POLYSOMAL RIBOSOMES COMPLEXED WITH ELONGATION FACTOR G CAN ENGAGE IN THE PEPTIDYL TRANSFER REACTION

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Received 19 July 1976

1. Introduction

The 50 S subunit of prokaryotic ribosomes carries the peptidyl transferase centre, responsible for peptide bond formation during protein synthesis ([1], for review). On this same subunit there is a site for interaction with elongation factor G (EF-G), a supernatant protein involved in ribosomal translocation [1]. While early experiments involving antibiotics failed to detect a functional interdependence between these two sites [2,3], more recent studies have suggested such a relationship since occupancy of the donor site of the peptidyl transferase centre by peptidyl-tRNA decreases the capacity of the EF-G-site to interact with the factor [4,5]. Moreover, work aimed at elucidating the role of individual ribosomal proteins has shown that some are related to both peptidyl transferase centre an EF-G-site ([6], for review). Consequently, the two sites may be close neighbours on the ribosomal surface.

To study further the functional relationship between these sites, we have investigated the ability of *E. coli* polysomal ribosomes with the EF-G-site occupied by the factor to engage in the peptidyl transfer reaction. The results indicate that occupancy of the EF-G-site does not interfere with the transfer of the nascent peptidyl chains of polysomes to the acceptor substrate puromycin.

Abbreviations: Gpp(NH)p, guanylylimido diphosphate; Gpp(CH_2)p, guanylylmethylene diphosphonate; EF-G, EF-Tu and EF-2, elongation factors G, Tu, and 2 (eukaryotic); P- and A-site, ribosomal donor and acceptor site; Pur, puromycin; Thios, thiostrepton.

2. Materials and methods

Preparation of 1 M NH_4Cl-washed *E. coli* ribosomes, EF-G, and N-acetyl-[^{14}C]Phe-tRNA (1050 cpm/pmol) has been described elsewhere [4,7]. *E. coli* MRE600 endogenous polysomes were prepared as described [5], except that the high-salt washings were replaced by sedimentation through sucrose cushions containing 100 mM NH_4Cl, 6.3 mM $Mg(acetate)_2$, 20 mM Tris–HCl pH 7.8, 0.3 mM EDTA, 6 mM 2-mercaptoethanol [8]. S100 extracts (supernatants from 100 000 × *g* spin) [9] were freed of nucleic acids, GTP, and other low molecular-weight components by streptomycin precipitation [10] and filtration through Sephadex G-25. Just prior to use, portions of these extracts in 60 mM NH_4Cl, 10 mM Tris–HCl pH 7.8, 2 mM dithiothreitol and 45% glycerol were incubated at 55°C for 50 min to diminish the retention of labelled guanosine nucleotides on nitrocellulose membranes. Specific activities of [^{3}H]GTP (470 cpm/pmol) and [^{3}H]Gpp(NH)p (590 cpm/pmol) were determined by isotopic dilution [11], and that of [^{3}H]puromycin (1075 cpm/pmol) by reaction with *N*-acetyl-[^{14}C]Phe-tRNA of well known specific activity and isolation of the synthesized *N*-acetyl-[^{14}C]Phe-[^{3}H]puromycin by extraction with ethylacetate at pH 4.0. Labelled compounds were from the Radiochemical Centre, Amersham.

EF-G was bound to ribosomes in reaction mixtures (74 to 166 μl) containing: 90 mM NH_4Cl, 10 mM $Mg(acetate)_2$, 10 mM Tris–HCl pH 7.8, 1 mM dithiothreitol, 110 μg/ml EF-G, either 2 mM fusidic acid plus 8.5 μM [^{3}H]GTP (preincubated with phosphoenol pyruvate and pyruvate kinase) or 32 μM [^{3}H]Gpp(NH)p,

