CHAPTER 3

Insights into Unfolded Protein Mediated
Ribosome Dissociation: Ribosome
Antiassociation activity of Unfolded Proteins
INTRODUCTION

As stated in the chapter 1 section III.2, earlier studies show that the interaction of unfolded protein with the 70S ribosome results in rapid dissociation of ribosomal subunits. We have studied this phenomenon in order to further understand the mechanism of unfolded protein triggered ribosome dissociation.

The dissociation of ribosome into its subunits is an important post-translational step during nascent polypeptide synthesis. This step ensures the availability of isolated subunits that is necessary to initiate a new round of translation. In bacteria the ribosome recycling factor along with EFG and GTP executes disassembly of post-termination complex, the mechanism of which has been outlined in chapter 1 section III.2. The following section highlights the fact that there are alternative pathways of ribosome recycling in eukaryotes and that this process is differentially regulated during cellular stress.

Comparison of ribosome recycling between the three domains of life

Eukaryotic organelles that contain own DNA, mitochondria and chloroplast have functional RRF homologues. It has been reported that RRF is essential for mitochondrial maintenance and chloroplast RRF binds with the ribosome at a 1:1 ratio. No obvious eukaryotic cytoplasmic RRF homologues have been found except for the organelle-localized RRF. Ribosome recycling process in eukaryotes and archaea are fundamentally different to that in bacteria. In all domains of life, translation termination begins with recruitment of a ternary complex of a class I release factor and a GTPase with GTP to the ribosomal A site. In bacteria release factor RF3, the GTPase trigger the release of class 1 release factors RF1 and RF2 from the ribosome by GTP hydrolysis, resulting in the post-termination complex (post-TC). By contrast, in eukaryotes and archaea, the GTPase (eRF3 in eukaryotes and aEF1α in archaea) ensures efficient peptide release by only
rearranging the conformation of the class I release factor eRF1 or aRF1, respectively. Release of the GDP-bound eRF3/aEF1α from ribosome yield a post-TC that still contains the class I release factor. In eukaryotes and archaea, ABCE1 is recruited to the post-TC after dissociation of eRF3/aEF1α. In eukaryotes, a combination of ABCE1-ATP with release factors eRF1 and eRF3-GTP is needed to split post-TCs efficiently. Beside ABCE1-dependent ribosome splitting, the eukaryotic translation initiation factors elf1, elf1A and elf3 can promote a passive splitting of the post-TC in vitro at low Mg\(^{2+}\) concentrations. The ABCE 1 protein can also recycle stalled ribosomes. Another aspect of eukaryotic translation is that it occurs by “closed loop” mechanism, in which ribosomes reaching the 3’ end of the mRNA and can reinitiate translation again on the 5’ end of same mRNA as two end of the mRNA are linked. The mRNA ends can interact to circularize the transcript and allow the recycling of terminating ribosomes back onto the same mRNA to commence another round of translation. The highly conserved recycling factor Rli1p (ABCE1 in mammal), that both bind to release factors on terminating ribosomes and interact with initiation factors to form the preinitiation complex provide additional assistance to this process. At translation termination the initiation factor elf4F interacts with the release factor complex (eRF1-eRF3). When eRF3 released from the complex, Rli1p interact with the termination complex to release the 60S ribosomal subunit, leaving the 40S subunit which still attached with the eRF1-Rli1p complex.
Ribosome recycling during cell stress

Translation may often stall due to various situations in a cell. For example, translation of mRNA without a stop codon (non-stop mRNA) does not terminate efficiently because release factor cannot work on the stop less mRNA. In cellular stress, translation may also stall at various positions on mRNA before reaching the stop codons. Translation stalling abruptly reduces the cytoplasmic free ribosome pool that can participate actively in translation process. Inside cell, the transfer messenger RNA (tmRNA) actively participates to rescue the stalled ribosomes. They work by providing a stop codon to allow completion of translation of a non-stop mRNA and consequently recycle the ribosome.

