Chapter 2

Comparison of nascent & chemically unfolded protein in the ribosomal environment
Introduction:

In the pioneering in vitro refolding studies, Christian B. Anfinsen (1973) denatured native protein by guanidine hydrochloride and then diluted out the denaturant to follow the formation of native structure of protein. He concluded that the information for correct folding of a protein lie in its linear amino acid sequence. Later on, chaperones were identified as a class of proteins (Ellis, 1987) that can reduce formation of protein aggregation by utilizing extra energy and thereby assist in production of functional proteins. When in vitro folding of protein is compared with the in vivo situation, the fundamental difference appears. In vitro, we observe folding of a synthesized protein whose functional native form has been disrupted chemically. But in vivo, the protein is synthesizing into its biologically active form within the cell. Theoretical considerations by Levinthal (Levinthal, 1968) dictated that the pathway to achieve the native form of a protein cannot be random, especially in the physiological time scale. However, diversity of amino acid sequences of proteins rendered it challenging to find a common mechanism (or code) of protein folding; neither chaperone assisted pathway could provide a proper answer to it. At this point, the finding that a large number of chemically unfolded proteins of diverse characteristics can be folded by specific interactions with the in vitro transcribed PTC RNA sequence (Samanta et al, 2008; Das et al, 2010)) helps us to imagine how randomness in the folding process can be drastically reduced. How far this in vitro interaction can be physiologically relevant – is the topic of discussion of this chapter.

In a number of previous studies (Nakatogawa et al, 2002; Seidelt et al, 2009) ribosome bound nascent proteins were isolated by using specific stalling sequences in the nascent chain. We used snap freezing followed by UV crosslinking to block the ongoing in vivo processes in the log phase growing bacterial cells; then isolated a tagged protein bound population of ribosome as the ribosome nascent chain complex (Basu et al, 2008a). Interestingly we isolated a large population of 50S ribosome to be associated with the full length nascent protein along with the relatively small 70S population (Basu et al, 2008a). This result perhaps indicated that the release of nascent protein through the 50S into the
cytosol is a slower process than the translation of a nascent protein and its termination. We further studied activity gain of synthesized full length protein to validate the above observation in presence and absence of ribosome associated chaperones like trigger factor, DnaK etc. Using western blot, toe printing and MALDI TOF MS/MS technique we analyzed interaction pattern of nascent protein with the PTC linked region of the rRNA. Results demonstrated that the nascent protein interacts with the PTC RNA region identically as the chemically unfolded analogue of the same native protein does in vitro with the transcribed PTC RNA sequence.
Materials & Methods:

Chemicals & strains:

Radioisotopes used were from the Board of Radiation Isotope (Govt. of India). ThermoScript RT kit was purchased from Invitrogen, USA. Other required chemicals were purchased from Sigma-Aldrich.

Association of nascent protein with the large ribosomal RNA:

The procedure to isolate the ribosome - nascent protein complex from the log phase growing bacterial cell has been described previously (Basu et al., 2008a). We selected a monomeric bacterial protein HspH as our test protein. Gene of C terminally His-tagged HspH from Bradyrhizobium japonicum was cloned in pET24b (Kan') vector. A single colony of a transformant resistant to Kan was grown over night and the culture was diluted in 500 ml LB (tryptone-1%, NaCl-1% and yeast extract-0.5%) at 37°C. At OD_{600} of about 0.4, IPTG was added to it to a final concentration of 0.4 mM to induce the synthesis of HspH protein. After 1 hour of induction at 37°C, protein synthesis in these cells was abruptly stopped by quick chilling. Cells were immediately UV-irradiated, harvested and subjected to French press, and the crude ribosomal extract was obtained by low and high-speed centrifugations. One half of the crude ribosome extract was subjected to affinity chromatography through a Ni-NTA column (Qiagen, USA) in which the full-length HspH protein is expected to bind by virtue of the six-histidine residues at the C-terminus. After applying the sample, the column was thoroughly washed with 10-60mM imidazole. The flow through and wash was collected. The bound protein was eluted with 250-350mM imidazole. Both the eluted fractions and the flow through with wash were centrifuged at 230,000g for 2 hours at 4°C to have ribosome and its subunits in the pellet, free or cross-linked to HspH. They were dispersed in suitable buffers and run separately in a 5%-20% sucrose density gradient with 30% sucrose cushion at the bottom. The crude...
ribosome extract and purified *E. coli* ribosomes (as control) were also subjected to the same density gradient centrifugation. Fractions were collected and $A_{260}$ was measured.

