Research Highlight

Role of the ribosome in protein folding

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In all organisms, the ribosome synthesizes and folds full length polypeptide chains into active three-dimensional conformations. The nascent protein goes through two major interactions, first with the ribosome which synthesizes the polypeptide chain and holds it for a considerable length of time, and then with the chaperones. Some of the chaperones are found in solution as well as associated to the ribosome. A number of *in vitro* and in *vivo* experiments revealed that the nascent protein folds through specific interactions of some amino acids with the nucleotides in the peptidyl transferase center (PTC) in the large ribosomal subunit. The mechanism of this folding differs from self-folding. In this article, we highlight the folding of nascent proteins on the ribosome and the influence of chaperones etc. on protein folding.

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Proteins are synthesized as unbranched linear polypeptide chains that fold into 3-D active states. If all the degrees of freedom of individual amino acids in the polypeptide chains are considered the calculated time scale of folding by random search becomes enormous [1] compared to the lifetime of a cell. Anfinsen experimentally followed the folding of the full-length enzyme ribonuclease by chemically unfolding it and then dialyzing out the chemical, observing the reappearance of its activity. Reappearance of crucial interactions like disulphide bond formation between specific amino acids was also observed. The conclusion from these experiments, as stated in his Nobel lecture, was that "the native conformation is determined by the totality of interatomic interactions and hence by the amino acid sequence, in a given environment".

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Abbreviations: BCA, bovine carbonic anhydrase; **IF-3,** initiation factor 3; **PTC**, peptidyl transferase center; **RRF,** ribosome-recycling factor

Measurements of relevant physical parameters, local conformations of polypeptides, etc. led to the following possible modes of protein folding:

(i) Rapid formation of the secondary structures through near-neighbor interactions between amino acids, followed by tertiary interaction among them to give rise to the native state.
(ii) Rapid hydrophobic collapse of the polypeptide chain upon removal of the denaturing chemical leading to the formation of a nearly globular state. The tertiary interactions among amino acids which subsequently take place slowly in the restricted state.
(iii) Adjacent residues forming a nucleus from which the native structure develops sequentially.

These schemes eventually confronted two things:

(i) The intracellular protein concentration is too high to allow individual protein molecules to fold freely without being influenced by the neighboring macromolecules.
(ii) A number of chaperones were discovered as proteins, which help others to fold or be rescued from misfolding and aggregation by protectively sequestering their hydrophobic patches [2]. This property is extremely relevant in the con-

Table 1. The recent estimation of the fraction of the total protein in the cell that can be folded by different chaperones (adopted from reference [16])

Folding of proteins by	Fraction of protein folded in prokaryotic cytosol (size range)	Fraction of protein folded in eukaryotic cytosol
DnaK-J	5-18% (>30 kDa)	
GroEL-ES	10-15% (~20-60 kDa)	
Unassisted	67%	
Hsp70/Hsp40		~ 15-22%
Prefoldin/CC		Actin/tubulin etc
Unassisted		Not known precisely

centrated protein solution in the cell. The apparently nonspecific chaperone-folding protein interaction often reiterates with ATP hydrolysis to unfold misfolded proteins for refolding and disassembling protein aggregates. This enhances self-folding of proteins without altering the intrinsic mechanism of this process.

The recent estimation of the fraction of the total protein in the cell that can be folded by different chaperones is given in Table 1 (adopted from reference [16]). The question is what happens to the vast majority of the proteins whose folding is not influenced by the chaperones.

1 The search for a protein-folding activity in ribosomes

Some of the experiments, designed to assess the role of chaperones on the folding of proteins still bound to the ribosome in *Escherichia coli*, involved termination of translation by puromycin or chilling the translating complex to delay the release of the nascent full-length proteins from the ribosome. It was found that the polypeptides with long, but not short peptide linkers at C termini [3], could fold without chaperones when linked to the ribosomes. If the ribosome was responsible for folding, the long linker probably ensured that the actual protein part could access the folding center of the ribosome while the short linker failed to do it if the linker ends were away from the folding center. Therefore, the full-length proteins folded on the ribosomes and the folding was post-translational. These experiments and the fact that on the average about 67% proteins in the cells could fold without assistance from the chaperone suggested strongly that a thorough search should be conducted to check if there is any intrinsic protein-folding activity in the ribosome.

In the past, considering the chaperones as the sole protein folding modulators, "unassisted" folding was estimated to cover the majority of the proteins in any cell. However, strangely, the ribosomes were not clearly implicated in the "unassisted" [4] and the "default" [4] pathway of folding, although (i) the full-length nascent proteins stay on the ribosomes for a considerable length of time; thus, their folding is likely to be influenced by the ribosome [4–7]; (ii) the length of protein chains in this group varies widely, from small to very large and they do not have any unusual structural feature that would allow them to fold without any assistance in the crowded environment of the cytosol, and, as mentioned above, (iii) the addition of a significant length (~25) of additional amino acids to the C termini of proteins made it possible for them to attain active state even when linked to the ribosomes [3, 8–10]. Addition of chaperones could enhance this folding in some cases [10].