8 to 12 A_{260} unit/ml of either polysomes or NH_4Cl-washed ribosomes (complexed with poly(U) [11]), and unless otherwise specified, 0.3 to 1.1 A_{280} unit/ml of heat-treated S100 extract. After 5 to 10 min of incubation at 30°C (time sufficient for the binding of EF-G to reach a plateau) 12 or 15 μl portions were analyzed for ribosome-bound guanosine nucleotide by filtration through nitrocellulose membranes [7]. The incubation was continued in the remaining reaction mixture and the stability of the [^{3}H]guanosine nucleotide·EF-G·ribosome complex was immediately assayed by adding either 10 μM thiostrepton or an excess of unlabelled guanosine nucleotide (0.4 mM GTP or 0.9 mM Gpp(NH)p) to block further binding of [^{3}H]guanosine nucleotide to ribosomes. After incubation at 30°C for the indicated time intervals, the remaining ribosome-bound [^{3}H]guanosine nucleotide was determined. Results were corrected for the retention of [^{3}H]guanosine nucleotide on nitrocellulose membranes observed in parallel mixtures without ribosomes or polysomes. Where indicated another portion of the reaction mixture containing the EF-G·ribosome complexes was supplemented with 10 μM [^{3}H]puromycin and, after incubation at 30°C for the indicated time intervals, the peptidyl-[^{3}H]puromycin formed was determined by measuring the radioactivity precipitable in cold trichloroacetic acid.

3. Results

Ribosomes carrying peptidyl-tRNA in the P-site have a decreased capacity to interact with EF-G [4,5]. However, in the presence of an S100 extract, GTP, and fusidic acid endogenous *E. coli* polysomes form relatively stable GDP·EF-G·ribosome·fusidic acid complexes [5]. As shown in fig.1 (points on the ordinate at −0.5 min), under these conditions approximately 80% of the polysomal ribosomes could form this complex, as measured by the ribosome-dependent retention of [^{3}H]GDP. Adding to these complexed ribosomes 10 μM [^{3}H]puromycin caused the rapid release of 35% of the polysomal nascent peptidyl chains, assuming that all ribosomes present possessed peptidyl-tRNA (fig.1, close circles). To interpret this experiment, however, requires investigation of the stability of the GDP·EF-G·ribosome·fusidic acid complex under the conditions prevailing during the puromycin reaction, specially since previous work has show that this complex can turn over [12]. Consequently, a portion of the reaction mixture containing the complex was mixed with 10 μM unlabelled puromycin and either a large excess of unlabelled GTP (fig.1A) or 10 μM thiostrepton (fig.1B) to block further binding of [^{3}H]GTP to the ribosomes. (Thiostrepton rapidly inactivates only those ribosomes that are not complexed with EF-G

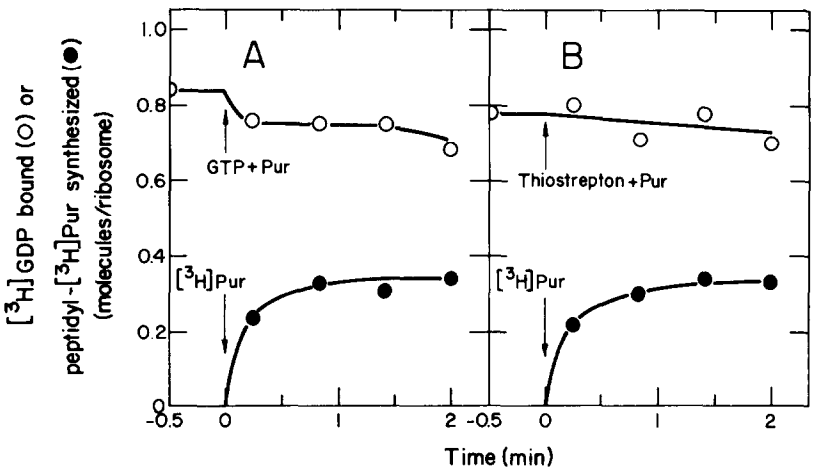

Fig.1. Stability of [^{3}H]GDP·EF-G·ribosome·fusidic acid complex formed on polysomes and reaction of peptidyl nascent chains with [^{3}H]puromycin. Experiment was performed as described under Materials and methods. After formation of EF-G·ribosome complex portions of the reaction mixture were assayed for complex stability, by addition of either 0.4 mM GTP (panel A) or 10 μM thiostrepton (panel B), and for peptidyl-[^{3}H]puromycin synthesis.

