Protein folding in *Escherichia coli*: role of 23S ribosomal RNA

Subrata Chattopadhyay 1, Saumen Pal, Debashis Pal, Dibyendu Sarkar, Suparna Chandra, Chanchal Das Gupta *

Department of Biophysics, Molecular Biology and Genetics, University College of Science, 92, A.P.C. Road, Calcutta-700009, India

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Abstract

Post-translational control of *Escherichia coli* ribosome on newly synthesised polypeptide leading to its active conformation (protein folding) has been shown in the case of the enzyme β-galactosidase. As expected, antibiotics chloramphenicol and lincomycin, which bind to 23S rRNA/50S subunit and kasugamycin and streptomycin which interact with the 30S subunit instantaneously inhibited protein synthesis when they were added to the growing cells. The increase in β-galactosidase activity, though stopped immediately after the addition of chloramphenicol and lincomycin, went on considerably in the presence of streptomycin and kasugamycin even after the stoppage of protein synthesis. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

We have shown a general protein folding activity in vitro in the ribosomes from various sources. In the case of *E. coli*, this activity was traced first to the 50S subunit and finally to the domain V of its 23S rRNA component [1–6]. This in vitro protein folding property of 23S rRNA was inhibited by antibiotics chloramphenicol, erythromycin lincomycin, etc. [3]. These antibiotics inhibit protein synthesis by blocking peptidyl transferase reaction. Mutations occur in the 23S rRNA in cells resistant to these antibiotics and these antibiotics bind to 23S rRNA in vitro [7–9]. Thus, the peptidyl transferase and protein folding activity overlap on the 23S rRNA (50S subunit). It may not be possible then to score a putative folding defective mutant of *E. coli* having normal peptidyl transferase activity. In addition to this problem, the presence of multiple 23S rRNA operons make the search for a protein folding defective mutant even more difficult. Under such circumstances, we asked whether a temporal non-overlap is possible between these two partially overlapping processes. If there is a measurable lag between finishing off the synthesis of a polypeptide and appearance of its activity, we can visualise that by inhibiting protein synthesis by antibiotics which bind to the 30S subunit of ribosome, when the 50S particle can still engage in folding the protein synthesised before adding these antibiotics. We used these antibiotics to stop protein synthesis in *E. coli* K12 cells 30 min after induction of β-galactosidase. Although the protein synthesis stopped immediately, the β-galactosidase activity increased considerably after the arrest of protein synthesis in cells treated...
with streptomycin and kasugamycin, which bind to 30S subunit, but not in cells treated with chloramphenicol and lincomycin. Therefore, the antibiotics which act on 23S rRNA to inhibit reactivation of denatured protein in vitro [3] also inhibit post-translational activation (folding) of the protein in vivo. The details of the experiments are given below.

2. Materials and methods

2.1. TGC medium

TGC medium contains 100 mM Tris-HCl, pH 7.5, 100 mM KCl, 8.5 mM NaCl, 20 mM NH₄Cl, 1 mM CaCl₂, 1 mM MgSO₄, 0.3% casamino acid, 15 mM sodium pyruvate, 1 mM potassium phosphate pH 7.5 and 0.2% glycerol. FeSO₄ (0.2 g) and 10 g thiamine were added to a litre of this medium. L-Galactosidase, mouse polyclonal antibody against it, anti-mouse IgG peroxidase conjugate and substrate O-phenylenediamine dihydrochloride were purchased from Sigma, USA.

2.2. Estimation of protein synthesis by 35S-methionine uptake

E. coli K12 was grown to log phase in TGC medium. β-Galactosidase was induced by adding IPTG and 35S-methionine was added to the growing cells. The antibiotics were added to the cells at appropriate times. Samples were withdrawn at different times as mentioned in the text and the 35S-methionyl tRNA in the cells were destroyed by incubating the samples in the presence of 1 M NaOH, and 0.5 M H₂O₂ at 40°C for 30 min [10]. Finally, the 5% TCA precipitable protein was trapped on glass fibre (GFC Whatman 25 mm) filters. The filters were dried and the 35S counts were taken in a scintillation counter.

