CHAPTER 4

Coaggregation of Ribosome during Protein Aggregate Formation: Contribution of Antichaperone activity of ribosomal RNA
INTRODUCTION

The ability of the molecular chaperones to govern the integrity of the proteome and play essential roles in cellular homeostasis has been discussed in chapter 1. They are involved in diverse functions ranging from protein folding, trafficking, connecting signaling pathways with protein homeostasis and even degradation of misfolded proteins that fail to refold [McClellan AJ et al, 2005; Young JC et al, 2003]. A defining characteristic of molecular chaperones is their ability to bind protein folding intermediates that is crucial for suppression of misfolding and aggregation. It has also been reported that several chaperones, depending upon the nature of the substrates and the environment, are also capable of facilitating aggregation of proteins [Puig A et al, 1994; Carrió MM et al, 1998; Primm TP et al, 1996; Kim PS et al, 1992; Bova MP et al, 1999]. Chaperones like protein disulfide isomerase, the Hsp70 class of chaperone BiP and β crystallin can facilitate aggregation of the client protein, an ability that has been termed as their antichaperone activity. In fact, it has been suggested that some chaperones can reduce accumulation of toxic soluble, oligomeric intermediates by facilitating the aggregation of disease associated proteins into benign amyloid-like aggregates [Douglas PM et al, 2009]. Recent additions to this list are nucleic acids that are also being considered as chaperone mediators. These molecules are capable of facilitating structural modification of proteins through conversion of α-helix to β-sheet [Biro JC, 2005]. The RNA chaperones also have been suggested to play a role in protein misfolding and aggregation. Noncoding, highly structured RNAs have been shown to facilitate the generation of cytotoxic oligomers both in vitro and in vivo [Gomes MP et al, 2008; Kampers T et al, 1996; Adler V et al, 2003; Silva JL et al, 2008; Lin H et al 2005]. Interestingly, a recent study has demonstrated that the antiprion drugs 6-aminophenanthridine and guanabenz can significantly reduce ribosome’s chaperoning ability by
selectively binding to the specific positions on the RNA1 subdomain that are necessary for interaction with the unfolded proteins (chapter 1 section III.3). The interaction of these drugs with the rRNA domain is also believed to be significant for the implementation of their antiprion activity, although the exact mechanism still needs to be elucidated. Also, our studies have demonstrated that the interaction of the partially folded proteins with this rRNA domain can interfere with aberrant intermolecular interactions and thereby suppress protein aggregation (chapter 2). Although extensive work has been performed to understand the ability of the ribosome to direct proteins to their right refolding pathway, further studies are necessary to elucidate the effect of ribosome and its 23S rRNA domain on the process of protein aggregation.

The aggregation of lysozyme and insulin under reducing conditions is well documented and is often used a model system for protein aggregation studies [Tomoyasu T et al, 2010; Bhattacharyya J et al, 1998]. Insulin is the primary hormone responsible for controlling the cellular uptake, utilization, and storage of glucose, amino acids, and fatty acids. Bovine insulin is composed of two peptide chain, A and B. The A and B chains are joined by two interchain disulfide bonds. The molecular weight of A chain is 2.4 kDa and B chain is 3.4 kDa. The B chain of insulin is prone to aggregation at higher protein concentration under reducing or in basic conditions. Our present study was aimed at investigating the effect of the E. coli ribosome and the domainV of its 23S rRNA on lysozyme and insulin aggregation under reducing conditions. Experiments were also performed with non-disulphide containing protein, bovine carbonic anhydrase II that is known to form aggregation prone molten globule like intermediate during its refolding [Semisotnov GV et al, 1987; Uversky VN et al, 1996]. Surprisingly, our studies revealed that interaction of the proteins with the ribosome initiates coaggregation of the ribosomal components leading to formation of ribosome-protein coaggregates. The
coaggregation process is suppressed when tRNA is bound to the ribosome which suggests that ribosome aggregation is initiated due to specific ribosome-protein interactions. Further, under these conditions the domainV of 23S rRNA displayed antichaperoning ability and stimulated aggregation of the proteins due to the formation of RNA-protein coaggregates. Such sustained nucleic acid-protein interaction that was observed with this rRNA domain within the intact ribosome might initiate the process of ribosome aggregation. The ability of the ribosome to direct proteins to their right refolding pathway under conditions permissive to protein folding is widely reported in literature. Our studies demonstrate a new aspect of interaction of the nontranslating ribosome with proteins under conditions that facilitate their aggregation and the potential of domainV of 23S rRNA to act as a nucleic acid aptamer that can stimulate protein aggregation. This study also provides a possible explanation for several recent observations that have shown the presence of ribosomal proteins in cellular aggregates both under physiological and diseased conditions. The ability to initiate aggregation of ribosomal components also indicates at a new aspect of cellular toxicity of protein aggregates.
MATERIALS AND METHODS

Materials

Bovine carbonic anhydrase II (BCAII), hen egg white lysozyme, bovine insulin, GuHCl, DTT, Tris-base and total E.coli tRNA were purchased from Sigma. Heparin and the yeast Saccharomyces cerevisiae 80S ribosome were kind gifts from the laboratories of. Dr.S.N.Kabir and Dr. Jayati Sengupta (IICB). Nitrocellulose filter was purchased from Millipore and reagents for molecular biology like T7 RNA Polymerase and RNase free DNase I was purchased from Fermentas. All other chemicals were local products of analytical grade. All data analysis was performed using OriginPro8 software.

Buffers

The buffers used in this study are the following: Buffer A: 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 100 mM NaCl; Buffer B: 50 mM potassium phosphate (pH 7.5); Buffer P, 50 mM Tris-HCl (pH 7.5), 7 mM MgCl₂, 30 mM KCl, 70 mM NH₄Cl.

Methods

Preparation of ribosome and the ribosomal subunits

Ribosomes were purified from E. coli MRE 600 cells and the ribosomal subunits were prepared as described in chapter 5.