**Isolation of ribosomal RNA-nascent protein complex:**

In order to separate ribosomal proteins from the ribosomal RNA, the ribosomal particles (both UV crosslinked and non-crosslinked) were treated with 3M urea in 50 mM tris pH 7.5, 10 mM magnesium chloride and 100 mM sodium chloride in ice for 3 hours, followed by gel filtration in the same buffer using BioGel P-100 (BIORAD) column. rRNA was obtained in the excluded volume. RT-PCR was done on the purified rRNA using the primers specific for PTC linked region of the rRNA: 5'N (5'GAGAAAGAGAAGCTTGTACCCGCGGAAGA3') and BG32 (5'CCGAATTCCGGATCCGCGCCCACGGCAGATCTG3'). The plasmid, where the same PTC RNA region was cloned, was also used to amplify the region using the above two primers, and the product served as the length standard. The identity of lengths of the two PCR products confirmed that this stretch of RNA was free from ribosomal proteins (even in the case of UV-crosslinked 70S), which would otherwise block the PCR reaction.

**Western Blot to confirm presence of nascent protein in the ribosomal RNA:**

The rRNA-HspH complex after treated with RnaseA was run through a 10% SDS-PAGE along with purified native HspH protein; transferred onto a Hybond P+ membrane (Amersham Biosciences) with the aid of Mini Trans-Blot system (Bio-Rad). The membranes were incubated with anti- Penta-His antibody (QIAGEN, USA) and detected with Western Blotting Luminol Reagent (Santa Cruz Biotechnology).

**Unfolding of test proteins:**

Unfolding of the test proteins BCA (Bovine carbonic anhydrase), Bacterial HspH, Lysozyme, Ovalbumin, MDH (Malate dehydrogenase) and LDH (Lactate
dehydrogenase) were carried out as reported earlier (Samanta et al, 2008; Chattopadhyay et al, 1994; Pal et al, 1999; Chowdhury et al, 2002; Sanyal et al, 2002). In short, all native proteins were denatured using 6M guanidine hydrochloride for specified time as reported at 25°C temperature. Losses of secondary structures were verified by CD spectrum (Das et al, 2011; Ghosh et al, 2003).

**UV cross-linking of PTC RNA sequence - refolding protein complexes (in vitro):**

300 μl of sample, containing 125 nM domain V RNA and 600 nM unfolded protein (to ensure that all RNA molecules were bound to proteins), was irradiated on a glass dish in ice with 254 nm UV (GS GENE LINKER, BIORAD) at a distance of 6 cm for 2 min. The irradiated samples were precipitated by salt-ethanol and washed with 70% ethanol.

**Identification of the rRNA stretches that interact with the nascent and the chemically unfolded proteins:**

The toe printings (primer extensions) were done on the PTC-nascent protein or PTC-refolding protein complexes according to the procedure reported earlier (Samanta et al, 2008). Primer BG32 (5’ACCCCGGAATTCGCGCCCACGGCAGATAGG3’) was annealed with protein cross-linked PTC RNA. Annealed primers were labeled by [α-32P] dCTP following the 3-deoxynucleoside triphosphate method at 55°C using ThermoScript Reverse Transcriptase (Invitrogen). Labeled primers were extended at 58°C after the addition of all the four dNTPs in excess, by the same enzyme for about 45 minutes. The products were precipitated and washed with 70% ethanol and analyzed on a 6.5% polyacrylamide gel in 8 M urea, next to a sequencing ladder of domain V rDNA. It was obtained using the same primer by Thermo Sequenase DNA Polymerase (Thermo Sequenase™ Cycle Sequencing Kit, USB; USA).
Sample preparation for mass-spectrometry