2.3. Estimation of β-galactosidase protein in the cell by ELISA

Cells were grown in TGC medium to log phase as before and β-galactosidase was induced with IPTG. At appropriate times, antibiotics were added to the growing cells. Samples were withdrawn at the desired times. The cells were spun down and suspended in M9 buffer and lysed with toluene. The amount of β-galactosidase protein in the extracts was estimated by ELISA. Polyclonal mouse antibody against β-Galactosidase, anti-mouse IgG peroxidase conjugate and substrate O-phenylenediamine dihydrochloride tablets were obtained from Sigma.

2.4. Assay for β-galactosidase

β-Galactosidase activity in the cell extracts was measured after tolune treatment of the cells. The assay buffer contained 100 mM sodium phosphate, pH 7.0, 10 mM KCl, 1 mM MgSO₄, 50 mM β-mercaptoethanol and 1 mg/ml ONPG. To 1 ml of the assay mixture an equal volume of tolune extracts of cells grown under different conditions was added and incubated for 30 s at 37°C. The reaction was stopped by adding 1 ml 1 M sodium carbonate and enzyme activity was measured by recording the A₄₂₀.

The experimental results described below are the average of at least five independent observations and the standard deviations are indicated in the figures.

3. Results

3.1. Inhibition of in vitro refolding of β-galactosidase by antibiotics

β-Galactosidase (100 nM) was unfolded in the presence of 4 M guanidine hydrochloride and 50 mM β-mercaptoethanol for 1 h. The secondary structure was completely destroyed as shown by far UV CD spectrum (not shown). The denatured protein was allowed to refold after 50-fold dilution in the refolding buffer in the absence and in the presence of 10 nM 23S rRNA. After refolding, the enzyme was diluted 5-fold in the assay buffer, its activity was measured and expressed as percentage of the activity of the native protein. Fig. 1 shows that 23S rRNA could refold the denatured enzyme. The refolding could not be inhibited by antibiotics streptomycin and kasugamycin even at concentrations higher than what would completely stop protein synthesis in the cell, but chloramphenicol and lincomycin, when added to the 23S rRNA [11,12], progressively inhibited refolding of the enzyme with increasing concentrations until it was brought down to the level.
of unassisted folding. The 23S rRNA assisted folding was completely eliminated by about 500 μM chloramphenicol and 50 μM lincomycin.

Once we showed that the antibiotics chloramphenicol and lincomycin inhibited in vitro protein folding by 23S rRNA, whereas streptomycin and kasugamycin failed to do so, our objective was to look at the effect of all these antibiotics (which inhibit protein synthesis) on the in vivo activity of the enzyme β-galactosidase. It must be noted that none of these antibiotics influences the assay of β-galactosidase activity.

Although these antibiotics are well known inhibitors of protein synthesis, we checked again to make sure that they instantaneously stop protein synthesis at the concentrations at which the in vitro folding of β-galactosidase was inhibited.

3.2. Inhibition of protein synthesis by antibiotics

E. coli K12 cells were grown in TGC medium at 37°C with shaking to log phase (A550 = 0.25) and β-galactosidase was induced in IPTG. At the same time 35S-methionine was added and after 7 min of growth, the cells were divided into five equal portions. The antibiotics chloramphenicol (150 μg/ml, 464 μM), lincomycin (44 μg/ml, 99 μM), streptomycin (25 μg/ml, 17 μM) and kasugamycin (83 μg/ml, 193 μM) were added to the first four portions and the fifth was left to grow without antibiotic. As expected, the A550 of the cells did not change after the addition of the antibiotics. These are the concentrations of the four antibiotics normally used to inhibit protein synthesis in E. coli. An equal volume of cells was then withdrawn from each portion at 7.5 and 15 min. 35S-methionine labelled tRNAs were destroyed as described in [10], and then 5% chilled TCA precipitable 35S counts were taken. Fig. 2 shows the 35S counts in proteins in each of the above cases. Whereas in the control sample, protein synthesis continued linearly, it came to a halt in all the samples containing the antibiotics. This observation is in complete agreement with earlier observations on the effect of these antibiotics on protein synthesis. Although faulty protein synthesis starts in the presence of streptomycin if one waits for a longer time [7], protein synthesis stops immediately after adding streptomycin and no translation is expected to occur within 10–15 min [9].