Preparation of ribosomal RNA (rRNA) from subunits

The isolated ribosomal subunits 50S and 30S were incubated in 4M lithium chloride for 16 hours, centrifuged at 12,000 rpm for 25 minutes and the clear pellets were then dissolved in Buffer A. This rRNA solution was used for the lysozyme aggregation study in 1:1 molar ration to the protein.
**Binding of free 70S with total *E.coli* tRNA**

The binding of total *E. coli* tRNA to the free or empty ribosome was performed in Buffer-P as described earlier [Mondal S et al, 2014]. This tRNA bound 70S ribosome was then used for the aggregation studies of both lysozyme and insulin.

**Light scattering measurements**

Partially unfolded lysozyme and β chain of insulin aggregate under reducing condition and are widely used models for protein aggregation as stated earlier. In our studies the aggregation behavior of these proteins in presence of the ribosome and bDV RNA was monitored by turbidity measurements at 450 nm in Hitachi spectrophotometer (U-1900) and by light scattering measurements in a Hitachi spectrofluoremeter (F-2700) with excitation and emission set at 450 nm respectively. Aggregation studies with lysozyme and insulin were performed in Buffer A and Buffer B respectively in presence of 20 mM DTT.

**Studies with the ribosome**

Bovine insulin (20 μM) and of egg white lysozyme (10 μM) were incubated in 20 mM DTT with 70S ribosome (0.2 μM or 0.1 μM respectively). For measuring the effect of tRNA binding, the tRNA bound ribosome (stated above) was used for the aggregation studies.

**Studies with bDV RNA**

To study the effect of bDV RNA on lysozyme or insulin aggregation, 2 μM of the proteins were incubated with various concentrations (0.5 μM – 2 μM) of bDV RNA in presence of DTT in the respective buffers as stated above. To study the specificity of bDV RNA mediated protein aggregation stimulation, the proteins in 20 mM DTT were incubated for 45 min with equimolar concentrations of heparin (a nonspecific polyanion), total *E.coli* tRNA, *in vitro* transcribed
RNA1 and RNA2, model mRNA [Mondal S et al, 2014] and mDV RNA and the increase in scattering intensity was compared to that observed in presence of bDV RNA.

In the aggregation studies with BCAII, 200 μM of BCAII was denatured with 6 M guanidine hydrochloride and 3.5 mM EDTA for 3 hours at room temperature. Denatured BCAII was diluted to a final protein concentration of 2 μM in the absence or in presence of ribosome (0.02 μM) or bDV RNA (2 μM ) and the aggregation was monitored as stated above.

The light scattering experiments performed with the ribosome and bDV RNA were repeated five times and the data represents the average of all these experiments.

**Gel electrophoretic analysis of insoluble aggregate**

The proteins (lysozyme, insulin, BCAII) were incubated with the chaperones (70S ribosome, 80S ribosome, bDV RNA) for 60 minutes (insulin, lysozyme) or for 20 minutes (BCAII). The insoluble aggregates formed were separated by centrifugation at 21,380g for 20 minutes. The insoluble pellet were washed, resuspended in the respective buffers and subjected to 12% SDS – PAGE (lysozyme, BCAII) or 15% SDS-PAGE (insulin) for analysis of the proteins constituting the aggregates. The RNA incorporated in the aggregate was analyzed by 1% agarose gel electrophoresis of the resuspended pellet.

**Studies on protein-RNA interactions**

*Filter binding studies*

Filter binding studies were performed as described earlier studies [Das D et al, 2012; Chowdhury S et al, 2002]. The $^{32}$P-labelled bDV RNA was obtained by *in vitro* transcription (stated above) in presence of a $^{32}$P-UTP followed by DNaseI digestion and precipitation using ethanol. Lysozyme or insulin was incubated with equimolar $^{32}$P-bDV RNA in the presence of 20 mM DTT for various time intervals and filtered through pre-soaked nitrocellulose filter paper (Millipore) with
pore size of 0.22 μm. The filters were dried and $^{32}$P counts were taken in a liquid scintillation counter (Perkin Elmer). The RNA bound to the protein was retained on the filter while the free RNA passed through it. The percentage of radioactivity retained on the filter paper, calculated considering the radioactive count incorporated in the total RNA to be 100%, was plotted against time.

**Polyacrylamide Gel Electrophoresis**

In earlier studies, in order to demonstrate complex formation between prion protein that is positively charged at physiological pH and highly structured RNA aptamers, a semi-denaturing PAGE was used to ensure that the ribonucleoprotein complexes enter the gel [Adler V et al, 2003]. Since lysozyme is also positively charged at pH 7.5 that is maintained in our experiment, a similar gel containing 4M urea was run at room temperature to study the presence of internally labeled RNA1 in insoluble aggregate formed in presence of lysozyme and substoichiometric concentrations of RNA1. Lysozyme (2 μM) was incubated with 2 μM, 0.2 μM and 0.02 μM of $^{32}$P-labeled RNA1 in Buffer A at room temperature for 1 hour, centrifuged at 21,380g for 20 min, the insoluble pellet was washed and analyzed on 4 M urea / 6% polyacrylamide gel in TBE buffer (50 mM Tris–borate (pH 8.3), 1 mM EDTA). Gels were dried and analyzed by phosphorimaging. The varied molar ratios of RNA1 and lysozyme are indicated where appropriate.

**Electron Microscopy**

Lysozyme or insulin were incubated with 70S ribosome or bDV RNA for 1 hour in presence of 20 mM DTT and the samples were diluted 10-fold in Buffer A or Buffer B respectively before preparation of EM grid as stated earlier [Pathak BK et al 2014]. Imaging of aggregation in the refolding samples was done by using a transmission electron microscope (FEI
Tecnai12BioTwin). Control experiments were performed in which, 70S ribosome incubated with 20 mM DTT for 1 hour was also observed under identical conditions. To visualize the time course of ribosome-lysozyme interaction, 10 μM of lysozyme was incubated with 0.1 μM ribosome, aliquots of aggregation mix were withdrawn at 1 minute, 5 minutes, 10 minutes and 30 minutes from the initiation of incubation, diluted 10-fold and EM-grids were prepared immediately.
RESULTS AND DISCUSSION

Study of the effect of ribosome on aggregation of lysozyme and insulin

Lysozyme and the β chain of insulin undergo aggregation when exposed to high concentration of DTT. Lysozyme (10 μM) or insulin (20 μM) was incubated at room temperature with 20 mM DTT in Buffer A or Buffer B and proteins aggregation was measured by following the increase in turbidity at 450 nm (Figure 4.1).