These points make it pertinent to check if the ribosomes from all the cells and sub-cellular organelles can contribute to protein folding.

Table 2. List of proteins folded by ribosomes (all proteins tested so far could be folded without exception)

In vitro	*In vivo*
Bacterial alkaline phosphatase	Beta galactosidase
Glucose 6-phosphate dehydrogenase	Beta lactamase
Glucose oxidase	Carbonic anhydrase
Lactate dehydrogenase	GFP
Malate dehydrogenase	DnaK
Bovine carbonic anhydrase	HspH
Human carbonic anhydrase	Luciferase
Beta lactamase	
Beta galactosidase	
Restriction endonuclease EcoR1	
Restriction endonuclease BamH1	
Restriction endonuclease HindIII	
DnaK	
HspH from *Bradirhizobium japonicum*	
GFP	
Ricin-A chain	
T7-RNA Polymerase	
Rhodanese	
Horseradish peroxidase	

2 Protein synthesis and folding, two activities of the ribosomes from all sources

A large number of proteins were found to refold from denatured states when incubated with ribosomes from varied sources like eubacteria (*E. coli* and *B. subtilis*) [11–13], archaebacteria [14], eukaryotes (rat liver, wheat germ) [15] and mitochondria (bovine, Leishmania) (unpublished data and [16]). The proteins are listed in Table 2. For each protein, the level of regain in activity with ribosome was around 80-100%. There was no bias in selecting the proteins and all the proteins tested so far could be folded by the ribosome. The ribosomes from *E. coli* used in these studies were isolated following protocols to purify chaperone-free ribosomes [10] and they were tested to be free from DnaK and GroEL [15].

On separation of the subunits, the activity was detected in the large ribosomal subunit from all these sources. When the ribosomal proteins were increasingly depleted from the large ribosomal subunit with higher and higher concentration of lithium chloride, the activity remained in the core, in its large RNA (*e.g.* 23S rRNA in case of *E. coli*). Finally, the region in the domain V of *E. coli* 23S rRNA having the peptidyl transferase activity (the peptidyl transferase center, PTC) was found to have protein-folding activity. In fact, the PTC in the ribosome from any source has protein-folding activity [5, 17–21]. Even when this segment of ribosomal RNA was transcribed *in vitro*, it could fold denatured protein equally well. Therefore, both the synthesis [22] and folding of proteins are functions of the PTC.

The antibiotics that bind to PTC to stop translation also inhibit protein folding *in vitro* [5]. This gave a good experimental system to assess the ability of ribosomes to fold protein post-translationally *in vivo*. Chloramphenicol and Lincomycin bind to the PTC in the 50S subunit to stop protein synthesis and folding. Kasugamycin and Kirromycin bind to the 30S subunit and EF-Tu, respectively, in *E. coli* to stop protein synthesis without affecting folding of proteins synthesized just prior to their addition. Thus, the difference in the activities of proteins in the presence of, say chloramphenicol and kasugamycin would be due to folding of proteins synthesized just prior to their addition. This assay showed that ribosomes fold proteins *in vivo* [5].

3 Steps in ribosome-mediated protein folding

From the results of investigations made in various laboratories to study protein folding *in vitro* and *in vivo* using eubacterial ribosomes, the following steps appear to be traversed by the nascent polypeptide before it becomes active:

(i) Synthesis of a polypeptide chain on the ribosome followed by the splitting of the ribosome into subunits [23–25].

(ii) The polypeptide chain anchoring on the large loop of the PTC (Fig. 1) in the 50S subunit to reach a folding competent state [24, 25].

(iii) Release of the folding competent protein from the PTC with the assistance from the part of the domain outside the large loop [19] in prokaryotic and eukaryotic ribosomes [22]. In ribosomes from organelles like mitochondria, chloroplasts, etc., the regions outside the large loop in the PTC in domain V counterparts are often too small to act in releasing folding competent proteins. Instead, some large subunit proteins substitute the RNA component and interact with PTC. These proteins are much larger than their eubacterial counterparts and assist the release of "folding competent" [16] proteins from the PTC (unpublished data). Therefore, both translation and folding activities of the PTC are assisted by other factors.

