset: The sephadex G-25 gel filtration profile.
Fig. 3.4. Reactivation of denatured lactate dehydrogenase (▪▪▪▪) and human carbonic anhydrase I (○○○○) with different amounts of 1:1 molar mixture of RNA1 and RNA2.
Fig. 3.5. 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 minutes after addition of RNA1 (-•••-), but there was no recovery of activity when RNA1 was added 15 minutes after addition of RNA2 (-○○○-). A further incubation of 30 minutes was done before measuring enzyme activity after adding RNA2 and RNA1 respectively in the above cases.
Fig. 3.6.(A) Recovery of activity of denatured carbonic anhydrase when RNA1 was added to it (□) followed by RNA2 at mole to mole ratio of RNA1:RNA2 of 1:1 (-O-), 1:1/2 (-■-■-), 1:1/4 (-Δ-Δ-) and 1:1/8 (-▲-▲-). Enzyme activity was measured after 30 minute incubation following addition of RNA2. (B) First order plot with the same symbols as in the time course of recovery shown in (A).
Fig. 3-7. Time course of recovery of activity of RNA1 bound refolding carbonic anhydrase when released by 0.2% Triton X-100(-■■-) and 3% ethanol (-Δ-Δ-).
Fig. 3.8. Sephadex G-100 gel filtration profile of (a) native carbonic anhydrase, (b) $^{32}$P labelled RNA1, (c) $^{32}$P labelled RNA1 bound refolding carbonic anhydrase and (d) carbonic anhydrase released from $^{32}$P labelled RNA1 after addition of RNA2. Carbonic anhydrase was labelled with fluorescent probe FITC and quantitated from fluorescence emission intensity at 520 nm when excited at 495 nm. FITC labelling was necessary to quantitate very small amount of enzyme used in these experiments. Open and filled circles
Fig. 3.9. Gel retardation assay on 5% polyacrylamide gel of refolding carbonic anhydrase bound RNA1. Lane 1: RNA1 only; lane 2: RNA1 bound to refolding enzyme and lane 3: sample of lane 2 treated with 1% SDS at 50°C.
Fig. 3.10. A. Nitro cellulose filter binding assay of refolding carbonic anhydrase bound RNA1 (−●−●−) and RNA2 (−■−■−). A fixed concentration of α\textsuperscript{32}P labelled RNA (30 nM) was incubated with different concentration of denatured carbonic anhydrase at 25°C for 15 minute in refolding buffer and filtered through nitrocellulose membrane (0.45μm).
Fig. 3.10.B. Time course of release of carbonic anhydrase from the RNA1-protein complex after adding (a) RNA2 at a mole to mole ratio of RNA1:RNA2 of 1:1 (-0-0-) and (b) 3% ethanol (-•-•-). Extent of RNA1-protein complex was measured by nitrocellulose (0.45μm) filter binding assay using α^32-P labelled RNA1.
Fig. 3.11. Gradual increase in (A) intensity of tryptophan fluorescence emission peak and (B) the activity of carbonic anhydrase after addition of RNA2 to RNA1 bound refolding 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 $F_0$. 
Fig. 3.12. Lehrer plot of acrylamide quenching of tryptophan fluorescence of carbonic anhydrase. Native enzyme (--●--●--), self folded enzyme (--0--0--), RNA1 bound refolding enzyme (--Δ--Δ--) 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).