The mass spectrometry (MS) samples were prepared as described (Urlaub et al., 2000; Urlaub et al., 2002). UV-irradiated samples were precipitated, air-dried and dissolved in 8 M urea, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA and 1 mM dithiothreitol, heated for 10 min at 70°C, and cooled to room temperature. The sample was diluted 10 fold with 50 mM Tris-HCl (pH 8.0) and then digested with trypsin (Gold MS grade, Promega) with an enzyme/substrate ratio of 1:25 for 16 hrs at 37°C. The samples were precipitated and dissolved in 50 mM Tris-HCl (pH 8.0) and 2 mM EDTA. They were then digested with RNase A and RNase T1 for 2 hrs at 50°C, followed by trypsin for 16 hrs at 37°C. Digestion was stopped by direct injection of 500 μl sample on to μRPC_C18_ST_4.6/100 column (GE Healthcare) mounted in an AKTAbasic 100 system and run using the unicorn software. For reverse phase chromatography the solvents were: A) 0.1% (v/v) trifluoroacetic acid (TFA) in double distilled H₂O and B) 0.085% (v/v) TFA in acetonitrile. The column was equilibrated with 5X column volume (CV) of solution B and the following gradients were applied in steps with a flow rate of 0.1 ml/min. (I) Isocratic elution of injection peak at the rate of 5% solution B for 2X CV, (II) 5% to 100% solution B for 20X CV, (III) Isocratic wash with 10% B for sometime till the baseline is reached. 500μl fractions were collected and the presence of cross-linked peptide:RNA was checked by absorbance at 250 nm. Peak fractions were pooled, vacuum dried and then mixed with the appropriate matrix for MS and MS/MS analysis.

Mass spectrometry

MS was carried out in an Applied Biosystems AB4700 MALDI ToF/ToF mass spectrometer. 0.5μl of each sample was mixed on a stainless steel plate with equal volume of either 10 mg/ml 2,5-dihydroxy benzoic acid (DHB) or 5 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA) (recrystallized and dissolved in 50% ACN, 0.1% TFA) matrix solutions. It was then dried at room temperature and subjected to MS and MS/MS. PMF were obtained in positive reflector mode. The instrument was operated in the delayed extraction mode with delay time of 200 ns. Spectra were obtained by
accumulation of 2500 and 6000 consecutive laser shots respectively in MS and MS/MS mode, and the laser intensities used were in the range 4000 to 5000. Close external calibration for MS was performed with 4700 Cal Mix (Applied Biosystems). Peak harvesting was carried out using 4000 series Explorer (Applied Biosystems) software. Collected spectra were processed with Data Explorer software V.4.6 for advanced baseline correction (Peak width 32, Flexibility 0.5, Degree 0.1), noise reduction (correlation factor 0.8) and deisotoping. Processed spectra were checked for the presence of tryptic peptides and their possible modifications for the representative proteins using MassSorter v.1.05.04 (Barnes et al., 2006). The search parameters included 1 missed cleavage, error tolerance of ±50 ppm for PMF and ±0.2 Dalton for MS/MS ion search. The toe printings (primer extensions) were done on the PTC-nascent protein or PTC-refolding protein complexes according to the procedure reported earlier (Samanta et al, 2008).

Identification of the amino acids of nascent and chemically unfolded proteins which bind to the PTC-RNA nucleotides:

MALDI TOF MS and MS/MS studies were done with the nascent and chemically unfolded protein complexes with the PTC-RNA as described earlier from our lab (Samanta et al, 2008; PhD thesis of Dr. Dibyendu Samanta, Calcutta University).