In addition during stress, bacteria including *E.coli* downregulate protein synthesis by the formation of 100S ribosome. The 100S ribosome is a dimer of 70S ribosomes, formed by ribosome modulation factor (RMF) binding to the ribosomes. Another protein factor called hibernation promoting factor (HPF), expressed during the stationary phase, also binds to
ribosomes and promotes 100S ribosome formation by RMF. The 100S ribosomes have no translational activity and are formed during the stationary phase. RMF inactivates ribosomes by blocking the peptidyl transferase centre and the entrance to the peptide exit tunnel. When stationary-phase cells are transferred to rich nutritious culture medium, RMF is immediately released from 100S ribosomes, which dissociate back into 70S ribosomes. The \textit{E. coli} strain with deleted \textit{rmf} gene cannot form 100S ribosomes and its lifetime is shorter than that of the wild strain [Yamagishi M et al, 1993]. These phenomena indicate that an interconversion system between 70S and 100S ribosomes is an important strategy for survival under stress conditions.

\textbf{Figure 3.2: Formation of 100S ribosome.} Schematic diagram showing the formation process of the 100S ribosome upon transition from the log Phase to the stationary growth phase of bacteria [Picture adapted from Kato T et al, 2010].

\textbf{Unfolded protein mediated subunit dissociation}

The ribosomes from eubacteria, archaea and eukaryotes have the ability to assist in folding of proteins both \textit{in vitro} and \textit{in vivo} [Das D et al, 2008; Das D et al, 2012]. The chaperoning centre of the ribosome resides at the peptidyl transferase centre (PTC) located on the domain V of 23S rRNA of the large ribosomal subunit. Another significant outcome of the unfolded protein-
ribosome interaction is the rapid dissociation of the ribosome into its subunits [Basu A et al, 2008b; Basu A et al, 2008a]. The subunit dissociation ability of unfolded protein led to the proposition that the proteins formed upon completion of polypeptide synthesis could itself act as the dissociation factor for ribosomal subunits in addition to the ribosome recycling factor RRF. A model was proposed in which the unfolded protein mediated subunit dissociation is followed by favourable interaction between the proteins with domain V of isolated 50S subunit rRNA and release of the protein in a folding competent state [Basu A et al, 2008b]. However, since the PTC, the binding site of unfolded protein does not directly participate in inter-subunit bridge formation; the mechanism of unfolded protein mediated subunit dissociation remained unclear. The relationship between subunit dissociation and the chaperoning ability of the ribosome was also not known. However, it has been proposed that the 50S subunit must be free to ensure optimum folding of the freshly synthesized polypeptide on the 70S ribosome. This is achieved if the folding protein dissociates the 70S particle in the process of accessing the domain V of 23S rRNA of the 50S subunit at a time when all the tRNAs dissociated from the ribosome on completion of polypeptide synthesis [Basu A et al, 2008b]. In the fourth step of translation, IF3 binds to the 30S ribosomal subunit as an antiassociation factor and prevents reassociation of subunits. The 50S subunit is sequestered by the unfolded or newly synthesized protein.

In earlier studies ribosome recycling been assayed by adding purified RRF and EFG along with GTP and initiation factor 3 (IF3). Ribosome subunit association or dissociation can be directly monitored by light scattering or ultracentrifugation methods [Antoun A et al, 2004]. Rayleigh light scattering techniques have been used to monitor rapid association of ribosomal subunits during initiation of eubacterial protein synthesis. Using this technique, the effects of recycling factor RRF, initiation factors IF1, IF2, IF3 and buffer conditions on subunit association have
been studied along with the role of GTP in this process. In contrast to the rapid and non-invasive light scattering techniques, ultra centrifugation methods provide little kinetic information on subunit association or dissociation, but have been used to monitor the extent of ribosome subunit association.