35S-Methionine uptake was measured to estimate total protein synthesis in the cells under different conditions of growth. In order to estimate the synthesis of β-galactosidase protein itself under similar experimental set-up, an equal volume of the cells was lysed with toluene and the β-galactosidase protein in

![Fig. 1. Effect of antibiotics on the 23S rRNA mediated recovery of activity of denatured β-galactosidase. Continuous lines and dotted lines represent RNA mediated and unassisted recovery of the enzyme activity, respectively. In both cases, the symbols represent recoveries of activity in the presence of streptomycin (○ - ○), kasugamycin (▲ - ▲), lincomycin (● - ●) chloramphenicol (○-○) and without antibiotic (□-□). The concentration of chloramphenicol is shown in the upper scale and the concentration of other antibiotics are shown in the lower scale.](image1)

![Fig. 2. Incorporation of 35S-methionine in E. coli K12 cells. 35S-Methionine was added at zero time. After 7 min of growth, cells were divided into five equal portions; the antibiotics were added to four portions and the fifth was left without antibiotic. Samples were taken at different times and 5% cold TCA precipitable counts were taken after destroying aminooacyl tRNAs by alkali. The symbols represent 35S-methionine incorporation in the presence of streptomycin (○ - ○), kasugamycin (▲ - ▲), lincomycin (● - ●) chloramphenicol (○-○) and in the absence of antibiotics (□-□).](image2)
cell extracts was estimated by the method of ELISA at the appropriate dilution as mentioned Section 2. As shown in Fig. 3, synthesis of β-galactosidase under different conditions essentially followed the same pattern as the total protein synthesis. Although the antibiotics instantly stopped protein synthesis, their effects on the activity of β-galactosidase were not the same as shown below.

3.3. Effect of antibiotics on β-galactosidase activity in E. coli

The E. coli K12 cells were grown to log phase exactly as before. β-Galactosidase was induced with IPTG and the level of induction was seen by measuring the enzyme activity for up to 30 min. At this point, the culture was divided in five portions. The four antibiotics were added to the four portions, while the fifth was allowed to grow as control as mentioned earlier. Then aliquots were taken from each of these samples at different times, the cells were extracted with toluene and the β-galactosidase activities of the extracts were measured. While the enzyme activity immediately stopped increasing in cells treated with chloramphenicol and lincomycin, the activity continued to increase for about 10 min in cells to which streptomycin and kasugamycin were added (Fig. 4). The increments were not negligible and could be as high as 25% of the enzyme activity present at the time of adding the antibiotics. This rise in enzyme activity in spite of inhibition of protein synthesis could be due to post-translational activation of the enzyme molecules by the 50S ribosome particles which was not inhibited by streptomycin and kasugamycin, but was inhibited by 50S specific antibiotics. The E. coli ribosome, its 50S subunit and 23S rRNA have been shown to be able to reactivate unfolded proteins in vitro [2–4]. It appears, therefore, that the 50S subunit folds the newly synthesised polypeptides on the ribosomes in the cell and the antibiotics chloramphenicol and lincomycin inhibit this folding process. The protein concentrations in the toluene extracts of the cells grown in the presence of all the four antibiotics were checked to ensure that they were the same and remained unchanged with time.