In vivo and in vitro protein folding assay:

For in vivo protein folding assay, *E. coli* K12 cells were grown at 37°C in Tris-Glycerol-Casamino acid (TGC) medium: 100 mM Tris-HCl (pH 7.5), 100 mM potassium chloride, 8.5 mM sodium chloride, 20 mM ammonium chloride, 1 mM calcium chloride, 1 mM magnesium sulphate, 0.3% casamino acid, 15 mM sodium pyruvate, 1 mM potassium phosphate (pH 7.5), 0.2% glycerol, 0.2 μg/liter ferrous sulphate and 10 μg/liter thiamine. In the log phase (OD₆₀₀ = 0.2) cells, β-galactosidase was induced with IPTG, and ³⁵S-Methionine was added (t=0 min). After 8 minutes of growth, cells were divided into six equal portions. Antibiotics chloramphenicol (150 μg/ml, 464μM), lincomycin (44μg/ml, 150 μg/ml, 464μM), lincomycin (44μg/ml,
99μM), streptomycin (25μg/ml, 17μM), kasugamycin (83 μg/ml, 193μM) and kirromycin (200μM) were added to five, while the sixth one did not receive any antibiotic. At different time points after addition of antibiotics, aliquots were withdrawn from the growing cells and were lysed with toluene to measure their β-galactosidase activities. To 200 μl of the assay buffer [100 mM sodium phosphate (pH 7.5), 10 mM potassium chloride, 1 mM magnesium sulphate, 50 mM β-mercaptoethanol and 1 mg/ml ONPG], an equal volume of toluene extracts of cells grown under different conditions were added, and the mixture incubated for 30 seconds at 37°C. The reaction was stopped by adding 200 μl of 1M sodium carbonate and the enzyme activity was measured by recording OD420. β-galactosidase activities were normalized with respect to cell concentration before plotting as a function of time. The enzyme activity was similarly measured from the E. coli tig (trigger factor) mutant and dnaK, dnaJ, tig triple mutant cells, except that the cells were grown at 28°C instead of 37°C. To measure protein synthesis after addition of antibiotics, samples were withdrawn at different times and 35S-Methionyl tRNA in the cells were destroyed by incubating the samples in the presence of 1 M sodium hydroxide and 0.5 M hydrogen peroxide at 40°C for 30 minutes (Chattopadhyay et al, 1996). Finally 5% TCA precipitated protein was trapped on glass fiber (GFC Whatman 25mm) filters. After drying the filters, 35S counts were taken in the scintillation counter.

For in vitro refolding experiments, β-galactosidase and bovine carbonic anhydrase were denatured using guanidine hydrochloride, as described (Samanta et al., 2008). Recovery of enzymatic activity was measured following dilution of guanidine hydrochloride followed by incubation with protein folding modulators (Samanta et al., 2008).
Results:

Interaction of nascent protein with the PTC of the ribosomal RNA:

Ribosome nascent protein complexes (RNC) have been isolated by a number of groups (Nakatogawa et al, 2002; Seidelt et al, 2009) to study protein folding inside the ribosome. Most of them used specific sequences of amino acids to stall nascent protein in the ribosome. Very few (Gilbert et al, 2004)) of those studies were attempted to freeze the ribosome during protein synthesis and trace the nascent protein inside. We used a C-terminal histidine-tagged rhizobacterial protein HspH as the model protein to isolate ribosome nascent chain complex (Basu et al, 2008a). HspH is a monomeric bacterial protein. We did snap freezing of the growing cells which was followed by UV crosslinking to trap the full length protein on the ribosome (Fig.2.1).

![Diagram](image)

Fig.2.1: Experimental scheme to analyze nascent HspH – rRNA interaction in living E.coli cell.
UV-induced crosslinking gave a snapshot of the intracellular association pattern of nascent protein with the ribosome. By applying the entire ribosome population from the UV-crosslinked cells to the Ni column, we eluted a significant amount of 50S and a comparatively small 70S population cross-linked to the full length HspH (Fig. 2.2) (Basu et al, 2008a).

