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Reduced puromycin sensitivity of translocated polysomes after the addition of elongation factor 2 and non-hydrolysable GTP analogues

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Treatment of reticulocyte polysomes with elongation factor eEF-2 and GTP led to an increased sensitivity of peptidyl-tRNA for puromycin as a result of the translocation from the ribosomal A-site to the P-site. Upon addition of an excess of the non-hydrolysable GTP analogue, Guo*PP*[CH_2]*P*, the puromycin sensitivity decreased rapidly. The decrease in sensitivity required high concentrations of eEF-2 with half maximal effect at an eEF-2 concentration of around 1 μM. The data suggest either that peptidyl-tRNA had re-translocated back to the A-site due to the higher affinity of eEF-2 for the pre-translocation than for the post-translocation ribosome, or that the eEF-2–Guo*PP*[CH_2]*P* complex blocks the peptidyl-transferase activity.

Elongation factor; Eukaryote; Protein synthesis; Puromycin; Reticulocyte; Translocation

1. INTRODUCTION

Translocation of mRNA 1 codon forward during the protein synthesis elongation cycle is stimulated by elongation factor eEF-2 (EF-G in prokaryotes) and is coupled to the movements of peptidyl-tRNA from the ribosomal A- to the P-site and of deacylated tRNA from the P- to the E-site [1]. The driving force for the reaction is believed to be the increased stability of the peptidyl-tRNA–ribosome interaction after translocation, whereas the affinity of deacylated tRNA for the ribosome is only slightly decreased after transfer to the E-site [2]. This suggestion is supported by the observation that translocation occurs in the absence of the elongation factor [3]. Thus the functional role of the elongation factor seems to be to reduce the activation energy required for translocation [4]. This function is apparently not coupled to the factor-dependent hydrolysis of GTP as the hydrolysis most likely follows after translocation. Accordingly, translocation occurs in the presence of non-hydrolysable GTP analogues [5,6] and the hydrolysis of GTP is promoted by post-translocation ribosomes [7,8].

It has been proposed that the GTP hydrolysis leads to a release of the factor from the post-translocation ribosome, suggesting that the factor is less-stably bound to the ribosome in the presence of GDP than when complexed to GTP [9]. However, the affinity of eEF-2 for post-translocation ribosomes is similar in the presence of both non-hydrolysable GTP analogues and GDP [10], and the only difference observed is the higher affinity of eEF-2 for pre-translocation ribosomes in the presence of GTP or GTP analogues [10]. This increased affinity apparently counteracts the energy gained from the net increase in stability of the tRNA ribosome interactions after translocation. Thus, the function of the GTP hydrolysis seems to be to reduce the ability of eEF-2 (EF-G) to reverse the translocation. To test this hypothesis, we have determined the effect of a non-hydrolysable GTP analogue, Guo*PP*[CH_2]*P*, on the translocation reaction in the presence of various concentrations of eEF-2. The addition of Guo*PP*[CH_2]*P* led to an eEF-2-dependent decrease in puromycin sensitivity of the polysome-bound nascent polypeptide. We suggest that the result is due to a reversed translocation, although the possibility that eEF-2–Guo*PP*[CH_2]*P* bound to post-translocation ribosomes blocks the peptidyltransferase can not be completely excluded.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Glass fibre filters GF/C were from Whatman Biochemicals Ltd. (Maidstone, Kent, UK. [^{35}S]Methionine was purchased from Amersham International, UK. GTP and Guo*PP*[CH_2]*P* were from Boehringer-Mannheim (Germany). Puromycin, pyruvate kinase and phospho*enol*pyruvate were from Sigma Chemical Co. (St. Louis, MO, USA). eEF-2 was isolated from rat livers as previously described [11].

2.2. *Preparation of reticulocyte lysates*

Rabbit reticulocytes were prepared as previously described [12]. The reticulocyte lysate was incubated with [^{35}S]methionine (50 μCi/ml) for

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Abbreviations: Guo*PP*[CH_2]*P*, guanosine 5′-[β,γ-methylene]-triphosphate; eEF, eukaryotic elongation factor; EF, prokaryotic elongation factor.

5 min at 30°C according to Pelham and Jackson [13] in order to label the nascent polypeptides. The reaction mixture was rapidly cooled to 0°C and layered on top of a 2-ml cushion of 0.75 M sucrose, 25 mM KCl, 20 mM Tris-HCl, pH 7.6, 2 mM $Mg(CH_3COO)_2$ and 5 mM 2-mercaptoethanol. The labelled polysomes were pelleted by centrifugation at 40,000 × g_{av} for 16 h. The polysomes were resuspended in 0.25 M sucrose, 70 mM KCl, 30 mM HEPES/KOH, pH 7.6, 2 mM $Mg(CH_3COO)_2$ and 1 mM dithiothreitol to a concentration of 2.2 pmol/μl.

