RIBOSOMAL TRANSLOCATION PROMOTED BY GUANYLYLIMIDO DIPHOSPHATE AND GUANYLYL-METHYLENE DIPHOSPHONATE

Juan MODOLELL, Tomás GIRBÉS and David VÁZQUEZ
Instituto de Biología Celular, Velázquez 144, Madrid 6, Spain

Received 13 October 1975

1. Introduction

It has been known for a number of years that ribosomal translocation requires the participation of elongation factor G (EF-G) and the hydrolysis of GTP ([1], review). However, the significance of this hydrolytic reaction, a fundamental question in protein biosynthesis, has not yet been satisfactorily explained. It has been traditionally assumed that the energy liberated in the reaction assists the rearrangements of tRNA, mRNA and ribosomal subunits that result in the movement of mRNA and, probably, of peptidyl-tRNA [1]. Very recently, however, an alternative view has been proposed [2,3]: the binding of EF-G plus GTP to the ribosome promotes these rearrangements, and the hydrolysis of GTP merely allows completion of the translocation process by inducing the release of EF-G from the ribosome. This second alternative is supported by the finding that the non-hydrolyzable analog of GTP guanylyl-methylene diphosphonate (Gpp(CH_2)p) can, under some conditions, replace GTP in translocation [2,3]. However, since earlier research showed Gpp(CH_2)p to be incapable of promoting translocation [4–6], we have reexamined its effect on this process using ribosomes complexed with *N*-acetyl-Phe-tRNA and poly (U), and endogenous *E. coli* polysomes. Moreover, to study further the role of GTP hydrolysis in translocation we have investigated the effect of a different non-hydrolyzable analog [7]: guanylylimido diphosphate (Gpp(NH)p). With both in vitro systems, Gpp(NH)p promotes translocation almost as efficiently as GTP, but at concentrations approx. 2 orders of magnitude higher than those of GTP. Gpp(CH_2)p is less effective than Gpp(NH)p and even higher concentrations are required to promote translocation.

Abbreviations: Gpp(CH_2)p, guanylylmethylene diphosphonate; Gpp(NH)p, guanylylimido diphosphate; EF-G, elongation factor G; Pur, puromycin.

2. Materials and methods

Preparation of 1 M NH_4Cl-washed *E. coli* ribosomes and polysomes, EF-G, and *N*-acetyl-[^{14}C]Phe-tRNA (1010 cpm/pmol) has been described elsewhere [4,8]. Gpp(CH_2)p was either purchased from Miles Laboratories (England) or prepared in our laboratory [9] and purified by chromatography on either DEAE-cellulose columns [9] or on PEI-cellulose thin-layer plates [10]. Gpp(NH)p was from Boehringer Mannheim (West Germany) and was further purified by DEAE-cellulose chromatography [9]. [^{3}H]Puromycin (1670 cpm/pmol) was from the Radiochemical Centre, Amersham.

Translocation of *N*-acetyl-[^{14}C]Phe-tRNA was performed in mixtures (20 or 105 μl) containing: 20 mM NH_4Cl, 50 mM KCl, 12 mM Tris–HCl pH 7.8, 12 mM Mg(acetate)$_2$, 1 mM dithiothreitol, 0.5 mM puromycin, 9.2 A_{260} unit/ml of ribosomes complexed with poly (U) and containing 8.2 pmol/A_{260} unit of ribosomes of *N*-acetyl-[^{14}C]Phe-tRNA previously bound to the ribosomal A-site [4], and EF-G, fusidic acid and guanosine nucleotide as indicated. Translocation of peptidyl-tRNA in purified polysomes was carried out in mixtures (20 to 140 μl) containing: 100 to 170 mM NH_4Cl, 12 mM Tris–HCl pH 7.8, 12 mM