![Figure 4.1: Effect of ribosome on aggregation of lysozyme and insulin. a) Time course of change in turbidity of lysozyme (10 μM) in 20 mM DTT, incubated in absence of 70S ribosome (■), in presence of 0.1 μM 70S ribosome (▲) and in presence of 1 μM 70S ribosome (●). The control experiment was done by incubating 70S ribosome in 20 mM DTT without lysozyme as marked as 70S ribosome (▼) in the figure. b) Time course of change in turbidity of insulin (20 μM) in 20 mM DTT, incubated in absence of 70S ribosome (■) and in presence of 0.2 μM 70S ribosome (●).](image)

As shown in Figure 4.1a, a significant increase in turbidity was observed in presence of lysozyme and the 70S ribosome even when the concentrations of ribosome present in the experiment were 10-fold (1μM) or 100-fold (0.1 μM) lower with respect to lysozyme (10 μM). No increase in turbidity was observed when the ribosome at these concentrations was incubated alone under the conditions stated above. Incubation of insulin with the 100-fold lower
concentration of 70S ribosome also showed significant stimulation of turbidity compared to that observed only in presence of the protein (Figure 4.1.b).

To analyze the composition of the insoluble aggregate denaturing polyacrylamide gel electrophoresis was performed (as stated in Materials and Methods) formed in presence of the proteins, the ribosome and 20 mM DTT (Figure 4.2).

Surprisingly, a significant fraction of the ribosomal proteins appeared in the pellet (aggregate) and a concomitant reduction in ribosomal proteins was observed in equivalent amount of the supernatant (Figure 4.2). Since several ribosomal proteins co-migrate with either the insulin β chain or lysozyme in SDS-PAGE, the intensity of the protein band does not reflect any change in

Figure: 4.2: SDS-PAGE analysis of protein aggregates. a) 10 μM of lysozyme was incubated in 20 mM DTT in the presence and in the absence of 70S ribosome for 1 hour at room temperature in Buffer A. After incubation both the samples were centrifuged, the insoluble aggregate washed and suspended in 40 μl of the same buffer. The supernatant was concentrated to a volume 40 μl using Amicon Ultra-10K filter. The resuspended pellet and supernatant were loaded on the 12% SDS-PAGE. Lane 1, pellet of the 70S + lysozyme; lane 2, pellet of self (absence of 70S); lane M, protein MW marker; lane 3, supernatant of self; lane 4, supernatant of the 70S + lysozyme.

b) 20 μM of insulin was incubated in 20 mM DTT in the presence and in the absence (self) of 70S ribosome and processed in similar way as describe for lysozyme. The samples were finally loaded on the 15% SDS-PAGE. Lane M, protein MW marker; lane 1, 20 μM native protein; lane 2, pellet of the insulin + 70S; lane 3, pellet of self ; lane 4, supernatant of the 70S + insulin; lane 5, supernatant of self.
level of aggregation of these proteins in presence of the ribosome. However, the contribution of the large number of ribosomal proteins per ribosome in the aggregate did explain the observed increase in turbidity even in presence of sub-stoichiometric ribosomal concentrations (1:100) as stated above. The relative partitioning of the ribosomal components into the insoluble and soluble fractions was however different in case of insulin and lysozyme.

To analyze the ribosomal RNA in the aggregates, agarose gel electrophoresis was performed as shown in Figure 4.3.

![Agarose gel electrophoresis of aggregates.](image)

**Figure 4.3: Agarose gel electrophoresis of aggregates.** a) 10 μM of lysozyme was incubated in 20 mM DTT in the presence of 0.1 μM 70S ribosome for 1 hour at room temperature, pellet down and the resuspended pellet loaded on the 1% agarose gel. b) 20 μM of insulin was incubated in 20 mM DTT in the presence of 0.2 μM 70S ribosome for 1 hour, pellet down and loaded on the 1% agarose gel.

Agarose gel electrophoresis of the aggregate obtained upon centrifugation showed that ribosomal RNA is also a component of the aggregate formed in presence of both lysozyme (Figure 4.3.a) and insulin (Figure 4.3.b).

The aggregation of ribosomal components in presence of lysozyme and insulin was visualized by electron microscopy. Bovine insulin (20 μM) and of egg white lysozyme (10 μM) were incubated in 20 mM DTT with 70S ribosome (0.2 μM or 0.1 μM respectively) and electron microscopy was performed as stated in Materials and Methods (Figure 4.4). The electron
microscopic image of the ribosome under reducing conditions was also included in the given figure (Figure 4.4.c).

Figure 4.4: Electron micrographs of proteins in presence of ribosome. a) Negative staining transmission electron micrographs of lysozyme incubated in 20 mM DTT in presence of 70S ribosome for 1 hour. b) Electron micrograph of insulin incubated in 20 mM DTT in presence of 70S ribosome for 1 hour. c) Electron micrograph of 70S ribosome incubated with 20 mM DTT for 1 hour. The samples were diluted 10-fold before preparation of EM-grid.

As shown in Figure 4.4, in case of lysozyme (Figure 4.4.a) a large amount of aggregates was observed in presence of ribosome while in the case of insulin (Figure 4.4.b), the presence of the ribosome triggers formation of structures that appear short, filamentous and organized. In
addition in both the cases no intact ribosomal particles were seen in the micrograph. The electron microscopic image of the ribosome under reducing conditions (Figure 4.4.c) showed that the appearance of the ribosome remains unaltered in presence of 20 mM DTT, thus implying that the observed ribosomal aggregation was induced by interaction with the proteins and was not due to the effect of reducing conditions used in the experiment. Figure 4.5 shows the EM image of self-aggregation of lysozyme and insulin in presence of 20 mM DTT.