2.3. *Puromycin release*

Polysomes (0.88 μM) were incubated at 30°C with indicated concentrations of eEF-2 in the presence of 100 mM KCl, 4.5 mM $Mg(CH_3COO)_2$, 12 mM HEPES/KOH, 8 mM Tris-HCl, pH 7.6, 0.4 mM dithiothreitol, 6 mM 2-mercaptoethanol, 4% (by volume) glycerol, 100 mM sucrose, 40 μM EDTA and 10 μM GTP. After 5 min, 100 mM Guo*PP*[CH_2]*P* was added to a final concentration of 5 mM. Samples were taken at indicated times and rapidly cooled to 0°C. Puromycin was added to a final concentration of 100 μM and the samples incubated at 0°C for 1 h. The samples were thereafter diluted with 200 μl ice-cool buffer containing 0.25 M sucrose, 70 mM KCl, 30 mM HEPES/KOH, pH 7.6, 2 mM $Mg(CH_3COO)_2$ and 1 mM dithiothreitol, and the tRNA-bound radioactivity was precipitated with *N*-cety-*N,N,N*-trimethylamoniu m bromide as described by Darnbrough et al. [14]. Acid-unstable radioactivity was removed by heating for 10 min in 10% (by weight) trichloroacetic acid as described by Mans and Novelli [15].

3. RESULTS AND DISCUSSION

The position of peptidyl-tRNA on the ribosome can be monitored by the release of P-site-located peptides by puromycin [16]. This reaction requires that the peptide is located in the ribosomal P-site and that the A-site is unoccupied [17,18]. The puromycin release can occur at 0°C, thus allowing an identification of the position of peptidyl-tRNA under conditions that do not permit translocation [6]. Approximately 30% of the peptidyl-tRNA carried by isolated reticulocyte polysomes were sensitive to puromycin. This is in accordance with our previous results [19]. Additional 30% were rapidly transformed to a puromycin-sensitive state by incubation of the polysomes with eEF-2 and GTP, showing that eEF-2 promoted the translocation of peptidyl-tRNA from the A-site to the P-site (Fig. 1). The reaction was rapidly completed and the puromycin sensitivity remained unaltered upon further incubation. However, eEF-2 was still able to convert the available GTP to GDP in the presence of the post-translocation ribosomes [7].

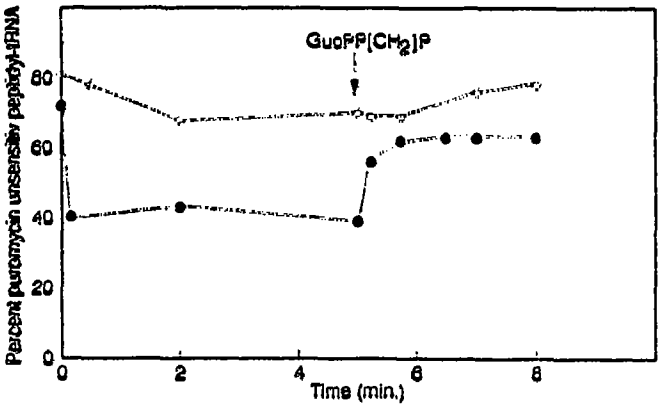

Fig. 1. Reduction of the puromycin sensitivity of the polysomes after addition of GuoPP[CH_2]P. Polysomes were incubated with 9.6 μM eEF-2 and GTP as described in section 2. Guo*PP*[CH_2]*P* was added after 5 min incubation. After indicated times samples were withdrawn and the puromycin sensitivity was determined in the presence (●) and in the absence (○) of eEF-2.

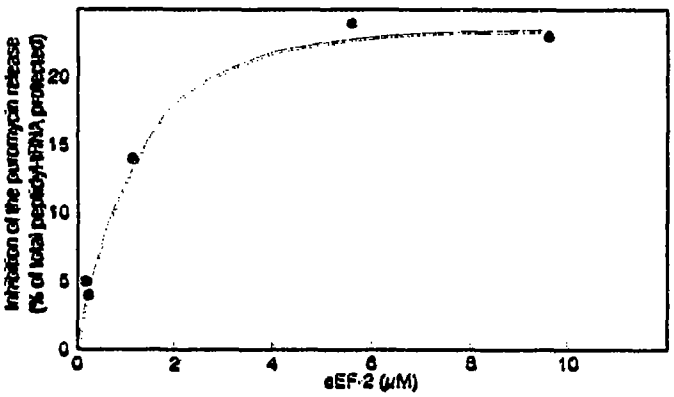

Fig. 2. The dependence of the eEF-2 concentration on the effect of GuoPP[CH_2]P on the reduction of puromycin sensitivity. Polysomes were incubated with GTP for 5 min as described in section 2 in the presence of the indicated concentrations of eEF-2. The puromycin sensitivity was determined from samples taken just prior and 0.5 min after the addition of Guo*PP*[CH_2]*P*.