Mg (acetate)$_2$, 1 mM dithiothreitol, 11 μM [^{3}H] puromycin, 10.8 A_{260} unit/ml of polysomes, EF-G, and other components as specified. In both cases incubations were at 30°C for either 10 min or the indicated time intervals, and were followed by determination in 20 μl aliquots of either *N*-acetyl-[^{14}C]Phe-puromycin [4] or peptidyl-[^{3}H]puromycin [8]. Controls without EF-G or guanosine nucleotide were run in parallel to account for either *N*-acetyl-[^{14}C]Phe-tRNA or peptidyl-tRNA bound to the ribosomal P-site at the start of the incubation (less than 20 and 25%, respectively, of that bound to the ribosomal A-site). Unless otherwise specified, their values were subtracted.

3. Results

To investigate the ability of Gpp(CH$_2$)p and Gpp(NH)p to promote translocation we used either ribosomes complexed with poly(U) and A-site-bound *N*-acetyl-[^{14}C]Phe-tRNA [4] or endogenous, purified *E. coli* polysomes carrying most of their peptidyl-tRNA in the ribosomal A-site [8]. As an index of translocation we measured the EF-G plus guanosine nucleotide-dependent reactivity of *N*-acetyl-[^{14}C]Phe-tRNA or peptidyl-tRNA with puromycin. Fig.1 shows that with both systems Gpp(NH)p promoted translocation of almost as many ribosomes as GTP, but at concentrations about 2 orders of magnitude higher than those of GTP. Gpp(CH$_2$)p was less effective, specially with the *N*-acetyl-[^{14}C]Phe-tRNA system, and only approximately 50% of the ribosomes translocated at saturating amounts of the nucleotide. However, for unknown reasons, in other experiments with polysomes Gpp(CH$_2$)p was almost as effective as Gpp(NH)p (fig.2B). We would stress that since Gpp(NH)p and Gpp(CH$_2$)p induce formation of stable EF-G–ribosome–guanosine nucleotide complexes on free ribosomes (ribosomes devoid of peptidyl-tRNA) [1,7], care was taken so that EF-G was present in molar excess over ribosomes in the experiments of Figs.1 and 2. Since the EF-G preparations used might have been partly inactivated, the presence of excess EF-G was verified by including 2.5 mM fusidic acid in all reaction mixtures containing GTP. Fusidic acid is a powerful inhibitor of translocation in systems containing catalytic amounts of EF-G, while it has no effect on those with an excess of the factor [4,11].

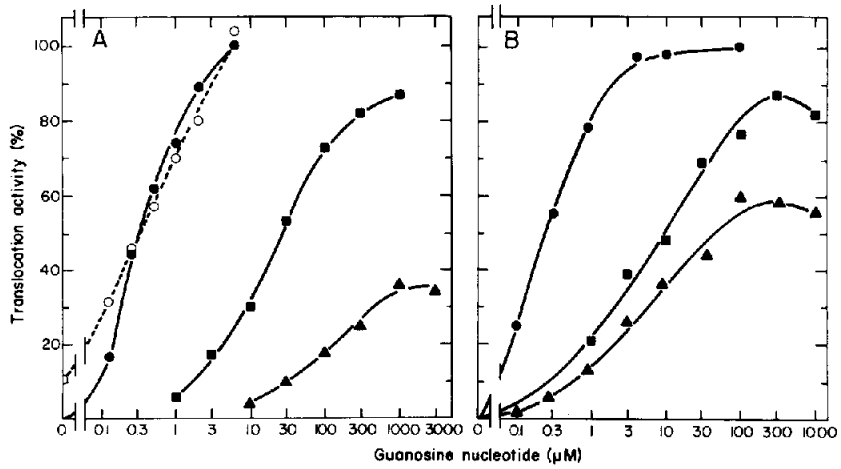

Fig.1. Effect of increasing concentrations of GTP (●), Gpp(NH)p (■), and Gpp(CH$_2$)p (▲) on translocation of *N*-acetyl-[^{14}C] Phe-tRNA (A) and peptidyl-tRNA (B). Experiments were carried out as described under Materials and methods. Final concentrations of EF-G were (A) 90 and (B) 70 μg/ml. Translocation promoted by a mixture of 0.1 M Gpp(CH$_2$)p and different concentrations of GTP (without fusidic acid) is indicated by (○). In this experiment Gpp(CH$_2$)p was added to the reaction mixture before GTP. 100% represents the amount of translocation promoted by 6 μM (A) or 100 μM (B) GTP, that is, 4.4 (A) and 6.6 to 9.3 (B) pmoles of puromycin derivates/A_{260} unit of ribosomes. Experimental points in panel B are averages of four experiments.