Using these techniques our present studies demonstrates that the unfolded proteins can act as antiassociation factor for the 50S subunit and this ability might contribute to its subunit dissociation ability. Studies performed with ribosome bound to PTC and aminoglycoside antibiotics indicate at a communication between the intersubunit bridges and domain V of 23S rRNA and the specific involvement of helix 69 of 23S rRNA (a component of the central intersubunit bridge B2a). Subunit dissociation studies with partially or intrinsically unfolded proteins are also detailed below.
MATERIALS AND METHODS

Materials

The protein bovine carbonic anhydrase II (BCAII), Guanidine hydrochloride (GuHCl) and the antibiotics (chloramphenicol, blasticidin, paromomycin, neomycin), GTP were purchased from Sigma. 70S ribosome and the isolated 50S and 30S subunits from *E.coli* MRE600 were prepared as described in chapter 5. All other chemicals were local products of analytical grade. All data analysis was performed using OriginPro 8 software. Purified RRF, EFG, IF3 and HYPK were kind gifts from the laboratories of C. Dasgupta Univ. of Calcutta and D. Mukhopadhyay of SINP, Calcutta.

Methods

Dissociation of ribosomes and reassociation of ribosomal subunits

Buffers used for 70S dissociation and reassociation studies with ribosomal subunits were as Basu A et al, 2008b; 50 mM Tris-HCl (pH 7.5), 100 mM NaCl (MgCl₂ concentration was 7.5 mM or as stated in figure legend).

Light scattering studies

Dissociation of 70S ribosome by translation factors or in presence of unfolded proteins was measured by following ribosomal light scattering (Hitachi F-3010 Fluorescence Spectrophotometer (excitation: 5mm slit; emission: 5 mm slit; wavelength at 350 nm at 90°angle) at temperatures 20°C. Translation factor mix, unfolded BCAII, Huntingtin-interacting protein K (HYPK) or BCAII denatured with different concentrations of Guanidine hydrochloride (1M-6M) were mixed in stoichiometric amounts as specified in figure legends. Mixture A containing either factors (40μl) or BCAII (2 μl) was mixed manually with mixture B (160 μl or 198 μl) containing 0.1 μM of the 70S ribosome preparation in buffer as specified in the materials
and methods. The mixture (200 μl) was placed in a cuvette and the intensity of the scattered light was continuously recorded beginning at 20 seconds after the mixing. For antiassociation studies isolated 30S subunit (0.1 μM) was added with the isolated 50S subunit (0.1 μM) either in the presence of uBCAII (0.025 μM - 0.5 μM) or in the absence of it.

**Sucrose density gradient centrifugation**

0.1 μM or 0.5 μM unfolded BCAII (uBCAII) or HYPK was incubated with 0.1μM of vacant or antibiotic bound 70S ribosome for 5 min. In another set 0.1μM of vacant or antibiotic bound 70S ribosome was incubated with RRF, EFG, IF3 and GTP for 5 min minutes where the concentration of the factors were RRF= 20 μM, EFG = 20 μM, IF3 = 4.5 μM, and GTP =360 μM [Hirokawa G et al, 2005]. After incubation, 100 μl of reaction mixtures were applied on the 5 ml of a 17% to 25% sucrose gradient prepared in the 70S dissociation buffer containing MgCl₂ concentrations as mention in the figure legend. Samples were centrifuged at 1, 92,000 g (Thermo SW52 rotor) for 2 hours and 30 minutes at 4°C. Fractions were collected from top to bottom of the tube and absorbance at 260 nm was measured.

**Effects of denatured BCAII on in vitro luciferase production**

*In vitro* transcription-translation for luciferase synthesis was performed using Promega kit: *E.coli* S30 Extract System for Circular DNA. Luciferase was synthesized by following manufacturer’s instructions. The amount of luciferase produced after 60 minutes of incubation at 37°C, was assayed using luciferase assay reagent in a F-7000 spectrofluorometer in luminescence mode (excitation: wavelength at 350 nm and emission scan set at 380nm-700nm). Experimental data was analyzed by comparing the area of total fluorescence obtained in the presence and in the absence of uBCAII.
RESULTS AND DISCUSSION

Mg$^{2+}$ dependence of unfolded protein mediated subunit dissociation

Physiologically, magnesium ion concentration plays a vital role in the determining the equilibrium between the ribosome and its subunits. The 70S ribosomes are transiently dissociated into subunits by RRF and EFG, and that the subunits are then stabilized by IF3 that acts as an antiassociation factor for the 30S subunit. Earlier studies with ribosome recycling factors RRF, EFG, GTP,IF3 show that very little dissociation takes place at high Mg$^{2+}$ concentration [Hirokawa G et al, 2005] while the ribosome dissociation activity of IF3 was only observed under very low Mg$^{2+}$ ion concentration [Kaempfer R et al, 1972].