The remaining puromycin-insensitive polysomes (40%) seemed unable to translocate. We have previously shown that this population co-varies with the ribosomal population that has bound eEF-1, and we suggested that the insensitivity was due to A-site-bound aminoacyl-tRNA [19]. The reason why this population is unable to be transferred to a puromycin sensitive state is presently under investigation.

After the addition of an excess of the non-hydrolysable GTP analogue, Guo*PP*[CH_2]*P*, the puromycin sensitivity rapidly dropped from approximately 60% to less than 40% (Fig. 1). The effect of Guo*PP*[CH_2]*P* was mediated by eEF-2 (Fig. 1) and the puromycin sensitivity decreased with increasing concentrations of eEF-2 (Fig. 2). This result could either be due to an eEF-2–Guo*PP*[CH_2]*P*-dependent inhibition of the peptidyltransferase activity, or an eEF-2–Guo*PP*[CH_2]*P*-promoted reversed translocation. The former explanation requires that the eEF-2–post-translocation ribosome complex is more stable in the presence of Guo*PP*[CH_2]*P* than in the presence of GDP, or that the conformations of eEF-2 in the two complexes differ. We have previously shown that eEF-2–GDP and eEF-2–Guo*PP*[CH_2]*P* have similar affinities for the post-translocation ribosome [10], and that the conformation of eEF-2 shows the same protease sensitivity in the presence of both nucleotides [20]. We therefore suggest

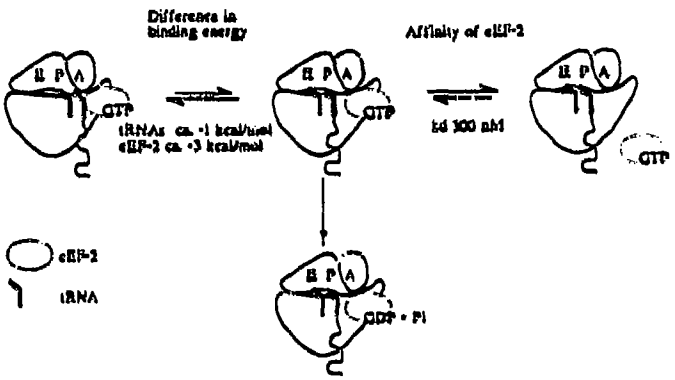

Fig. 3. Illustration of the different eEF-2 -ribosome complexes. The differences in binding energies are taken from [2] for tRNA and [10] for eEF-2. The affinity of eEF-2-GTP for post-translocation ribosomes are taken from [10].

that eEF-2-Guo*PP*[CH_2]*P* promotes a reversed translocation. Since eEF-2-Guo*PP*[CH2]*P* can promote the forward translocation reaction when present in stoichiometric concentrations [6], it is tempting to suggest that ribosome-bound eEF-2-Guo*PP*[CH_2]*P* allows free diffusion of the peptidyl-tRNA between the A-and the P-sites. The translocatable peptidyl-tRNA is protected to approximately 60% from puromycin after the addition of Guo*PP*[CH_2]*P* at saturating concentrations of eEF-2 (Fig. 2). This should indicate that the eEF-2-Guo*PP*[CH_2]*P*–pre-translo cation ribosome complex is energetically favoured over the complex containing post-translocation ribosomes. This is in accordance with calculations based on available data from studies in both eukaryotic and prokaryotic systems (Fig. 3).

Translocation is supposed to gain its energy from the increased stability of the two ribosome-bound tRNAs after translocation [2]. Movement of peptidyl-tRNA from the A- to the P-site decreases the dissociation constant from 10^{-7} to 10^{-9} M, whereas the shift of deacylated tRNA from the P- to the E-site slightly increases the K_d from 10^{-8} to 2×10^{-7} M. Thus, the gain in energy has been estimated to approximately 1 kcal/mol [2]. We have shown that eEF-2-Guo*PP*[CH_2]*P* forms an approximately 100-times more stable complex with pre-translocation than with post-translocation ribosomes [10]. The gain in energy from the translocation of peptidyl-tRNA is therefore counteracted by the loss in stability of the ribosome–eEF-2-GTP complex (see Fig. 3). Based on these calculations, eEF-2 should be expected to hinder and in fact revert the translocation, provided that (i) the hydrolysis of the eEF-2-bound GTP or GTP analogue is blocked, (ii) the concentration of eEF-2 is high enough to allow formation of the weak complex between post-translocation ribosome–eEF-2–GTP, and (iii) the translocation is reversible. This is in line with our observations. Reversible translocation has been suggested by Tanaka et al. [6] based on a small and slow effect of eEF-2-Guo*PP*[CH_2]*P* on the puromycin reactivity. The modest effect is in line with our results since they used relatively low concentrations of eEF-2, but we can not explain why the reaction demanded long incubation times. In our experiments we could not show any dependence on incubation time as the protection from puromycin reached an equilibrium already within a few seconds.

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