(iv) The folding competent protein takes the final shape when the ribosome releases it in the cytosol. This is the slowest of all the steps [4, 5] and largely influenced by the composition of the cytosol, especially in respect to proteins, which have hydrophobic patches exposed. Such proteins, like the chaperones, interact with the folding competent state because it may still have some hydrophobic amino acids exposed. Chaperones, like DnaK-J, Trigger factor, etc., are known to associate with ribosomes [2, 16] and interact with nascent proteins on their release from the ribosome. This interaction works positively if the chaperone can assist the folding of the protein. As a result, we see a contribution of the chaperones in protein folding. However, the effect is likely to be negative if the chaperone binds, but cannot assist folding. There are ample examples of proteins that cannot be folded by specific chaperones [26–28] and interaction with them generally leads to a loss of protein activity. The implications of all these findings are explained later in the review. First, we shall describe what is known about the mechanism of ribosomal PTC-mediated protein folding.

Biotechnol. J. 2008, 3, 999–1009

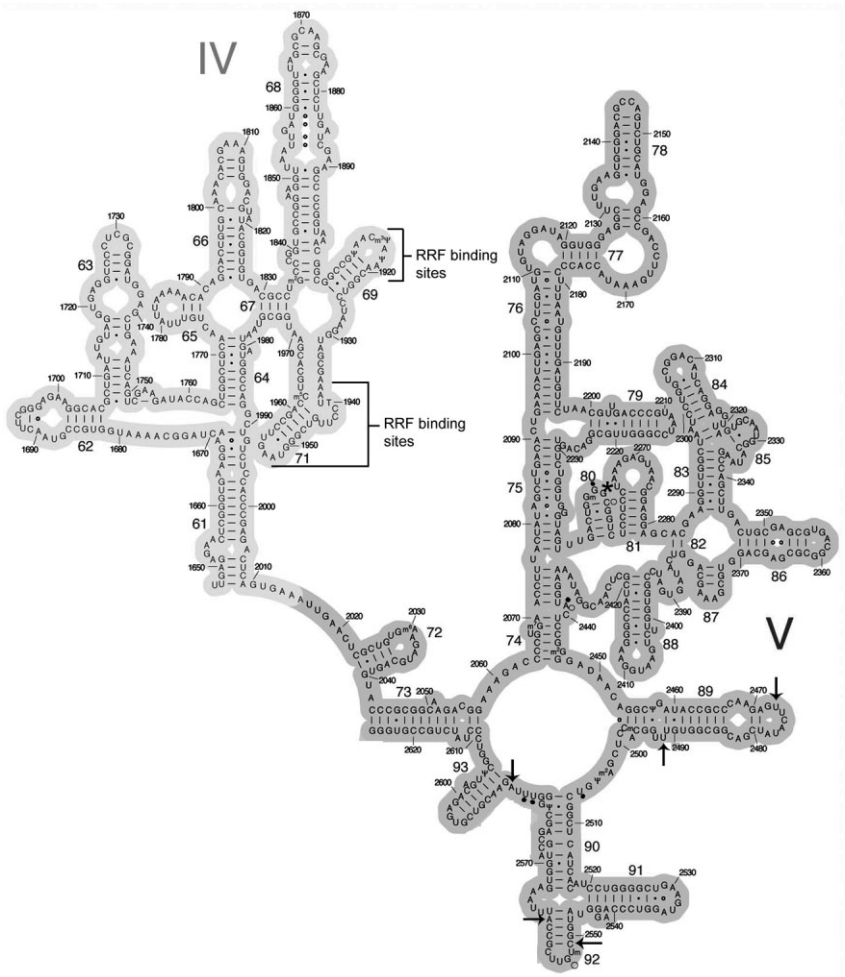

Figure 1. Secondary structure of domain IV and V of *E. coli* 23S rRNA showing sites for interaction of RRF (multiple sites of interaction in bracket and single site marked with *), unfolded protein (arrows) and P (closed circle) and A-site (open circle) tRNA.

4 A general protein-folding property suggests the presence of specific folding mold in the ribosome

The presence of a general protein-folding activity in ribosomes suggests the existence of a specific protein-folding mold. The folding pathway of a protein in the presence of a ribosome is different from its self-folding [29]. Any general function of a nucleic acid polymer (DNA or RNA) is always related to particular sequence/sequences. The protein-folding activity was also related to specific nucleotides in the large loop of the PTC in the ribosome [21]. These nucleotides were identified by primer extension analysis with reverse transcriptase, after the unfolded protein bound to the PTC was cross-linked to it and the end-labeled oligodeoxynucleotide primer was annealed towards the 3′-end of the PTC. The primer extension products terminating at cross-linked sites were run next to a sequencing ladder of the PTC ribosomal DNA to identify the cross-linking sites. The set of nucleotides interacting with the folding proteins were thus identified. In the case of *E. coli* PTC, the same primer extension patterns in the large loop region of the PTC were seen for all the proteins studied so far. The proteins were bovine carbonic anhydrase (BCA), human carbonic anhydrase (HCA), lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and lysozyme. The large loop in the PTC from each of *B. subtilis*, bovine mitochondria and mitochondria from *Leishmania donovani* also had distinct sets of nucleotides that interacted with all folding proteins (unpublished observations, this lab.). Thus, a tentative folding mold was detected in the PTC in each of these ribosomes and compilation of these data would possibly provide a comprehensive scheme to figure out the shape of the mold that could be ideal for protein folding. It may be mentioned here that with additional primers annealed to regions upstream from the 3′-end of the PTC, we could detect more binding sites for proteins (unpublished observation, Ph. D. thesis by Saheli Chowdhury, 2002). However, these interactions