Figure 4.5: Electron micrographs of protein aggregates. Electron micrographs of lysozyme (10 µM) (a) and insulin (20 µM) (b) after incubation in 20 mM DTT for 1 hour.

Electron micrograph shows the appearance of protein aggregate in reducing condition for both the protein lysozyme and insulin.

It was then necessary to assess whether protein induced ribosome aggregation is a universal phenomenon or is unique to the bacterial ribosome. For that we performed the lysozyme aggregation study in presence of yeast 80S ribosome (Figure 4.6).
Figure 4.6: Aggregation study with 80S ribosome. a) Time course of change in turbidity of reduced lysozyme (10 μM) in absence of yeast 80S ribosome (■), in presence of 0.1 μM 80S ribosome (●). b) 10 μM of lysozyme was reduced in the presence of 0.1 μM 80S ribosome and in the absence of 80S ribosome for 1 hour at room temperature in Buffer A, pellet down and dissolved in 40 μl of the same buffer and loaded on the 12% SDS – PAGE. Lane M, protein MW marker; lane 1, 10 μM native lysozyme; lane 2, lysozyme + 80S.

Incubation of lysozyme (10 μM) with substoichiometric concentration (0.1 μM) of yeast 80S ribosome in presence of 20 mM DTT also led to stimulated increase in turbidity (Figure 4.6.a) and SDS-PAGE analysis of the insoluble aggregate obtained after centrifugation showed the presence of ribosomal proteins (Figure 4.6.b). This study demonstrated that the eukaryotic yeast ribosome is also susceptible to co-aggregation with lysozyme under reducing conditions.

Effect of lysozyme concentration on the protein-ribosome coaggregation process

Further studies were conducted on the effect of protein concentration on lysozyme self-aggregation and protein-ribosome coaggregation under reducing conditions (Figure 4.7).
Lysozyme at a concentration of 65 μM rapidly aggregates leading to a large increase in turbidity at 450 nm. Although in the presence of 100-fold lower concentration of ribosome the increase in turbidity is comparable to that observed in absence of the ribosome (Figure 4.7.a), SDS-PAGE analysis of the aggregate revealed the presence of ribosomal components in the insoluble fraction (Figure 4.7.b). Hence, the ribosome-protein interaction leading to ribosomal aggregation persists even when protein-protein interaction is increased due to an increase in protein concentration. We also followed the time course of the ribosome coaggregation process by SDS-PAGE analysis of the insoluble aggregate obtained from aliquots of aggregation mix withdrawn at different time intervals after incubation of the protein with the ribosome (Figure 4.8).
Figure 4.8: Time course of ribosome coaggregation. 10 μM of lysozyme (20 mM DTT) was incubated in presence of 0.1 μM of 70S ribosome. After different time intervals from the initiation of the reaction, sample was withdrawn and centrifuged and resuspended pellet loaded on the 12% SDS – PAGE. Lane M, protein MW marker; lane 1, pellet after 1 minute; lane 2, pellet after 5 minutes; lane 3, pellet after 10 minutes; lane 4, pellet after 30 minutes.

As shown in Figure 4.8, appearance of ribosomal components in the aggregate is initiated as early as 1 minute after initiation of the aggregation process.

The time course of the ribosome lysozyme coaggregation process was also studied by electron microscopy (Figure 4.9).
Figure 4.9: Micrographs showing the time course of ribosome-lysozyme coaggregation. Micrographs were prepared from samples withdrawn at different time intervals from the initiation of incubation with DTT, a) after 1 minute; b) after 5 minutes; c) after 10 minutes; d) after 30 minutes of incubation.

The electron microscopic study was performed on the time course of lysozyme-ribosome coaggregation (Figure 4.9.a to 4.9.d) as stated in Materials and Methods. This study shows an initial clumping of the ribosome at 1 minute, followed by progressive loss of integrity of the ribosome from 5 minutes to 10 minutes time interval, ultimately culminating in aggregation of the ribosomal components by 30 minutes after initiation of interaction.
To test whether a preformed protein aggregate is also capable of triggering ribosome coaggregation process, 65 μM lysozyme was incubated in 20 mM DTT for 30 minutes (Figure 4.10).

Lysozyme undergoes almost complete aggregation under these conditions since the intensity of the protein band in the insoluble aggregate obtained upon self-aggregation is comparable to that of the total protein present in the experiment (Figure 4.10: lanes 1, 2). The aggregated lysozyme was incubated with 0.65 μM ribosome for one hour and analyzed by SDS-PAGE. As shown in Figure 4.10 (lane 3) the lysozyme aggregates also had the ability to stimulate ribosome coaggregation.
Effect of ribosomal subunits and tRNA on ribosome protein co-aggregation

In order to identify the site of interaction of aggregating proteins with the ribosome, a comparison of the extent of ribosome-protein coaggregation was studied in presence of the lysozyme or insulin and the isolated 50S and 30S ribosomal subunits (Figure 4.11).

**Figure 4.11: Aggregation study with ribosomal subunits.** a) Bar diagram shows the changes of turbidity of reduced lysozyme when incubated separately with 30S, 50S, 70S ribosome and tRNA bound ribosome as indicated in the figure. b) The changes of turbidity of reduced insulin when incubated separately with 30S, 50S, 70S ribosome and tRNA bound ribosome as indicated in the figure. Bar graphs represent the mean values (± standard deviations) from five independent experiments.

The bar diagram shown in Figure 4.11.a and 4.11.b represents the turbidity measured 30 min after initiation of incubation. For both insulin and lysozyme, a comparable increase in turbidity was observed both with the 70S ribosome and the isolated 50S subunit while comparatively lesser turbidity increase was observed in presence of the 30S subunit. Next, the 70S ribosome was incubated with the total *E.coli* tRNA under conditions that is conducive to P-site tRNA binding (Materials and Methods). The increase in turbidity upon interaction of the proteins with the tRNA bound ribosome was measured. For both insulin and lysozyme, the turbidity increase is considerably suppressed with the tRNA bound ribosome when compared to that of the empty 70S ribosome. Since tRNAs have specific binding sites on the ribosome, this observation implied that the ribosome aggregation process is due to specific ribosome-protein interaction.
Antichaperoning effect of ribosomal RNA on lysozyme aggregation

To determine the specificity of interactions between ribosomal RNA and lysozyme, aggregation process was monitored in presence of total rRNA, chemically extracted from the ribosomal subunits as described in the Materials and Methods. Lysozyme aggregation in presence of total rRNA was shown in Figure 4.12.