**Fig 2.2:** Association of nascent full length bacterial protein HspH with ribosomal subunits (A) in vivo & (B) following in vitro translation: 5%-20% sucrose density gradient profile of Ni-column eluted fraction (■) from total ribosome extract (●) and control 70S, 50S, 30S particles ran parallel in a separate gradient (△).

So, we isolated a population of post-translational 50S that is bound to the full length nascent HspH and a population of post-translational 70S that just terminated translation of nascent protein whose C terminus is accessible to Ni.
Presence of nascent HspH in the ribosomal RNA bound population was confirmed by the western blot analysis done with anti-penta-his-antibody. Purified native HspH (C terminally His tagged) served as the length standard as shown in the fig 2.3.

**Fig 2.3:** Western Blot analysis showing band for nascent HspH (C-terminally His tagged) isolated from its rRNA bound population in lane 1; this was compared with the native HspH (C-terminally His tagged) purified from the same *E.coli* strain.

**Ribosome mediated protein folding: in vivo & in vitro:**

Isolation of a large population of full length nascent protein bound 50S, along with the relatively small 70S, indicated that the release of nascent protein through the 50S is a slow process than translation elongation or termination. To validate our notion, we compared activity gain of synthesized full length protein with that of the chemically unfolded form of same native protein as a measure of folding. We used different antibiotics, all of which inhibit protein synthesis. We compared the effect of the antibiotics which target the PTC with those which bind to other components of the translation apparatus, by measuring the activity of the nascent proteins synthesized under these conditions.

Using β-galactosidase as the model enzyme (due to its convenient assay as a measure of folding), its synthesis was induced in log phase *E. coli* K12 cells. After 8 minutes of induction, the culture was divided into equal portions and one antibiotic was added to each of them except the one used as control. The cells growing in presence of streptomycin and kasugamycin [bind to 30S (Carter et al, 2000; Schuwirth et al, 2006)] showed increase in β-galactosidase activity which saturated after 12 minutes (Fig. 2.4A). In contrast the cells growing in presence of chloramphenicol and lincomycin [bind to the PTC of 50S (Long et al, 2003; Douthwaite et al, 1992)] showed little increase in the
activity. The effect of kirromycin that binds to the translation factor EF-Tu (Wolf et al, 1974) was similar to that of the 30S specific kasugamycin.

Fig 2.4: In vivo and in vitro protein folding.

(A) β-Galactosidase activities in induced *E. coli* K-12 cells in the absence and presence of different antibiotics. In the control sample, the enzyme activity increased linearly (■). The enzyme activity stopped increasing almost immediately in cells treated with chloramphenicol (○) and lincomycin (▲) but continued to rise for about 10 minutes in cells treated with kasugamycin (▼), streptomycin (●) or kirromycin (▲).
Effect of antibiotics on protein synthesis was measured by uptake of $^{35}$S-Met in the absence (■) and in the presence of chloramphenicol (☉), streptomycin (●) or kasugamycin (▼).

Folding of β-Galactosidase (1) and BCA (2): Chemically unfolded proteins were folded in the absence (□) and in the presence of different modulators like the 70S ribosome (a), the 50S ribosomal subunit (a), the 23S rRNA (a) and the domain V (PTC) of 23S rRNA (□). Bar graphs represent the mean folding values (± standard deviations) from three independent experiments.

Effect of antibiotics on the 23S rRNA mediated recovery of chemically unfolded β-Galactosidase activity: The symbols represent recoveries of activity in the presence of streptomycin (☉), kasugamycin (●), chloramphenicol (▲) and lincomycin (▲). The concentration of chloramphenicol is shown in the upper scale and the concentrations of other antibiotics are shown in the lower scale.