were related to the release of folding competent proteins and further studies are required to explain the mechanism of this process. In this review, we concentrate on what we know about proteins binding only to the large loop of PTC to make them folding competent [19]. In addition, the identification of nucleotides crucial for protein synthesis and folding is essential to understand the relation between these two basic functions of ribosomes.

5 The interaction sites of the ribosomal folding mold on the proteins

The next obvious step was to identify the amino acids in proteins that interact with the specific nucleotides on the ribosomal folding mold. This needs investigation with a number of proteins to see if there are specific amino acids that interact with each of the specific nucleotides on rRNA. If such a relation exists, then Anfinsen's search for folding code in the primary amino acid sequence becomes fathomable. With two proteins, BCA and lysozyme, five single amino acids were identified which interacted with five specific nucleotides in the *E. coli* PTC to achieve folding competent state [21]. The following characteristics of these interactions were observed:

(i) Two of the five nucleotide-amino acid combinations (asparagine-cytosine and glutamine-adenine) were identical for the two proteins. For the remaining three combinations, the amino acids were positively charged and polar (only lysine, asparagine, glycine and leucine). As expected, there was no negatively charged amino acid in this group [21].

(ii) All these amino acids were in the random coils on the surface of the crystal structures of the proteins. They were not in direct contact with the active sites of the proteins, not even remotely connected to the active sites through consecutive interaction with amino acids leading to the active sites [21].

(iii) A very important revelation comes when this binding pattern is compared with the amino acids of the protein folding intermediates (partially folded protein) which preferably interact with the chaperone DnaK. The binding motif to DnaK consists of a hydrophobic core of four to five residues enriched particularly in leucine, and also isoleucine, valine, phenylalanine and tyrosine and two flanking regions enriched in basic residues. Acidic residues are excluded from the core and disfavored in flanking regions [30].

Having two out of five interactions identical for only two proteins looked at so far, one would expect that studies on such interactions with a few more proteins would give the consensus, the best combination of nucleotide-amino acid for ribosome-assisted folding. Secondly, the complementarity in the amino acid preference of the PTC and DnaK for binding with folding proteins explains the difference and possible synergy in their mode of action. The fact that DnaK is displaced from the PTC when the latter is engaged in protein folding [16] suggests an interaction of the nascent protein with the PTC and DnaK in the cell.

6 Interaction of chaperones and ribosomes with nascent protein chains

The interactions of chaperones and ribosomes with nascent protein chains are basically protein-protein and protein-nucleic acid interactions. The interaction with membranes and polysaccharides will become subjects of studies in future.

The fate of the proteins released from ribosomes in folding competent states in the cytosol would be decided by their concentrations and environmental effect. The chaperones in the cytoplasm will participate in it depending on their affinity for these late folding intermediates.

In such a scenario, the specific activity of a protein would vary widely from host to host and it would vary with the growth conditions of the cell, especially if the concentrations of the protein we are looking at (mainly in case of over-expression of the proteins) and the molecular chaperones inside the cell vary widely. The wide variation in the amount of active proteins due to variation in the conditions following post-translational folding on ribosomes has never been seriously studied. We checked the influence of DnaK and Trigger factor on the activity of β-galactosidase subsequent to its ribosome-mediated folding using wild-type *E. coli* and mutants deficient in DnaK and Trigger factor. The effects of mutations are striking and should be kept in mind when purifying proteins from *E. coli* to make sure that optimal specific activity is ensured, that is, that proteins folded optimally. The entire scenario becomes evident from Fig. 2.