As shown in Figure 4.12, comparable increase in turbidity was observed both with the total rRNA from 50S subunit and the rRNA from 30S subunit while comparatively lesser turbidity increase was observed in presence of rRNA from 30S subunit.
Antichaperoning effect of domain V of 23S rRNA on protein aggregation

As stated above with both insulin and lysozyme, the comparable increase in ribosome-protein coaggregation was observed with the 50S subunit and 70S ribosome and the coaggregation was suppressed upon tRNA binding. The rRNA isolated from ribosomal subunit can also increase the light scattering in reducing condition.

The peptidyl transferase center that resides in the domainV of the 23S rRNA forms the major site of interaction of the tRNA with the 50S subunit [Polacek N et al, 2005]. In addition earlier studies have also demonstrated that the ability of domainV of 23S rRNA in an intact ribosome to interact with unfolded proteins which form the basis for the ribosomal chaperoning action [Das D et al, 2012]. Our next objective was therefore to study the effect of in vitro synthesized bDV RNA on the aggregation of insulin and lysozyme under reducing conditions (Figure 4.13).
Figure 4.13: Lysozyme and insulin aggregation in presence of bDV RNA. a and c) Time course of change in light scattering intensity of lysozyme (a) and insulin (c) (in 20mM DTT) at three different protein concentrations, 2 μM, 4 μM and 6 μM in the absence of bDV RNA (self) and in presence of equimolar bDV RNA as marked in the figure by different color code and bullets. b and d) The bar diagrams in the figure shows the change in light scattering intensity of lysozyme (b) and insulin (c) in presence of different molar ratios of bDV RNA.

When lysozyme or insulin was incubated at room temperature in 20 mM DTT at concentration of 2 μM, the proteins aggregated into particles sufficiently large to scatter light at 450 nm. The aggregation observed at this protein concentration was however moderate. In contrast, incubation with bDV RNA leads to stimulated aggregation of both the proteins (Figure 4.13.a and 4.13.c).
However, unlike the increase in turbidity that was observed even in presence of sub-stoichiometric concentrations of ribosome (Figure 4.1.a and 4.1.b) with respect to the protein, the presence of at least stoichiometric concentrations of the bDV RNA was necessary for protein aggregation stimulation (Figure 4.13.b and 4.13.d). In addition, while the antichaperoning effect of the bDV RNA was observed over a wide range of lysozyme concentrations, similar effect on insulin aggregation is highly concentration dependent and was observed only at 2 μM protein concentration.

The effects of bDV RNA on lysozyme and insulin aggregation process under reducing conditions was visualized by electron microscopy (Figure 4.14). Insulin and lysozyme (2 μM) were incubated in presence of equimolar concentration of bDV RNA and 20 mM DTT as stated above.

![Image](image_url)

**Figure 4.14: Electron micrographs in presence of bDV RNA.** a) Negative staining transmission electron micrographs of lysozyme (2 μM) in presence of 20mM DTT and bDV RNA (2 μM). b) Negative staining transmission electron micrographs of insulin (2 μM) in presence of 20mM DTT and bDV RNA (2 μM).

As shown in Figure 4.14, a significant increase in number of protein aggregates was observed in presence of the bDV RNA compared to that in its absence (Figure 4.5). The appearance of both amorphous protein aggregates and thread like structures (that is characteristic of images of
nucleic acid associated with proteins) were observed in presence of bDV RNA and either of the two proteins. However, unlike lysozyme, the thread-like structures are predominant in the micrographs of the sample containing insulin and bDV RNA.

**Studies with non-specific and ribosome binding polyanion**

Several earlier studies have proposed that non-specific electrostatic interactions between polyanions and proteins can influence protein-protein interactions, protein folding and their stabilization [Jones LS et al, 2004]. Hence the effect of nonspecific polyanion heparin or polyanions associated with the ribosome (tRNA, model mRNA; [Mondal S et al, 2014]), the RNA1 and RNA2 sub-domains of bDV RNA and mDV RNA on insulin and lysozyme aggregation were compared to that observed with bDV RNA (Figure 4.15).

![Figure 4.15: Studies with non-specific and ribosome binding polyanion](image)

*Figure 4.15: Studies with non-specific and ribosome binding polyanion* The bar graph represents the light scattering intensity of lysozyme (blue) or insulin (orange) in presence of bDV RNA, mDV RNA, tRNA, mRNA, heparin, RNA1 and RNA2 sub-domain of bDV RNA and also in the absence of any modulator (self). Bar graphs represent the mean values (± standard deviations) from five independent experiments.
As shown in Figure 4.15, the presence of stoichiometric concentrations of nonspecific polyanions heparin, total *E.coli* tRNA or a model mRNA, had no effect on lysozyme or insulin aggregation. As stated earlier, truncations in the RNA2 sub-domain of mDV RNA delays the release of the bound protein from mDV RNA compared to bDV RNA [Das A et al, 2011]. However, the comparable antichaperoning effect of mDV RNA and bDV RNA on protein aggregation implied that the antichaperoning abilities of the RNA molecules are not affected by the delayed release of protein from the mDV RNA. Further, the RNA corresponding to the RNA1 sub-domain alone could stimulate lysozyme but not insulin aggregation. No stimulation of aggregation was observed in the presence of RNA2 sub-domain for both insulin and lysozyme. Control experiments were also performed to confirm that the RNA themselves did not precipitate or scatter light at the wavelength in which the experiments were performed.