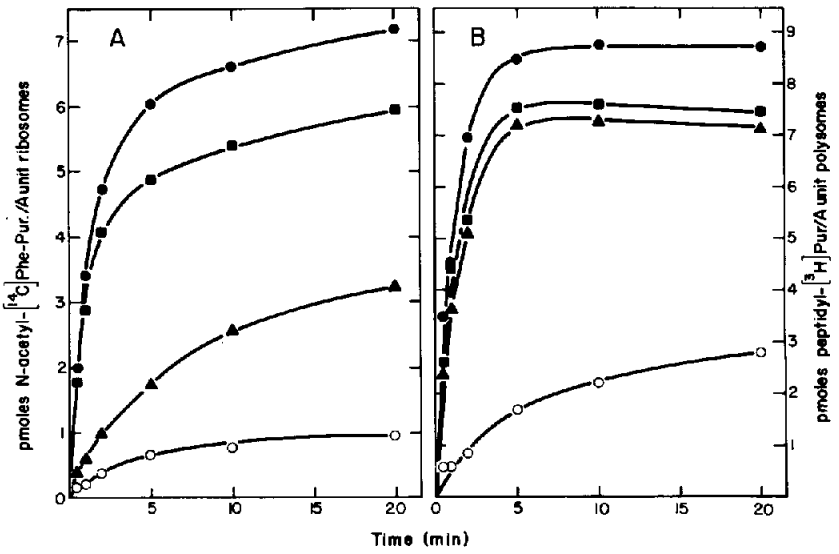

Fig.2. Time course of translocation promoted by GTP (•), Gpp(NH)p (■) and Gpp(CH_2)p (▲) of *N*-acetyl-[^{14}C] Phe-tRNA (A) and peptidyl-tRNA (B). Experiments were performed as described under Materials and methods. Final concentrations not specified in the text were: 150 μM Gpp(CH_2)p, and 4.5 and 5 μM GTP, 100 and 150 μM Gpp(NH)p, and 0.13 and 0.07 mg/ml EF-G for experiments in (A) and (B), respectively. Controls without EF-G and guanosine nucleotide (○) were not subtracted.

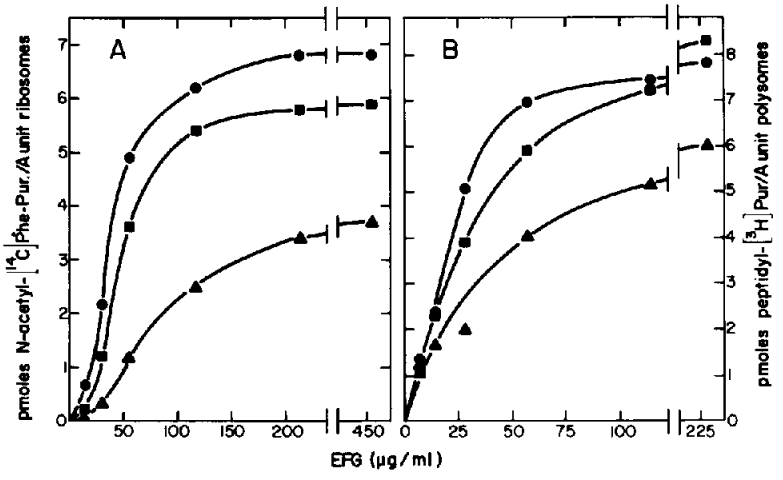

Fig.3. Effect of EF-G concentration on translocation promoted by GTP (•), Gpp(NH)p (■) and Gpp(CH_2)p (▲) of *N*-acetyl-[^{14}C]-Phe-tRNA (A) and peptidyl-tRNA (B). Experiments were performed as described under Materials and methods. Final concentrations not specified in the text were: 6 and 11 μM GTP, 0.95 and 0.09 mM Gpp(NH)p and 1.0 and 0.08 mM Gpp(CH_2)p for experiments in (A) and (B), respectively.