The cells were induced at A_{600} = 0.2 in early log phase and grown throughout at 30°C. The activities are normalized for the same amount of cells (respective to A_{600}) throughout the entire length of time shown. Therefore, the graphs are representatives of the increase in enzyme activity per bacterium (and not in increased mass of cells). At each of two time points in each cell population, two

Biotechnol. J. 2008, 3, 999–1009

aliquots were withdrawn and antibiotics chloramphenicol and kasugamycin were added to them. The time points were early (~15 min.) when enzyme activity was linearly rising and late (~45 min.) when it started saturating. Chloramphenicol binds to PTC in the 50S subunit and stops both protein synthesis and folding. Kasugamycin binds to the 30S subunit and only stops protein synthesis. Therefore, there was considerable increase in enzyme activity after adding kasugamycin, because the protein synthesized just prior to adding the antibiotics could still fold on the 50S subunit. The rise in activity saturated after ~10 min, the time taken by newly synthesized protein to fold on 50S. There was no increase in activity following the addition of chloramphenicol, because it stopped protein folding. The difference in activities in presence of kasugamycin and chloramphenicol measured the folding of protein synthesized in a few minutes prior to addition of the antibiotics. The graphs highlight that, (i) at low enzyme concentration (~15 min) there was significantly better folding of enzyme in dnaK$^-$ and tig$^-$ cells compared with the wild-type, and (ii) at higher enzyme concentration (~45 min), there was almost no folding (activity in presence of antibiotics less than in their absence) in case of dnaK$^-$ cells, but it was better with the wild-type *E. coli*. In tig$^-$ cells, enzyme activity did not saturate in an hour in the control, thus the folding continued in presence of kasugamycin. Therefore, the enzymes released from the ribosome at low concentration, could avoid aggregation and fold themselves independently in absence of chaperone in the cytosol. When released at high concentration, the folding competent enzymes tend to aggregate, which can be prevented in the wild-type cells better than in chaperone mutant. For individual protein molecules, interaction with the chaperone would reduce self-folding, but for crowded proteins, chaperone-mediated uncrowding would enhance it.

So far, we have studied the role of amino acid and nucleic acid polymers in shaping the proteins. Some of these experiments were inspired by and substantiated genetic observations. Others were biochemical experiments that collectively established that chaperones interact with unfolded proteins in general, but can also assist or even inhibit folding [26–28] Many proteins remain associated with membranes and polysaccharides. These 1- and 2-D polymers also influence protein structure-function that surely would generate brisk research activity in the future [31].

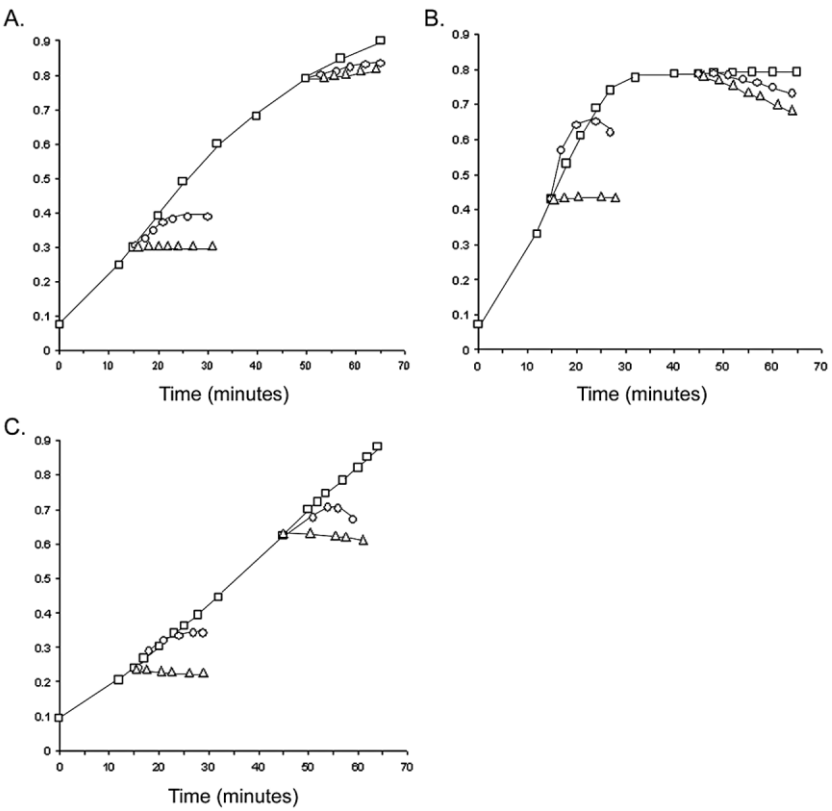

Figure 2. Rise in β-galactosidase activity after induction at 0 min for *E. coli* wild-type (A), dnaK (B) and tig (C) mutants, respectively. The cells were induced at $A_{600} = 0.2$ in early log phase and grown throughout at 30°C. The activities are normalized for the same amount of cells (respective to A_{600}) throughout the entire length of time shown. Therefore, the graphs are representatives of the increase in enzyme activity per bacterium (and not in increased mass of cells) (open squares). It should be noted, that the DnaK, tig double mutant *E. coli* is viable at temperatures below 30°C. Antibiotics added at 15 and 40 min were Chloramphenicol (open triangles) and Kasugamycin (open circles).