**Sustained nucleic acid-protein interaction under reducing conditions**

**Filter binding studies**

As stated above, earlier studies on the mechanism of bDV RNA chaperoning activity had shown that it is a two-step process involving the two subdomains RNA1 and RNA2 and the protein (BCAIi) bound to bDV RNA is released within 180 seconds from the initiation of interaction [Das D et al, 2012]. In the present study, filter binding analysis was performed with two different concentrations (2 μM and 0.5 μM) of lysozyme or insulin and stoichiometric concentration of $^{32}$P labeled bDV RNA. The radioactivity retained on the filter represents the RNA-protein complex present at each time point after initiation of interaction. Figure 4.16 shows the results of filter binding study for both lysozyme and insulin under our experimental conditions.
Figure 4.16: Filter binding study. Time course of interactions of $^{32}$P bDV RNA with lysozyme (a) or insulin (b) at two different protein concentrations, 0.5 μM (●) and 2 μM (■) is shown in the figure. The molar ratio of protein: bDV RNA was 1:1.

As shown in Figure 4.16, the 1) binding of the proteins to bDV RNA is a slow process compared to that reported earlier [Das D et al, 2012], 2) the bound proteins were not released even after 1 hour, a situation that bears resemblance to the lack of release of reduced and denatured lysozyme from bDV RNA [Pathak BK et al, 2014] 3) comparable time course of RNA-protein interaction is observed at both protein concentrations used in the experiment.

**Gel electrophoresis studies of aggregates**

The protein composition of the insoluble aggregate obtained upon centrifugation (stated above) of lysozyme (2 μM) in presence of bDV RNA (2 μM) was analyzed by SDS-PAGE gel electrophoresis (Figure 4.17.a). Agarose gel electrophoresis of the aggregate was also performed in order to determine whether the RNA coaggregates with the protein under the experimental conditions (Figure 4.17.b).
As shown in Figure 4.17.a, the amount of lysozyme in the insoluble aggregate formed in presence of bDV RNA was comparable to the total protein, while marginal protein is observed in the aggregate in absence of the RNA thereby providing further evidence of RNA induced aggregation stimulation. The intensity of the RNA band in the precipitate observed upon ethidium bromide staining of the agarose gel was comparable to that of total bDV RNA present during the experiment thereby indicating that almost the entire RNA is precipitated as a RNA-protein aggregate (Figure 4.17.b). However when insulin (2 μM) was incubated with bDV RNA under identical conditions, no insoluble aggregate could be obtained by centrifugation at a speed of 21,380g used in this experiment.
It is to be noted that while both the bDV RNA and RNA1 fragment of bDV RNA stimulated lysozyme aggregation only when present at stoichiometric concentrations (Figure 4.15), stimulation of turbidity was observed even in presence of substoichiometric ribosome concentrations (Figure 4.1.a). In order to investigate whether RNA-protein aggregates were formed even in presence of substoichiometric concentrations of the RNA which was evaded detection in light scattering studies, the following experiment was performed. The RNA1 fragment of bDV RNA was synthesized by *in vitro* transcription in presence of $[\alpha-^{32}P]$ UTP. The RNA (2 μM, 0.2 μM or 0.02 μM) was incubated with lysozyme (2 μM) in presence of 20 mM DTT and the insoluble aggregate obtained by centrifugation was analyzed by 6% semi-denaturing polyacrylamide gel (Materials and Methods) (Figure 4.18).

![Figure 4.18: Analysis of lysozyme-RNA1 aggregate by urea-PAGE. 2 μM of lysozyme was reduced in the presence of different molar ratio of $[\alpha-^{32}P]$ UTP labeled RNA1 for 1 hr at room temperature, centrifuged, pellet washed and resuspended in 20 μl of Buffer A and loaded on the 6% urea (4M) PAGE. Lane 1, 2 μM RNA1 as control; lane 2, pellet of 2 μM lysozyme + 2 μM RNA1; lane 3, pellet of 2 μM lysozyme + 0.2 μM RNA1; lane 4, pellet of 2 μM lysozyme + 0.02 μM RNA1.](image-url)
Under these conditions, the internally labeled RNA1 was observed in the pellet even when present at a 100-fold lower concentration with respect to the protein (Figure 4.18). The binding of RNA to lysozyme (2 μM) however did not result in the retarded migration of RNA1, possibly because the ribonucleoprotein complex was dissociated in presence of 4M urea present during electrophoresis.

**Effect of ribosome and bDV RNA on BCAII aggregation**

It was then necessary to investigate whether the formation of protein-ribosome coaggregates (observed with lysozyme and insulin) is unique to disulfide containing proteins or is a general phenomenon. The protein bovine carbonic anhydrase II (BCAII) was used for this study. The refolding yield of this protein, when folded from its completely unfolded state, is low due to the formation of aggregation prone molten globule intermediate formed during its refolding [Semisotnov GV et al, 1987; Uversky VN et al, 1996]. The time course of denatured BCAII aggregation is shown below (Figure 4.19). When BCAII was denatured with 6M GuHCl and diluted 100-fold such that the final protein concentration is 2 μM, the protein undergoes aggregation as revealed by both the increase in turbidity and light scattering at 450 nm (Figure 4.19).
Figure 4.19: Effects of ribosome and bDV RNA on BCAII aggregation. a) Time course of change in turbidity of unfolded BCAII diluted in Buffer A to maintain final protein concentration 2 μM in absence of 70S ribosome (■) and in presence of 0.02 μM 70S ribosome (●). The control experiment was done by incubating 70S ribosome in the presence of 0.06 M guanidine hydrochloride without BCAII as marked as 70S (▼) in the figure. b) Time course of change in light scattering intensity of unfolded 2 μM of BCAII in absence of bDV RNA (■) and in presence of 2 μM bDV RNA (●).

As with lysozyme and insulin, a significant increase in turbidity at 450 nm was observed when BCAII was incubated in presence of 0.02 μM ribosome. A stimulated increase in light scattering at 450 nm was also observed when BCAII (2 μM) was incubated along with stoichiometric concentration of bDV RNA (Figure 4.19.b).