The influence of magnesium ion concentration upon uBCAII mediated 70S ribosome dissociation, followed by light scattering studies, is shown in Figure 3.3.

![Figure 3.3: Unfolded protein mediated subunit dissociation. Light scattering studies of dissociation of 70S ribosome (0.1 μM) in the presence of 0.1 μM uBCAII in buffer containing different MgCl$_2$ (○) 7.5 mM, (□) 8.5 mM, in presence of 0.5 μM uBCAII with different MgCl$_2$ (■) 7.5 mM, (▲) 8.5 mM and (●) 10 mM are shown in the figure.](image-url)
In earlier studies significant subunit dissociation was observed with 10 mM Mg£º2 in presence of fivefold molar excess of uBCAI. In our studies however, only marginal ribosome subunit dissociation is observed at 8.5 mM Mg£º2 (a concentration at which complete dissociation is observed in presence of RRF, EFG, GTP and IF3; [Hirokawa G et al, 2005]). Dissociation was observed only at 7.5 mM Mg£º2 when uBCAI: ribosome was 5:1. In addition, when uBCAI and ribosome is present in stoichiometric concentration, dissociation increases with decreasing magnesium concentration.

**Effect of partially unfolded and intrinsically unstructured protein on ribosome subunit dissociation**

The ribosome dissociation experiments were also performed in presence of different unfolded forms of uBCAI and the Huntingtin-interacting protein K, HYPK. Experimental evidences and theoretical analyses of amino acids composition of HYPK predict that HYPK is an intrinsically unstructured protein with premolten globule like conformation [Raychaudhuri S et al, 2008]. HYPK is a potential interaction partners for the Huntingtin protein and reduces Htt polyglutamine (polyQ) aggregation upon overexpression. In presence of increasing concentration of Ca£º2+, HYPK shows conformational alterations as well as concomitant reduction of hydrodynamic radius. Light scattering studies on ribosome subunit dissociation obtained with the protein HYPK and different forms uBCAI is shown in Figure 3.4.
Hence, HYPK, a protein that is intrinsically unstructured irrespective of the presence of a denaturing agent [Connery CP et al, 1993] was also capable of inducing subunit dissociation. The ability to split the ribosome into its subunits also increases with increase in unfolding of BCAII. Taken together these studies demonstrate that dissociation into subunits is a general phenomenon dependent on interaction of regions of unfolded protein with the 70S ribosome.

**Sucrose density gradient centrifugation study for ribosome dissociation using uBCAII and HYPK**

Subunit dissociation process was also analysed by sucrose density gradient centrifugation (SDGC) in presence of uBCAII and HYPK as shown in Figure 3.5.
Figure 3.5: Sedimentation profile with uBCAII and HYPK. Sedimentation analysis of ribosome (0.1 μM) dissociation into its subunits in absence of (1) uBCAII and in presence of (2) 0.5 μM uBCAII and (3) 0.5 μM HYPK in buffer containing 7.5 mM MgCl$_2$ are shown here. The dotted line in the figure represents the position of 70S ribosome peak and position of 50S and 30S subunits are also shown in the figure.

**Unfolded protein is an antiassociation factor for 50S subunit**

In our study the kinetics of subunit association was monitored by the increase in light scattering after mixing of naked 50S and 30S subunits in presence of uBCAII. Earlier studies have demonstrated that uBCAII preferentially binds to the 50S subunit. As shown in Figure 3.6, binding of uBCAII to 50S subunit showed a strong inhibition on its ability to associate with the 30S subunit.
Figure 3.6: uBCAII as an antiassociation factor for 50S subunit. Light scattering analysis of reassociation of isolated 50S and 30S (each 0.1 μM) ribosomal subunits in (■) absence of uBCAII and in presence of (*) 0.025 μM, (▲) 0.05 μM, (▼) 0.1 μM and (●) 0.5 μM uBCAII in refolding buffer containing 7.5 mM MgCl₂.