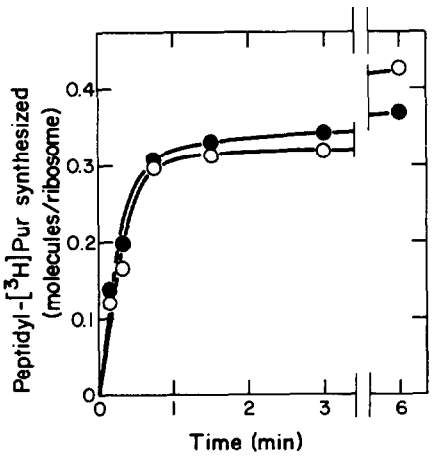

Fig.2. Peptidyl-[^{3}H]puromycin synthesis in the presence (•) or in the absence (○) of fusidic acid. Experiment was performed as described under Materials and methods.

and prevents subsequent interaction with the factor [12].) Figure 1 (open circles) shows that, with both treatments, the complex remained stable during the puromycin reaction. Furthermore, fig.2 shows that omitting fusidic acid from the reaction mixture, a condition that prevents stable binding of EF-G to the ribosome [13], did not modify the rate or the extent of the puromycin reaction. The results thus strongly suggest that ribosome-bound EF-G does not interfere with the reaction of polysomal nascent chains with puromycin.

The destabilizing effect of peptidyl-tRNA on EF-G·ribosome complexes [4,5,13] suggested to us an alternative way to examine the puromycin reaction of ribosomes complexed with EF-G, since the stability of the guanosine nucleotide·EF-G·polysomal ribosome complex should increase if the peptidyl nascent chain was released after formation of the complex Figure 3A shows that, indeed, puromycin increased the stability of a [^{3}H]GDP·EF-G·polysomal ribosome·fusidic acid complex, measured by addition of thiostrepton. Moreover, this stabilization required the presence of peptidyl-tRNA, since puromycin did not modify the decay of a complex formed on 70 S ribosomes bearing only mRNA (poly(U)) (fig.3B). It is thus clear that puromycin could react with peptidyl chains of ribosomes complexed with EF-G. The experiments depicted in fig.3A and B were performed in the absence of S100 extract, but the stabilizing effect of puromycin was observed both in the presence of this extract and when the decay of the complex was measured by addition of an excess of unlabelled GTP (not shown). Furthermore, the nonhydrolyzable analog of GTP guanylylimido diphosphate (Gpp(NH)p) promotes binding of EF-G to ribosomes [14], and fig.3C shows that puromycin also inhibited the decay of a [^{3}H]Gpp(NH)p·EF-G·poly-

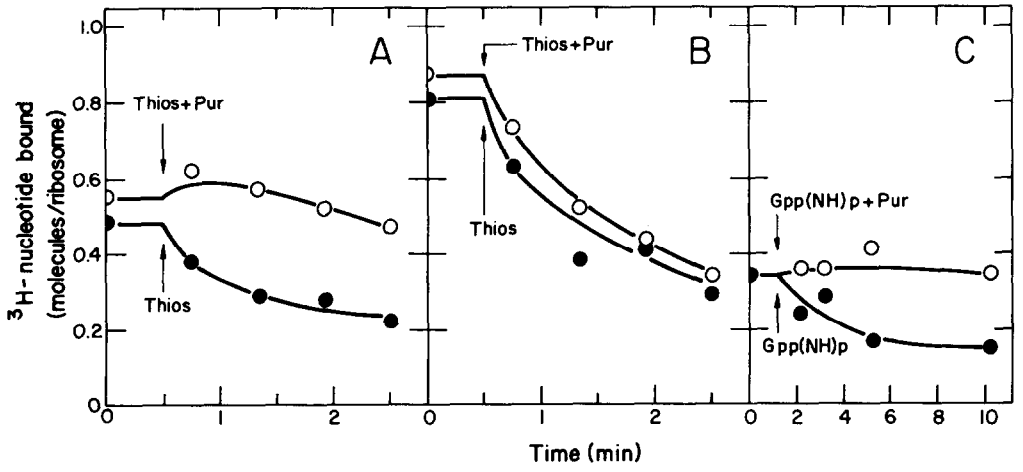

Fig.3. Effect of puromycin on the stability of preformed [^{3}H]GDP·EF-G·ribosome·fusidic acid (panel A and B) and [^{3}H]Gpp(NH)p·EF-G·ribosome complex (panel C). Experiments were carried out as described in the text, except that S100 extract was omitted and two parallel reaction mixtures were used to form the fusidic acid-stabilized complex in A and B. Polysomes were replaced by 70S ribosomes in B. Where indicated, puromycin was used at 0.36 mM.