SDS-PAGE analysis was also performed for the study of BCAII aggregation in presence of 70S ribosome or bDV RNA (Figure 4.20).
Figure 4.20: SDS-PAGE analysis of BCAII aggregates. a) Unfolded BCAII was diluted 100 times to maintain final protein concentration 2 μM in Buffer A in presence or absence of 70S ribosome, incubated for 20 minutes centrifuged, pellet washed and resuspended in 40 μl of the same buffer. The supernatant was concentrated to a final volume 40 μl. Resuspended pellets and supernatant were loaded on the 12% SDS-PAGE. Lane 1, pellet of 2 μM BCAII self (absence of 70S); lane 2: pellet of 2 μM BCAII + 70S ribosome; lane 3, supernatant of the 70S + BCAII; lane 4, supernatant of self.
b) Unfolded BCAII (2 μM) was incubated in the absence or presence of bDV RNA (2 μM) for 20 minutes in Buffer A. After incubation sample was centrifuged, and resuspended pellet loaded on the 12% SDS – PAGE. Lane 1, pellet of 2 μM BCAII self (absence of bDV RNA); lane 2, pellet of 2 μM BCA + bDV RNA.

SDS-PAGE showed that ribosomal components are present in the insoluble aggregate (Figure 4.20.a). SDS-PAGE analysis of the insoluble precipitate confirmed the bDV RNA stimulated aggregation of BCAII (Figure 4.20.b).
CONCLUSIONS

The ability of the ribosome to assist in folding of proteins is widely reported in literature. Extensive work has been performed on the mechanism by which the domain V of the 23S rRNA engages in transient specific interaction with unfolded proteins and releases them in their folding competent states [Das D et al, 2008; Das D et al, 2012]. The chaperone is also capable of protecting partially folded proteins from aggregation [Pathak BK et al, 2014]. Our present study is the first report that a) demonstrates a new aspect of ribosome-protein interaction in which the interaction of the ribosome with partially folded proteins triggers ribosome-protein coaggregation and b) highlights the antichaperoning ability of bDV RNA and hence the potential of this RNA domain to act as a nucleic acid aptamer that can induce protein aggregation.

Coaggregation of proteins with the ribosome

The coaggregation of ribosomal components leads to the formation of insulin - or lysozome - ribosomal proteins-rRNA coaggregates. The coaggregation process was monitored by turbidity measurements at 450 nm with time and the insoluble aggregate components were confirmed by SDS-PAGE and agarose gel electrophoresis. Experiments with lysozyme in presence of the 80S ribosome and with BCAII in presence of *E. coli* 70S ribosome showed that the coaggregation process is a universal one and occurs a) with both prokaryotic and eukaryotic ribosomes and b) upon interaction of the ribosome with both disulfide and non-disulfide containing proteins. Visualization of protein-ribosome coaggregates by electron microscopy revealed the appearance of the coaggregates and the absence of any ribosome-like particles. Although both insulin and lysozyme induce ribosome coaggregation, the appearances of the aggregates formed with the two proteins are distinctly different. The appearance of the ribosomal particles however remained unaltered in presence of 20 mM DTT. This, together with the fact that the ribosome retains its
translational ability even in presence of 30 mM DTT [Horan LH et al, 2007], implies that aggregation of the ribosomal components is not due to the effect of the reducing environment on the ribosome but is mediated by its interaction with proteins under the conditions used in our study. Electron microscopic analysis of lysozyme induced ribosome coaggregation showed that the process is initiated early and proceeds in a stepwise manner starting from clumping of the ribosomes to the gradual loss of integrity of the ribosome structure and ultimately to the aggregation of ribosomal components. This process is depicted in the model proposed for lysozyme-ribosome coaggregation (Figure 4.21).

![Figure 4.21: Ribosome-protein coaggregation model Sustained](image)

**Figure 4.21: Ribosome-protein coaggregation model Sustained** Noncovalent interactions between the domainV of 23S rRNA of bacterial large subunit and lysozyme initiates ribosomal disassembly. Ribosome-protein coaggregation proceeds in a stepwise manner starting from clumping of the ribosomes to the gradual loss of integrity of the ribosome structure and ultimately to the aggregation of ribosomal proteins and rRNA.
Our studies with lysozyme and the ribosome under reducing conditions also showed that the coaggregation process can also be induced upon incubation of the ribosome with preformed lysozyme aggregates. The significant suppression of turbidity observed upon tRNA binding to 70S ribosome however implied that protein-ribosome coaggregation is not a nonspecific process and occurs due to specific ribosome-protein interaction. The stimulation of lysozyme aggregation in presence of ribosomal RNA extracted from isolated 50S and 30S subunits indicated that the rRNA derived from 50S subunit can stimulate protein aggregation greater than that derived from the 30S subunit. The domain V of 23S rRNA of the large ribosomal subunit interacts with both the 3’-CCA end of tRNA during translation [Polacek N et al, 2005] and with the unfolded proteins during ribosomal chaperoning activity [Das D et al, 2012]. Hence the interaction of proteins with this RNA domain might be involved in the ribosome-protein coaggregation process.