7 The unfolded protein splits the ribosome into subunits for folding and ribosome recycling

So far, we have not discussed (i) the possible conformational flux of the polypeptide chain during its synthesis on the ribosome and (ii) the events that might be taking place between the termination of polypeptide synthesis and its folding on the ribosome. The former has been discussed elsewhere in many reviews and papers on protein folding on the ribosome [32]. Some important observations came out of our laboratory which connect termination of protein synthesis and its folding, which are discussed below.

As yet, no role has been envisioned for the newly synthesized full-length protein as far as interaction with the ribosome and its associated factors are concerned. One of the reasons is probably the enormous variability in protein conformation, which would tend to preclude the possibility of any unique interaction between them and a constant set of components including the ribosome and associated factors.

A crucial observation from this laboratory was that the unfolded proteins split the 70S ribosome *in vitro* into the 50S and 30S subunits and attach to the 50S subunit to fold itself [24]. Within the cell, the newly synthesized proteins could be shown to remain bound largely on free 50S subunits [25]. The rate of splitting of purified 70S ribosome was found to be much faster with unfolded proteins than with combined EFG-GTP, ribosome-recycling factor (RRF) and initiation factor (IF3) known to be involved in post-translational ribosome splitting (Fig. 3). Although the presence of these translation factors on and in the vicinity of the post-translation ribosome remains obvious, it may be reiterated that the association of nascent unfolded protein with the post-translation ribosome is also a reality and it is more unfolded than the flexible RRF [33]. It is no wonder that being present in the identical niche in 23S rRNA, the unfolded proteins bind ribosomes at a faster rate and supersedes the combined effect of RRF-EFG-GTP in splitting the ribosomal subunits.

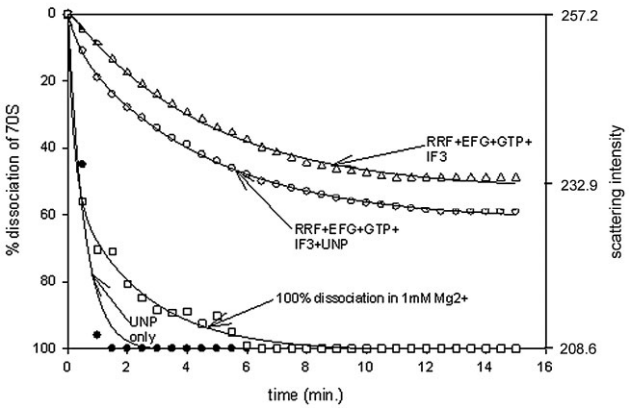

Figure 3. Light scattering experiments (at 18°C) to compare the rate of dissociation of 70S ribosome by unfolded protein (UNP), IF3, EFG and RRF. Experiments were performed in the Hitachi F-3010 Fluorescence Spectrophotometer (excitation: 3-mm slit; emission: 3-mm slit; wavelength at 350 nm at 90° angle) using subunit dissociation buffer (10 mM Tris-HCl, pH 7.5; 80 mM NH_4Cl; 10 mM $MgCl_2$; 0.2 mM DTT). The UNP and translation factors were added at five times the molar concentration of 70S and scattering intensity recorded within 5 s of mixing. In the total reaction volume of 400 µL, 70S concentration was 0.1 µM. Index ~ 70S + unfolded BCA (closed circles); dissociation of 70S ribosome at 1 mM Mg^{2+} - taken as 100% dissociation (open squares); 70S + RRF + EFG - GTP + IF3 + unfolded protein (open circles); 70S + RRF + EFG + GTP + IF3 (open triangles).

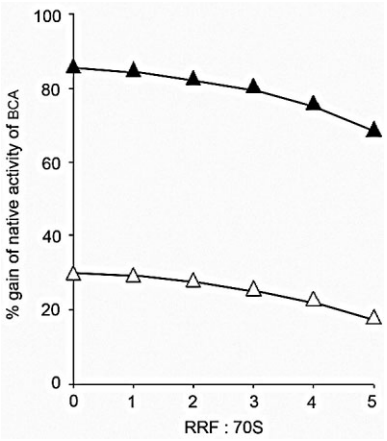

Figure 4. Folding of denatured BCA with 70S ribosome pre-bound with RRF at different molar ratio (closed triangle). The effect of increasing concentration of RRF on self-folding (without ribosome, open triangle) confirms that RRF does not inhibit ribosome-mediated protein folding.

A few experimental observations become relevant at this point, that can provide directions for further enquiry into the ribosomal coupling of protein synthesis and folding. These are as mentioned below:

(i) The RRF interacts with the P-site(s) in the PTC. However, it mainly interacts with the helices 69 and 71 of 23S rRNA [34], which are involved in inter-subunit bridge formation and probably break the bridge to dissociate the ribosome into subunits even without assistance from EFG-GTP and IF3. On the other hand, the newly synthesized proteins compete with tRNA binding to mainly P- and partly A-sites. RRF in association with EFG-GTP can displace P-site tRNA. Unfolded protein also can displace P-site tRNA with assistance from EFG-GTP (Fig. 3).Therefore, although they do not act on the same sites, RRF and the unfolded protein take part in removing P-site tRNA and dissociate the ribosome into subunits.