A dose dependence of the antiassociation activity on the concentration of the unfolded protein present is also observed. This, together with the observation that low Mg²⁺ concentrations facilitates uBCAII mediated subunit dissociation, indicates that the dissociation ability of the unfolded protein requires the presence of isolated ribosomal subunits and the antiassociation activity of protein on isolated 50S subunit might contribute to the dissociation process.

Experiments performed using equilibrium SDGC also indicated stable antiasociation activity of unfolded protein on isolated 50S subunit as shown in Figure 3.7
The antiassociation activity becomes further probable based on earlier studies that indicates long association of folding protein with the 50S subunit [Basu A et al, 2008b]. Western blot analysis performed in our studies with 50S peak however did not show presence of BCAII protein indicating that uBCAII does not bind 50S subunit stably enough to withstand sucrose density gradient centrifugation.

**Effects of uBCAII on luciferase in vitro transcription-translation**

Several studies have shown that the addition of ribosomal dissociation or antiassociation factors is capable of affecting translational activity [Benelli D et al, 2009; Pavlov MY et al, 1997]. However our preliminary studies with uBCAII show no effect of addition of the unfolded
protein, at concentrations indicated in the figure, on luciferase activity in vitro transcription-translation system (Figure 3.8).

Figure 3.8: Effect of uBCAIi on luciferase production. Bar diagram shows the total fluorescence obtained by luciferase reaction (1) in absence of uBCAIi and in presence of (2) 3 μM and (3) 5 μM uBCAIi.

In an earlier study it had also been stated that uBCAIi induced subunit dissociation is inhibited in presence of P/E site(s) tRNA on ribosome [Basu A et al, 2008b]. This implied that an actively translating ribosome might be refractory to the presence of unfolded proteins.

Effect of antibiotics on unfolded protein mediated dissociation of subunits

Blasticidin S and Chloramphenicol are antibiotics targeted to the peptidyl transferase center. Interaction of the antibiotic blasticidin with the peptidyl transferase center (PTC) mimics the interaction between 3’–CCA end of P/P-site tRNA with the PTC. The crystal structure of Blasticidin S with the H. marismortui 50S subunit shows two molecules of the antibiotic positioned so as to mimic C74 and C75 of a tRNA at the P-site by making interactions with the P-loop residue. Two binding sites at the PTC have also been reported for the antibiotic chloramphenicol. One site of the bound antibiotic suggests that it hinders substrate binding
directly by interfering with the positioning of the aminoacyl moiety in the A site while the other chloramphenicol binding site lies in the hydrophobic crevice at the entrance to the peptide exit tunnel suggesting that chloramphenicol inhibits protein synthesis by perturbing the egress of nascent polypeptides into the exit tunnel.

Studies in our laboratory had demonstrated that the chaperoning ability of the ribosome can be inhibited in a dose dependent manner upon binding to both blasticidin [Mondal S et al, 2014] and chloramphenicol (as shown in Figure 3.9).

Figure 3.9: Effects of blasticidin and chloramphenicol on BCAII reactivation. Ribosome mediated BCAII reactivation inhibited increasingly with increasing concentration of (a) blasticidin or (b) chloramphenicol.

PTC antibiotics

To assess the effect of PTC binding antibiotics chloramphenicol and blasticidin on unfolded protein mediated dissociation of ribosomal subunits, the antibiotics were bound to the empty ribosome under appropriate conditions (as describe in chapter 5) and the dissociation of antibiotic bound ribosome followed by light scattering and sucrose density gradient centrifugation studies. The binding of both blasticidin and chloramphenicol at concentrations
necessary to inhibit of ribosome’s chaperoning ability also completely inhibits both unfolded protein induced and RRF induced ribosome subunit dissociation (Figure 3.10).

**Figure 3.10: Effects of blasticidin and chloramphenicol on subunit dissociation.** Light scattering analysis of unfolded BCAII (0.5 μM) induced dissociation pattern of (■) 70S ribosome, (●) 70S bound chloramphenicol, and (▲) 70S bound blasticidin in refolding buffer containing 7.5 mM MgCl$_2$. Translation factor mediated ribosome subunit dissociation in the same buffer is also represented in this figure; ( △) RRF-EFG-IF3-GTP, (○) RRF-EFG-IF3-GTP + 70S bound chloramphenicol and (□) RRF-EFG-IF3-GTP + 70S bound blasticidin. Final reaction concentration of 70S ribosome is 0.1 μM and the concentration of translation factors are RRF = 20 μM, EFG = 20 μM, IF3 = 4.5 μM, GTP = 360 μM.