Incubation of insulin and lysozyme (in presence of 20 mM DTT) or refolding BCAII (unfolded with guanidine hydrochloride) with stoichiometric concentrations of bDV RNA lead to stimulation of aggregation and formation of RNA-protein aggregates. The comparable aggregation stimulation with both bDV RNA and mDV RNA and the filter binding studies with bDV RNA and insulin or lysozyme indicated that sustained RNA-protein interaction is involved in the antichaperoning activity. Presence of the RNA1 sub-domain in the insoluble aggregates formed in presence of 100-fold lower concentrations of $^{32}$P labeled RNA1 and reduced lysozyme showed that RNA-protein interactions occurs even at substoichiometric RNA concentrations that is comparable to the ribosome: protein ratio present during lysozyme induced ribosome aggregation. Since the intact ribosomes require numerous protein-protein and protein-RNA interactions to maintain its integrity, such stable interactions of the partially folded proteins with
domainV of 23S rRNA might act as an initial trigger in the disruption of its normal structure resulting in the formation of protein-ribosome coaggregates. However the reason behind the increase in turbidity observed upon incubating insulin and lysozyme with the 30S subunit, albeit lower than that observed with the 70S ribosome or the 50S subunit, still remains to be identified. Some aspects of the ribosome-protein coaggregation reported here needs further investigation. Although lysozyme, insulin and BCAII undergo ribosome-protein coaggregation and show stimulated aggregation in presence of domainV of 23S rRNA, certain protein specific differences have been observed with respect to the partitioning of the ribosomal proteins into soluble and insoluble fractions and morphology of the ribosome-protein coaggregates. With respect to the antichaperoning effect of bDV RNA, insulin and lysozyme differ in the effect of protein concentrations and the ability of the RNAI fragment to stimulate protein aggregation. Whether the difference in size and net charge of the β chain of insulin (that undergoes aggregation), BCAII and hen egg white lysozyme under our experimental conditions is responsible for the observed variations needs to be further analyzed. Further, as stated earlier, the ability of the bacterial ribosome (when present in stoichiometric concentrations) to significantly improve the refolding yield of proteins is well established. It is to be noted that in these studies the proteins were present in low concentrations in an environment that is conducive to protein folding while in our studies the proteins have a tendency to aggregate due to the environment, the intrinsic nature of the protein folding intermediates or the protein concentrations present during the experiment. Although the domainV of 23S rRNA might be involved in both aspects of ribosome-protein interaction, further studies are needed to define the conditions which lead to either protein folding or ribosome aggregation. The biological relevance of the observations presented here is discussed below.
**Biological relevance of formation of protein-ribosome coaggregates**

Several recent studies point towards the ability of non-coding, highly structured RNA to initiate pathogenic transformation of proteins both *in vitro* and *in vivo*. *In vitro* synthesized small, highly structured RNA can initiate the conversion of the prion protein from the Prp\(^C\) to Prp\(^sc\) form and their expression in transgenic *Drosophila melanogaster* leads to the aggregate accumulation *in vivo* and a neurodegenerative phenotype [Adler V et al, 2003; Savvateeva-Popova E et al, 2007]. Further, the total RNA extracted from yeast or bovine liver can promote the assembly of the Tau protein into the Alzheimer’s disease associated paired helical filament (PHFs) like structures and cellular RNA isolated from neuroblastoma cells (N2a RNA) can induce accumulation of Prp-RNA aggregates *in vivo* [Gomes MP et al, 2008; Kampers T et al, 1996]. The antichaperoning ability of bDV RNA observed in our study, also demonstrates the potential of this rRNA domain to act as aggregation inducing RNA aptamer. Incidentally, recent studies have implicated this rRNA domain to be the principal target for antiprion drugs, 6-aminophenanthridine and guanabenz [Tribouillard-Tanvier D et al, 2008; Pang Y et al, 2013].

The observations presented here are also relevant to several recent reports suggesting that the disruption of the translation apparatus might be a widespread phenomenon. These studies show that the ribosomal proteins might represent important targets that must be protected against the formation of non-native protein structures and aggregates during stress conditions [Maisonneuve E et al, 2008; Mirzaei H et al, 2006; Ding Q et al, 2005]. The *in vivo* accumulation of protein aggregates that are predominantly composed of ribosomal components has also been reported. Mutant yeast cells lacking gene for Tsa1 protein (a ribosome associated molecular chaperone having peroxidase activity; [Weids AJ et al, 2014]) were found to accumulate aggregated proteins, and this was exacerbated when the cells were subjected to reductive stress in presence
of 20 mM DTT. The analysis of the protein aggregates revealed that they are predominantly composed of ribosomal proteins [Trotter EW et al, 2008; Rand JD et al, 2006]. In addition, studies on the effect of deletion of ribosome associated chaperone network (Hsp70/Hsp40 system SSB-RAC and the nascent chain polypeptide associated complex) in yeast cells, also demonstrated a strong reduction in 80S ribosome and polysome peaks together with aggregation of ribosomal biogenesis factors and ribosomal proteins [Koplin A et al, 2010]. Further, neuronal death that occurs during focal brain ischemia has been attributed to the irreversible aggregation of translational complex components like the ribosomes, its associated nascent polypeptides, translational initiation factors and co-translational chaperones [Zhang F et al, 2006; Liu CL et al, 2005]. Although it has been proposed that disruption of the assembly and processing of new ribosomes or the cotranslational aggregation or misfolding of the nascent proteins is responsible for these observed phenomena [Weids AJ et al, 2014; Trotter EW et al, 2008; Rand JD et al, 2006; Koplin A et al, 2010; Zhang F et al, 2006; Liu CL et al, 2005], the exact mechanism of ribosomal aggregation still remains to be identified. Our studies suggest that the misfolded aggregation-prone proteins, whose cellular levels are likely to increase under all the aforementioned conditions, could themselves target the non-translating ribosomes or its subunits and trigger the aggregation of ribosomal components. Also, the ability of protein aggregates to target multiple cellular processes in both prokaryotic and eukaryotic cells forms the basis of their toxicity [Stefani M et al, 2003]. The possibility of formation of the protein-ribosome coaggregates between partially folded proteins and non-translating ribosome as observed in this study might also form an important aspect of protein aggregate induced cellular toxicity.

In chapter 2 we show that the ribosome and domain V of 23S rRNA can interact with BCAII molten globule (BCAII-m) and increase the reactivation yield. These chaperones can also
prevent aggregation during refolding of BCAII-m and lysozyme from its reduced and denatured state. In contrast studies presented in this chapter (chapter 4) shows that the ribosome and its domain of 23S rRNA displays antichaperone activity and facilitates the aggregation process of lysozyme and BCAII under specified conditions (i.e reducing condition for lysozyme and in higher protein concentration for BCAII). Earlier studies with protein disulphide isomerase and alpha crystalline had demonstrated the ability of these chaperones to both stimulate and suppress protein aggregation. Further studies are necessary to explain the mechanism of diverse role of the ribosomal chaperone on the same set of client proteins and the role of experimental conditions in determining the outcome of ribosome-protein interaction.