The ability of all the antibiotics to block protein synthesis was confirmed by addition of $^{35}$S-methionine to the cells growing in absence and presence of the antibiotics. None of the antibiotic containing cells incorporated significant counts during the time when the enzyme activity increased in the presence of 30S-specific antibiotics (Fig. 2.4B). Hence this increase of β-galactosidase activity could be exclusively for the population of enzyme that was just synthesized as full length product prior to the addition of antibiotics. Similar results were observed when β-lactamase, GFP, DnaK and firefly luciferase (Das et al, 2008) were used as test proteins. Using appropriate mutant cells, we also showed that the ribosome-mediated folding is independent of the ribosome-associated chaperones DnaK, DnaJ and Trigger factor (Ph.D thesis of Dr. Anindita Das, CU). Chloramphenicol binds to the A site of ribosome without causing much conformational alteration (Yonath, 2005) and streptomycin binds to the 16S rRNA to stabilize the ram state of ribosome (Carter, 2000). The above results probably suggest that the chloramphenicol binding region of ribosome is accessible to the nascent protein that is just synthesized and the region might have link with the increased activity gain of proteins.
In the purified system in vitro, when the denaturant guanidine hydrochloride was diluted out from the unfolded β-galactosidase, up to 30% of the native activity was regained upon spontaneous refolding (Fig. 2.4C). However in the presence of folding modulators, such as in vitro transcribed PTC-RNA, 23SrRNA, 50S subunit and 70S ribosome, up to 80% of the native activity could be regained (Fig. 2.4C). The 23SrRNA alone is capable of binding PTC specific antibiotics in vitro. When unfolded β-galactosidase was allowed to refold in the presence of the 23S rRNA along with 50S and 30S specific antibiotics, regain of activity decreased dramatically with the 50S-specific antibiotics only (Fig. 2.4D). The 30S-specific antibiotics showed no effect even at very high concentrations. Hence activity gain of a nascent protein is a much slow process compared to translation or its termination. Results were similar when instead of β-galactosidase, bovine carbonic anhydrase (BCA), beta lactamase, GFP or DnaK were used as the experimental enzyme. The identity of the in vitro and in vivo results also suggested that the in vivo behavior of PTC can be predicted from the in vitro folding experiments using transcribed PTC RNA sequence.

**Interaction of nascent protein with the PTC region of 23S rRNA:**

To check the nascent protein-rRNA interaction the Ni-column purified ribosome population was first gently freed from ribosomal proteins by prolonged incubation with low concentration of urea in ice. The 23S rRNA was purified by gel filtration, with the view that its PTC presumably cross-linked with the nascent HspH protein. It should be noted that none of the ribosomal proteins would crosslink with the PTC because they are not linked with it. We confirmed this by doing RT-PCR on the gel-filtered rRNA, similarly freed from r-proteins from UV irradiated non translating 70S ribosome, using appropriate PTC specific primer. A distinct band of desired length was obtained, indicating that the synthesis of DNA by RT-PCR was not blocked by any ribosomal protein cross linked to the PTC (Fig 2.5A, lane 3). The same length of RT-PCR product was obtained when 23S rRNA was purified from non-UV-exposed 70S by phenol extraction (Fig 2.5A - lane 2) or when PCR was done on the cloned PTC-DNA (Fig 2.5A - lane 4).  

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Fig 2.5(A): Control experiment to check accessibility of the PTC linked region in the total rRNA isolated from UV-crosslinked (lane 3) and non-crosslinked (lane 2) 70S particles. The rRNAs from both the 70S preparations produced distinct bands of the PTC region of 23S rRNA when PCR amplified with the same primer pairs. This is compared with the PCR product (lane 4) amplified using the same primers from the cloned PTC segment present in a plasmid under T7 promoter, it served as the length standard. Length of the PCR products was further compared with a DNA ladder ran in parallel in lane 1.

(B): Toe-printing on the PTC linked region of rRNA using specific $^{32}$P-labeled primer: The transcribed PTC-RNA was UV-irradiated in the absence (L2) and in the presence
(L3) of chemically unfolded protein (Samanta et al, 2008). In (L4) and (L1), PTC-RNA region of 23S rRNA was isolated from cells with nascent HspH crosslinked to it and from non translating UV-irradiated 70S respectively.