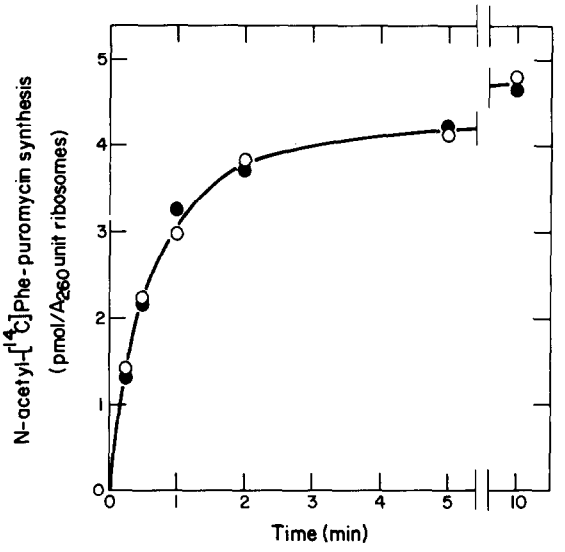

Fig.4. Failure of EF-G plus Gpp(CH_2)p to inhibit the reaction of *N*-acetyl-[^{14}C]Phe-tRNA nonenzymically bound to the ribosomal P-site with puromycin. Nonenzymic binding of *N*-acetyl-[^{14}C]Phe-tRNA at 6 mM Mg^{2+} was performed essentially as described [11]. Puromycin reaction was assayed in reaction mixtures (130 μl) containing: 20 mM NH_4Cl, 50 mM KCl, 6 mM Mg(acetate)$_2$, 1 mM dithiothreitol, 0.7 mM puromycin, 7.3 A_{260} unit/ml of ribosomes containing 5.3 pmol *N*-acetyl-[^{14}C]Phe-tRNA bound per A_{260} unit of ribosomes and with (•) or without (○) 80 μg/ml EF-G and 1 mM Gpp(CH_2)p. After incubation at 30°C for the times indicated, 20 μl portions were assayed for *N*-acetyl-[^{14}C]Phe-puromycin synthesized [7].

somal ribosome complex, as measured by the exchange of [^{3}H]Gpp(NH)p with unlabelled Gpp(NH)p. Similar results (not shown) were obtained with complexes induced by guanylyl-methylene diphosphonate (Gpp(CH_2)p).

In contrast to the present results, Otaka and Kaji [15], using a poly(U)-directed system, have reported that EF-G plus Gpp(CH_2)p inhibits the reaction of *N*-acetyl-Phe-tRNA, nonenzymically bound at 6 mM Mg^{2+} to the ribosomal P-site, with puromycin. We have attempted to reproduce this effect, but, as fig.4 shows, 80 μg/ml of EF-G plus 1 mM Gpp(CH_2)p in our hands did not affect the rate or the extent of the puromycin reaction. Moreover, increasing the concentration of EF-G to 160 μg/ml still produced no inhibition (not shown).

4. Discussion

The evidence presented here indicates that EF-G and peptidyl-tRNA can be bound simultaneously to the ribosome and that under these conditions peptidyl-tRNA truly occupies the donor site of the peptidyl transferase centre, as judged by its reactivity with puromycin. The simplest explanation of these results is that the EF-G- and the P-site, including the donor site of the peptidyl transferase centre, do not overlap, even though they may be close neighbours on the ribosomal surface [6]. There must, however, be mutual conformational influences between the sites, as suggested by the decreased ability of EF-G to interact with the ribosome when the donor site of the peptidyl transferase centre is occupied by peptidyl-tRNA [4,5,13], and by the partial inhibition of non-enzymic binding of acylated-tRNA to the P-site by ribosome-bound EF-G [16]. Non overlapping of EF-G- and P-sites reinforces our previous suggestion that during translocation peptidyl-tRNA reaches the P-site before EF-G is released from the ribosome [17]. In contrast, correct positioning of the acceptor substrate in the peptidyl transferase centre seems to require the release of EF-Tu [1].

Pranger and Van der Zeijst [18], and Otaka and Kaji [15] studying yeast and *E. coli* systems respectively, have described inhibition of the reaction of *N*-acetyl-Phe-tRNA with puromycin by EF-2 plus fusidic acid [18] and EF-G plus Gpp(CH_2)p [15]. Since we have been unable to reproduce this inhibition with the *E. coli* system (fig.4), we are at loss to explain the discrepancy between the results with the different systems. Nevertheless, all other considerations apart, it would seem likely that the results obtained with endogenous polysomes should more closely reflect the properties of the ribosomes engaged in protein synthesis in the cell.

Acknowledgements

We are grateful to Miss Pilar Ochoa for expert technical assistance. This work has been supported by Institutional Grants from 'Comisión Administradora del Descuento Complementario (Instituto Nacional de Previsión)' and 'Dirección General de Sanidad' (Spain).

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