In sedimentation studies, the antibiotic bound ribosomes were incubated with uBCAII or RRF/EFG/GTP at conditions stated in the legend and subjected to ultracentrifugation. A control experiment in which vacant ribosomes bound to PTC antibiotics were subjected to SDGC, at a concentration of 1 mM Mg$^{2+}$ (Figure 3.11).
Chloramphenicol and blasticidin completely inhibited both uBCAII and RRF/EFG/GTP/IF3 mediated dissociation of 70S subunits. Also, in the control experiment in which vacant ribosomes bound to PTC antibiotics were subjected to SDGC, at a concentration of 1 mM Mg^{2+} almost all the ribosomal subunits remained associated as 70S ribosomes. This indicates that the PTC antibiotics themselves exert a strong association activity on the ribosomal subunits under conditions used in the experiment. As discussed above, the antiassociation activity of protein on 50S ribosomal subunit might contribute to the uBCAII mediated subunit dissociation. Therefore, as isolated subunits are absent due to the association activity of chloramphenicol and blasticidin, the uBCAII mediated subunit dissociation might become inhibited.
**Aminoglycoside antibiotics**

Aminoglycoside antibiotics are broad-spectrum bactericidal agents that are used to treat Gram-negative bacterial infections. *In vivo*, the aminoglycoside antibiotics have been shown to allosterically alter the mechanism of aa-tRNA selection during mRNA decoding on the ribosome by inducing local rearrangements in ribosomal RNA (rRNA) within the highly conserved helix 44 (h44) decoding site of the small (30S) subunit. *In vitro*, these aminoglycosides also inhibit a range of distinct steps in the translation mechanism including mRNA and tRNA translocation and ribosome recycling, the process of subunit separation after the termination phase of protein synthesis. Recently, the aminoglycoside neomycin was shown crystallographically to bind to the bacterial ribosome within H69 of 23S rRNA in the large (50S) subunit. Binding at this site was hypothesized to provide a potential explanation for the inhibition of both ribosome recycling and substrate (mRNA and tRNA) translocation.

The binding of paromomycin and neomycin inhibits the RRF mediated displacement of helix 69 of 23S rRNA thereby preventing disruption of bridge B2a and hence subunit separation [Borovinskaya MA et al, 2007]. In the earlier study it has been reported that the rate of unfolded protein mediated ribosome subunit dissociation occurs much more rapidly than that observed in presence of RRF, EFG, GTP and IF3, the uBCAII mediated dissociation rate becomes significantly suppressed in presence of these ribosome recycling factors [Basu A et al, 2008b]. One possibility therefore is that although the binding sites of uBCAII and RRF are distinct, there might be a similarity between the mechanisms of subunit dissociation by these two factors. To investigate whether uBACII mediated ribosome dissociation also involves the bridge B2a, the effect of the antibiotics paromomycin and neomycin upon unfolded protein mediated ribosome dissociation was studied. The concentration of the antibiotics and their binding conditions to the
ribosome were according to that in which these antibiotics caused complete inhibition of RRF mediated ribosome dissociation upon binding to the low affinity site on helix 69 of 23S rRNA [Hirokawa G et al, 2007; Feldman MB et al, 2010]. The results of the experiment are shown in Figure 3.12.

**Figure 3.12: Effects of aminoglycosides on ribosome dissociation into subunits.** Light scattering studies of uBCAII induced dissociation pattern of (■) 70S ribosome, (●) 70S bound paromomycin, and (▲) 70S bound neomycin in refolding buffer containing 7.5 mM MgCl₂. Subunit dissociation also monitored in the presence of (∆) 70S+ RRF-EFG-IF3-GTP, (○) 70S bound paromomycin + RRF-EFG-IF3-GTP, (□) 70S bound neomycin + RRF-EFG-IF3-GTP. Antibiotics paromomycin (100 μM) and neomycin (10 μM) were bound to 0.1 μM of 70S ribosome and then mixed with the 0.5 μM of uBCAII or translation factor mix at concentrations stated in Figure 3.10.