To show that the limited increase in the activity of Fig. 3. ELISA to estimate β-galactosidase synthesis in E. coli K12 cells. Thirty minutes after induction of β-galactosidase, cells were divided in five portions. Antibiotics were added to four portions and the fifth was allowed to grow without antibiotics. Samples were taken at different times and the amount of β-galactosidase was estimated by immunoprecipitation with polyclonal mouse antibodies against β-galactosidase. Symbols represent the amount of the immunoprecipitable protein in the absence of antibiotics (○○○○) and in the presence of streptomycin (- - - -), kasugamycin (- - - -), lincomycin (- - - -) and chloramphenicol (- - - -).

Fig. 4. Thirty minutes after induction of β-galactosidase, cells were divided in five equal portions; four antibiotics were added to four portions and the fifth was left without antibiotics. Samples were withdrawn at different times, cells were lysed with toluene and β-galactosidase activities were measured. The ordinate shows the measured enzyme activity in A420. The symbols represent enzyme activities in the presence of streptomycin (- - - -), kasugamycin (- - - -), lincomycin (- - - -) chloramphenicol (- - - -) and in the absence of antibiotics (○○○○).
β-galactosidase in the presence of 30S specific antibiotics could be stopped at any point during this rise by chloramphenicol and lincomycin, whereas streptomycin and kasugamycin allow considerable increase in the enzyme activity for more than 5 min, although all these antibiotics would stop protein synthesis instantly. Considering the slow rate of synthesis of the polypeptide chains on the ribosome (15 amino acids per second), it is almost certainly true that the secondary structure of the proteins is formed by the time they are synthesised [13–17]. Ribosome may not play any active role in it. In fact, we have seen that about 10–15 s delay in adding ribosome to the refolding protein did not reduce the extent of reactivation (unpublished observation), although the secondary structures are formed by this time. The time taken by β-galactosidase activity to reach the saturation level in the presence of streptomycin and kasugamycin (5–10 min), should then be spent organising the tertiary and quaternary conformation of the protein and the ribosome must have a role to play in it.

Our experiments on in vitro folding of different multimeric proteins by ribosomal RNA showed a time lag of about 5–10 min between the release of the polypeptide from the RNA with the formation of the multimer and reappearance of activity (manuscript in preparation). So the ribosome/rRNA appear to be binding at specific region(s) of the polypeptide to prevent misfolding and releases it after proper nucleation is achieved so that there is no chance of misfolding any more. Formation of the active site takes place after release from ribosome/rRNA and it takes a considerable amount of time (5–10 min). The addition of 50S specific antibiotics prevents this nucleation step and the protein goes to fold by itself without proper nucleation so that most of it misfolds to inactive conformation.

The fate of β-galactosidase enzyme was followed for a short time of about 10–15 min after its synthesis was stopped by any of the four antibiotics. The activity reached its maximum within this time. All the antibiotics stopped protein synthesis instantaneously. Although a later release of error prone protein synthesis is found with streptomycin [7], there was complete inhibition of protein synthesis in the short span of our experiment. We have shown this in the case of total protein as well as β-galactosidase itself.
As mentioned earlier, none of these antibiotics influence the assay of β-galactosidase enzyme activity.

There is no reason to be sceptic in correlating the results on protein folding by the 70S ribosome, 50S subunit and the 23S rRNA [1–3,18]. Even in its native state within the 50S particle, the domain V of the 23S rRNA which we have shown to be mainly involved in this process [3,5], has a large number of nucleotides accessible by chemical agents, amino acyl tRNA, 30S subunit etc. More than 20 such nucleotides have been identified from biochemical and genetic experiments [8,18–23]. These could be the nucleotides in the three-dimensional structure of 23S rRNA in 50S subunit which play crucial role in protein folding.

Finally, a strong similarity in the 23S ribosomal RNA mediated folding of β-galactosidase in vitro and in vivo strengthens our point that within the cell, the ribosome plays a major role post-translationally in folding newly synthesised proteins. The folding is determined by ribosomes which initiate by giving it the correct direction and the polypeptide goes to complete the process (folding) by itself in a slow pace so that a considerably length of time is taken for the formation of active site and the chaperones could contribute to this step [16,17].

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