Fig.2 shows the time course of translocation promoted by 5 μM GTP, 0.10 and 0.15 mM Gpp(NH)p and 0.15 mM $Gpp(CH_2)p$. With the *N*-acetyl-[^{14}C]-Phe-tRNA system (fig.2A), GTP and Gpp(NH)p induced rapid bursts of translocation, complete within 5 min, followed by a slow, almost linear rate, which continued for more than 40 min (not shown). With $Gpp(CH_2)p$ the reaction proceeded more slowly and the two stages of the reaction were less apparent. With the polysomal system (fig.2B), translocation with each nucleotide was fast and was completed within 5 min, although it reached different plateaus.

The dependence of translocation on EF-G concentration is shown in fig.3. With both systems and at concentrations of the factor as high as 0.45 (panel A) and 0.23 (panel B) mg/ml, some of the ribosomes did not translocate with $Gpp(CH_2)p$ while most of them did with Gpp(NH)p.

The requirement of relatively high Gpp(NH)p and $Gpp(CH_2)p$ concentrations for translocation (fig.1) might be due to the low activity of these GTP analogs in promoting the binding of EF-G to the pretranslocated ribosome or in carrying out any of the subsequent step(s) of the translocation reaction. Since $Gpp(CH_2)p$ (0.1 mM) did not appreciably diminish the extent (fig.1A) or slow the rate (not shown) of the GTP (0.3 to 6 μM)-induced translocation of *N*-acetyl-[^{14}C] Phe-tRNA, it seems that $Gpp(CH_2)p$ was much less effective than GTP in promoting the binding of EF-G to the ribosome. Whether the analog was also less effective in the subsequent steps of translocation remains to be clarified.

4. Discussion

In the light of the present experiments the failure of $Gpp(CH_2)p$ to promote translocation in previous studies was evidently due to the use either of too low concentrations of the analog [4] or of catalytic amounts of EF-G [5,6]. Moreover, it is most unlikely that the analog-promoted translocation here reported might be due to contamination of the compounds with GTP since: (a) the preparations used appeared free of GTP when analyzed by thin-layer chromatography; (b) translocation occurred with two different analogs from three different sources; and (c) repurification of the analogs or preincubation under conditions that might cause hydrolysis of GTP did not diminish their activity (not shown).

Our results are consistent with the proposal that GTP hydrolysis is not necessary for translocation, but is only required for the detachment of EF-G from the ribosome after translocation has occurred [2,3]. With the analogs, however, it is not known whether EF-G is actually released after translocation and, if release occurs, which mechanism is responsible for it. Since ribosomes complexed with peptidyl-tRNA seem to have a low affinity for EF-G in the presence of the analogs, as indicated by the high concentrations necessary for translocation and the absence of competition between GTP and $Gpp(CH_2)p$ in the translocation of *N*-acetyl-[^{14}C]Phe-tRNA (fig.1), it is possible that the low affinity facilitates the release of EF-G with GTP hydrolysis. On the other hand, the rapid rates of translocation observed in the experiments represented in fig.2 indicate that physical removal of EF-G from the reaction mixture, to favor the release of EF-G [3], is not essential for translocation with the analog in our in vitro systems.

Acknowledgements

We thank Mr José María Buesa for expert technical assistance. This work was supported by a grant from 'Fondo Nacional para el Desarrollo de la Investigación Científica'.

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