(ii) Pre-binding the RRF to the ribosome does not affect protein folding because mainly the P-

site tRNA-binding nucleotides interact with protein for folding (Fig. 4). For the unfolded protein, dissociation of 70S and sequestering the 50S subunit for folding on it are independent events.

(iii) P-site-bound tRNA on 70S ribosome prevents *in vitro* protein folding. This tRNA can be displaced with concomitant subunit dissociation by EFG-GTP plus RRF or EFG-GTP plus unfolded protein. However, it cannot be displaced or subunit-dissociated by RRF or unfolded protein in the presence of EFG with a non-hydrolyzable GTP analog like GMPPCP.

In all the cases above, the unfolded proteins, by themselves or in combination with EFG-GTP, RRF and IF3 dissociate the ribosome much faster than the combination of RRF, EFG-GTP, IF3 on its own [35–37]. This suggests that, after one round of translation ending at a termination codon, the nascent polypeptide chain can act in catalyzing ribosome recycling in the presence of chain terminating and peptide releasing translation factors. There is a time lag following ribosome splitting when the nascent polypeptide sequesters the 50S subunit (see the symmetry with IF3, which sequester the 30S subunit) and spends minutes on it to reach the folding competent state [24, 25]. This is the rate-limiting step in the entire translation process. We would like to speculate that the RRF, on the other hand, should be essential for splitting the ribosomes when translation is blocked prematurely due to various reasons.

8 The roles of ribosome, chaperones and ribosomal tunnel in protein folding

The chaperones DnaK, TF, etc., can alternately bind to and dissociate from the ribosome. DnaK binds to the ribosome but dissociates from it when the unfolded protein binds the ribosome [16]. The ribosome-associated TF interacts with proteins released from the ribosome; releases them and reattaches to the ribosome [2]. The newly synthesized protein dissociates from the PTC in the ribosome irreversibly as soon as it attains the folding competent state. The interaction of unfolded protein with the ribosome could be more hydrophobic than the interaction of (largely folded) "folding competent protein" with the chaperone in the cytosol and this may be the overall strategy of sequential interaction among the three components of the protein-folding workshop. In fact, if we look closely at the nascent polypeptide chain, nothing prevents its growing N terminus already moving out of the PTC to interact with ribosome-associated proteins involved in translation and the ribosome-associated chaperones. There are reports on cotranslational folding of polypeptides into domains during translation [38, 39]. Nascent full-length rhodanese was enzymatically active if it was extended at its C terminus by at least 23 amino acids without stop codon [3]. Enzymatically active luciferase could also be formed by similarly extending its C terminus by 26 amino acids [40]. The DnaK-J, GrpE, and GroEL-ES doubled the folding in the former case but not the latter. Therefore, in both cases, there was folding without chaperone and assistance for folding was available from the ribosome. In fact, the DnaK, tig double mutant is viable at temperature below 30°C [41]. These studies supported the observation from this laboratory that the polypeptide segment folding on the ribosome should not be sterically hindered from the P-site (unpublished observation on the inhibition of folding by P-site-bound deacylated tRNA) in the PTC. In the above experiments, this was achieved by extension of polypeptides beyond the enzyme length and halting at the A-site, which could free the P-site tRNA. Blocking the A-site, for example with RRF was seen not to affect folding by the PTC (Fig. 4).

Proteins like rhodanese were known to be able to fold with assistance from the chaperone complex. There have not been any reports on the fate of proteins non-responsive to the chaperone-mediated folding, when locked as a peptidyl tRNA on the ribosomal A-site. Taking a queue from the experiment on the chaperone-independent folding of *in vitro*-translated luciferase, Svetlov *et al*. [8, 9] wanted to see if urea-denatured luciferase could be folded with S-30 translation extract. For reasons unknown, they added a different mRNA to the extract and could not detect folding. This is not surprising because access of denatured protein to the PTC for folding was denied by the other translating mRNA. From these studies we can draw the following conclusions:

The translating protein takes its shape on the ribosome. The chaperones can act on ribosome-associated polypeptides if they are free from the PTC (by C terminus extension). Secondly, the topology of folding did not demand the polypeptide chains to have free ends (the C terminus was on A-site) [9]. This agrees with the ribosome-assisted protein folding which requires its specific amino acids to clamp on the specific nucleotides in the PTC [21].