One of these primers after end-labeling was annealed to the RNA of the RNA-nascent protein complexes. This was extended by thermophilic reverse transcriptase at high temperature and the products of chain extension were run in a denaturing sequencing gel. Five distinct stops were detected and their positions were determined from the sequence of the same region of the RNA run in the same gel. Interestingly, positions of these stops fully agreed with those observed in the case of the in vitro folding experiments where chemically unfolded proteins were crosslinked with the in vitro transcribed PTC-RNA (Samanta et al, 2008) (Fig. 2.5B). Apart from the five stops (as marked in the Fig. 2.5B), we also found one at A2534 but this was neither seen reproducibly nor identified as RNA-protein complex in mass analysis that we performed to find out the amino acids of the nascent HspH that interact with the five strong binding sites in the PTC in vivo.

The MALDI TOF MS/MS studies were done on the UV-cross-linked RNA-nascent protein complexes, following the procedure reported earlier (Samanta et al, 2008). Result of this study was compared with the chemically unfolded protein counterparts, shown in Table 1. For HspH protein, there was complete agreement between the in vivo and in vitro binding sites in the RNA-protein complexes. The amino acids of different proteins that interacted with the same nucleotide in the RNA are not all identical but not random either (shown in the next chapter). In this small set of data, one RNA site (C2551) was recognized by the same amino acid (N) from all the five proteins, tested so far.
Interacting PTC Nucleotides (in vivo & in vitro) | Interacting amino acids of Unfolded proteins
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<th>Full length Nascent HspH (in vivo)</th>
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<tr>
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<tr>
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Table 2.1: Amino acids of nascent and chemically unfolded proteins that interact with the specific nucleotides of the PTC RNA are marked bold (identified using MALDI TOF MS/MS studies). A conserved interacting amino acid asparagine (N), (which was found to interact with C2551 in case of five other proteins) is written in red color.

The above results suggest that there exist conserved nucleotides for amino acid recognition in the PTC RNA sequence, and the in vivo components of this reaction could possibly be mimicked in vitro using chemically unfolded proteins.

Discussion:

In this chapter we have demonstrated that the nascent protein (unfolded) interacts with the PTC linked region of the rRNA identically as the chemically unfolded counterpart of the same protein does with the in vitro transcribed PTC RNA. We also found that during activity gain (as a measure of refolding), the folding protein behaves identically in presence of the in vitro transcribed PTC RNA sequence as the same nascent protein does while being translated by the PTC within the ribosome.
Anfinsen dictated that the information for correct folding of a protein lies in its linear amino acid sequence (Anfinsen, 1973); but the folding must be in the physiological time scale (Levinthal). A number of in vitro and in silico studies have been done to simulate the intracellular condition to search for a universal paradigm of protein folding, connecting the linear order of amino acids to the three dimensional structure of proteins. In such quest, transcribed PTC RNA sequence mediated folding of a number of unrelated proteins have been demonstrated from our lab previously (Samanta et al, 2008; Das et al, 2008; Das et al, 2010) and it was found that a number of such chemically unfolded proteins interact with the same five PTC RNA nucleotides (Samanta et al, 2008; Das et al 2010). This in fact demonstrates how the randomness of the folding process can be reduced. From the studies presented in this chapter, we have showed the physiological importance of these in vitro studies. As a corollary of the result, activity gain pattern of the nascent protein after it is synthesized and activity gain pattern of chemically unfolded protein in presence of the transcribed PTC RNA appears to be identical. We will utilize this information in the following chapters that the PTC associated behavior of nascent (unfolded) protein can be predicted from the in vitro behavior of chemically unfolded protein(s) when folding modulators like the 70S ribosome, the 50S subunit, the 23S rRNA or the transcribed PTC RNA sequence are present in the reaction mixture.