The chaperoning activity of paromomycin bound ribosome was also studied using reactivaton assay of uBCAII (Figure 3.13).
Figure 3.13: BCAII reactivation in presence of paromomycin. Bar diagram shows the percent of BCAII (0.3 μM) reactivation in the absence of (1) ribosome (self), in the presence of (2) 70S ribosome and (3) ribosome bound paromomycin. The concentration of 70S ribosome in the refolding mixture was 0.3 μM.

As shown in Figure 3.13, binding of paromomycin or neomycin to 70S ribosome caused complete inhibition of uBCAII mediated subunit dissociation. Paromomycin bound ribosome however retains complete chaperoning ability (Figure 3.13) thereby indicating that the binding to the aminoglycoside antibiotics does not interfere with the ribosome-unfolded protein interactions necessary for chaperoning function. However, the differential effect of paromomycin on subunit dissociation and chaperoning ability of the ribosome shows that these two phenomenon, both involving the unfolded protein can occur mutually independently of each other.
CONCLUSIONS

Both in vitro and in vivo studies have demonstrated that the unfolded protein interacts with the PTC located in the domain V of 23S rRNA. Although this rRNA domain is not involved in intersubunit bridge formation, the physical proximity and communication between domain IV and the PTC in domain V has been supported by several mutagenesis and cross-linking experiments [Leviev I et al, 1995]. Recent structural studies revealed that the lateral arm of domain IV of 23S rRNA, that contains the components of several intersubunit bridges like B2a, B2b, B2c, lies beneath the PTC and forms the floor of the cavity where the acceptor ends of the tRNAs are located (and which corresponds to the binding site of the antibiotics chloramphenicol and blasticidin) [Yusupov MM et al, 2001; Hansen JL et al, 2003]. Indeed, in our studies the binding to the PTC antibiotics tighten subunit association and inhibit both unfolded protein and translation factor induced subunit dissociation. It is to be noted that at the concentrations of the antibiotics used in our experiment both the binding sites of chloramphenicol and blasticidin are expected to be occupied [Hansen JL et al, 2003]. Our subsequent studies with aminoglycoside antibiotics indicate towards the specific involvement of bridge B2a in the dissociation process although the strengthening of subunit association upon paromomycin binding [Hirokawa G et al, 2007] might also have contributed to such inhibition. Although the binding site of RRF on the 50S subunit and the sites of interaction of the unfolded protein reported earlier (binding site of RRF, [Samanta D et al, 2008]) are distinct, our antibiotic inhibition studies indicate that there might be a similarity between the mechanisms by which subunit dissociation is induced by either of these factors.

The ability of the unfolded protein to act as an antiassociation factor for the 50S subunit is however distinct from the recycling factor RRF [Hirokawa G et al, 2005]. The facts that RRF,
together with EFG, has ribosome dissociation activity and that RRF binds to 50S subunits at the intersubunit face had prompted the examination of whether RRF by itself has anti-association activity like IF3. These studies however had clearly established that RRF does not act as an antiassociation factor for the 50S subunit. Earlier studies on the binding site of the antiprion drug 6AP on the domain V of 23S rRNA (sites that coincide with the interaction sites of the unfolded protein) had made the observation that these interaction sites form a pocket at the subunit interface (chapter 1 section III.3; [Banerjee D et al, 2014]). Whether the binding of unfolded protein to domain V of 23S rRNA directly inhibits subunit association or changes the conformation of the 50S subunit such that it is unable to associate with the 30S subunit, requires to be further investigated. It should also be noted that unlike unfolded protein mediated dissociation (Figure 3.3), a strong antiassociation effect is observed even when uBCAII and the ribosomal subunits are present at stoichiometric concentrations (Figure 3.6). This observation therefore implies that the antiassociation activity of unfolded protein on isolated 50S subunit might contribute to but not totally account for the subunit splitting induced by unfolded proteins.