Consideration of an unfolded state becomes relevant again when we find a tunnel or channel in different ribosomes [42–44], apparently as the conduit through which the nascent proteins are presented to the next stage, namely the cytosol, the

membrane or across the membrane. The tunnel in the 50S ribosomal subunit raised a very attractive possibility of maintaining the protein stretched until it has cleared the tunnel exit. The tunnel interior is envisaged to hide the nascent unfolded polypeptide from the attack on its hydrophobic patches by other proteins. The narrow (~20 Å) tunnel may allow just helices to go through, but at the same time, its interior is fairly aqueous. It is not quite easy to see how all the co-translationally folded peptide segments would conveniently go through the tunnel and how the peptidyl-tRNA could come out of the tunnel for co-translational folding [3, 45].

Another possibility that not all proteins might exit from the tunnel is suggested from the following observations. The folding of globin (heme binding site formation) takes place on the PTC. This intermediate is too large to go through the tunnel and has to leave via the interface between the two subunits [6, 46]. Some nascent peptide chains crosslink to the 16S rRNA [6]. The tail spike protein of phage P22 remains associated with the 30S after the subunits dissociate [7]. The nascent proteins could also be distributed in branches from the exit tunnel seen in the rear side of the large subunit [47]. In a cryo-electron microscopic study, three nascent proteins, Ig_1, Ig_2 and GFP were found to be largely concentrated in the entry cavity of the tunnel (PTC region) in *E. coli* ribosomes stalled following translation [48]. In these and in many earlier studies [49], the full-length protein is found to exit from a point, which is at a distance from the PTC. Whether the exit is from within or outside the tunnel thus remains unsettled. The conclusion from these findings can be that the protein, largely unfolded, can associate with different ribosomal components, even if it has to go through the tunnel. When the last P-site tRNA from the polypeptide is dissociated, nothing prevents the N-terminal region of the released nascent polypeptide to get to the P-site [48] of the PTC before its C terminus is out of the tunnel exit. From studies conducted in this laboratory, the P-site appears crucial for protein folding.

9 Protein folding problems for mutations in rRNA and in amino-acid binding to specific nucleotides

The studies on protein folding by ribosomes started systematically in the authors' and a few other laboratories relatively recently [11–13]. Especially encouraging is the report on the suppression of protein folding by ribosomes (and not translation) by drugs active against yeast and mammalian prions [50]. This makes the biology of ribosome-assisted protein folding even more relevant for research on a number of diseases related to protein folding defects. In short, our knowledge on the suppression and enhancement of ribosome-assisted protein folding is becoming increasingly relevant towards taking steps to contain diseases related to protein folding defects. Discovery of a host of antibiotics to inhibit protein synthesis gave a boost to treatment against bacterial and other microbial infection. Now that the role of the ribosome in protein folding is beginning to be understood, we are looking forward to the selection of drugs against protein folding and the possible use of them against diseases related to protein misfolding.

Additional evidence for protein folding by ribosomes is coming from genetic studies. When mutations were introduced in the five nucleotides in the PTC of *E. coli*, taking one nucleotide at a time and changing it to both similar (Pu → Pu, Py → Py) and dissimilar (Pu → Py) one, we found that all but one mutant were less efficient in protein folding. Whereas the wild-type PTC activated unfolded BCA to 60%, the mutants could not push it above 40% (manuscript in preparation). In addition, mutations were introduced in human carbonic anhydrase (HCA) to change one of its amino acids that binds to a specific nucleotide in the PTC. The amino acid is far away from the active site of the enzyme and well within the random coil in the periphery of the protein. The mutant proteins were grossly folding-defective and formed aggregates even at 30°C (manuscript in preparation). According to the literature on the same enzyme, only 1 mutation out of 12 led to folding defects if the changes were done randomly. This strongly supports our approach to find similar signatures in all the proteins that could be crucial for their folding.

Thus, the initial genetic information supporting the role of the ribosome in protein folding are encouraging and point towards the possibility of planning to design proteins.

Therefore, the entire process from amino acid condensation to the folded design of a protein takes place in two stages, controlled by the two functional states of the ribosome. This is similar to the roles of the RNA polymerase in initiating and terminating transcription, which are two aspects of the same processes, but very different from each other [51]. As the RNA polymerase initiates and carries on transcription and then recruits the nascent RNA transcript for the termination step [51, 52], the ribosome also initiates and carries on the condensation of the amino acid chains, but then recruits the nascent polypeptide to reformat itself so that it can fold the polypeptide to a functional protein.

Finally we can say that the thermodynamics of protein synthesis not only covers a set of diverse condensation reactions achieved at the cost of a huge energy released by GTP hydrolysis, but goes beyond that. Thanks to the ribosomes, the conformational entropic game plan encoded in the polypeptide during synthesis takes it from a compact meaningless folded chain to an articulated meaningful 3-D structure designed with a purpose.

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