In this context mention can be made of an important GTPase family protein, obgE that has been shown to regulate translation rate under certain stressed condition. The ObgE peptide plays an important role as a 50S based antiassociation factor, which inhibits the formation of 70S ribosomes from its isolated subunits [Feng B et al, 2014]. Cryo-EM studies showed that ObgE prevents the binding of the 30S subunit by inducing significant conformational changes at several intersubunit bridging contacts on the 50S subunit, including B1a, B2a, and B4. The antiassociation activity of ObgE can have vital implications in protein synthesis and bacterial physiology. Under stress conditions ObgE delays the maturation of the 50S subunit as well as sequesters a large number of mature 50S subunits from taking part into translation, thereby
lowering the number of active 70S ribosomes and thus, regulating the rate of total protein synthesis in the cell.

Thus unfolded protein mediated dissociation of the empty 70S ribosome can also have broader implications. As discussed earlier, ribosome subunit dissociation after termination is a crucial step in translational regulation. Both the translational status of the cell and the intracellular concentration of unfolded proteins are important factors in determining cell physiology [Richter K et al, 2010; Ashe MP et al, 2000; Uesono Y et al, 2002] and under diverse stress conditions there is a simultaneous increase in cellular concentration of unfolded proteins and translationally inactive ribosome [Richter K et al, 2010; Ashe MP et al, 2000]. It has been observed that under heat stress large granular depositions composed of incorrectly processed ribosomal RNAs and aggregating ribosomal proteins are formed [Richter K et al, 2010]. The accumulation of stalled ribosome during cold shock conditions also downregulate protein synthesis [Richter K et al, 2010]. The protein synthesis in yeast has been shown to be inhibited by the removal of carbon source (glucose or fructose) and this effect was reversed upon addition of carbon source to the growth medium [Ashe MP et al, 2000].

Degradation of the ribosomes and the stable ribosomal RNA is often associated with conditions of starvation. Thus, depletion of any one of a number of nutrients including phosphate, nitrogen, carbon, or even Mg$^{2+}$ can trigger the degradation process. Degradation of ribosomes during starvation can be rapid and quite extensive, amounting to >95% in some studies [Davis BD et al, 1986; Kaplan R et al, 1975; Ramagopal S et al, 1984]. Interestingly, ribosome degradation appears to be an “all or none” phenomenon in that once breakdown of a ribosome begins; it goes to completion, whereas residual ribosomes remain intact [Davis BD et al, 1986; Kaplan R et al, 1975; Ramagopal S et al, 1984]. During exponential growth the ribosomal subunits are actively
recycled into the translating ribosome. Starvation reduces the growth rate and increases the isolated ribosomal subunits in cytosol as the translation machinery is less actively engaged for the production of new proteins [Zundel MA et al, 2009]. Ribosomes that are not engaged in translation and consequently present as subunits are more sensitive to degradation by their inherent nature.

Hence, the pathway of ribosome degradation upon starvation appears to proceed from polysomes to monosomes to ribosome subunits [Kaplan R et al, 1975]. Ribosomal degradation experiment was carried out in presence of the antibiotic neomycin, an antibiotic reported to inhibit subunit dissociation in vivo [Zundel MA et al, 2009]. In the presence of neomycin, ribosome degradation was inhibited by more than 60% which strongly support the ribosomal degradation during starvation in *Escherichia coli* is indeed initiated by dissociation of ribosome into its subunits [Zundel MA et al, 2009]. However, the signals controlling the progression of this phenomenon are not known. Hence, any physiological condition increasing the amount of subunits would, therefore, lead to enhanced degradation. Whether the ability of unfolded proteins to act as 50S subunit antiassociation factor and thereby sequester the 50S subunits could trigger stress induced subunit dissociation remains to be further investigated. The dose dependent increase in both antiassociation and dissociation activity of uBCAII and an increase in subunit dissociation in presence of increasingly unfolded protein indicate that the dissociation process is sensitive to the concentration and folding status of the protein encountered by the ribosome. Whether the ability of unfolded protein to act as antiassociation factor 50S subunit can lead to translational inhibition or in toxicity of accumulated unfolded proteins needs to be further investigated.