PROPERTIES OF ELONGATION FACTOR G: ITS INTERACTION WITH THE RIBOSOMAL PEPTIDYL-SITE

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Summary: EF-G bound to poly(U)·ribosomes prevents enzymatic or nonenzymatic binding of charged tRNA not only to the A-site but also to the P-site. In turn, charged tRNA bound either to the P- or A-site prevents formation of EF-G·GMPPCP·ribosome complex. Ribosomes carrying newly synthetized peptidyl -tRNA in pretranslocative state are also unable to form stable complexes with EF-G. The functional implications of these observations are discussed and it is suggested that tRNA plays a regulatory role in the interaction of EF-G with ribosomes during the cyclic process of elongation.

Elongation of the peptide chain in bacterial systems requires the interaction of the ribosome with the soluble protein factors G (EF-G) and Tu (EF-Tu). Both factors show ribosome-dependent GTPase activities and are able to form stable complexes with ribosomes. EF-G GTPase is stimulated by aminoacyl-tRNA and mRNA but is also active in their absence (1). EF-G·ribosome complex is formed in the presence of GTP or GDP and is particularly stabilized by fusidic acid, while the nonhydrolyzable GTP analog GMPPCP induces alone formation of stable complexes (2-4). Recently, ribosomes have been shown to be unable to interact with EF-G and EF-Tu at the same time (5-8). Moreover, EF-G·ribosome complexes are unable to bind enzymatically or nonenzymatically Phe-tRNA^{Phe} to the aminoacyl-site (A-site)(6). In turn, Phe-tRNA^{Phe} bound to the A-site inhibits formation of stable EF-G·ribosome complexes (9).

In this communication we show that binding of EF-G to ribosomes and of tRNA to the peptidyl-site (P-site) are also mutually exclusive. These observations are discussed with respect to the mechanism of interaction of EF-G with ribosomes during elongation.

MATERIALS AND METHODS

Poly(U), GTP and GMPPCP were purchased from Boehringer; poly(A,U,G) (1:1:1) from Miles Labs; L-[^{14}C]phenylalanine (spec.act.840 cpm/pmol) from

The Radiochemical Center (Amersham). [^{3}H]GMPPCP (spec.act.182 cpm/pmol) was a gift from Dr. J. Modolell (Instituto de Biologia Celular, Madrid). NH_4Cl-washed ribosomes and homogeneous EF-G and EF-T (EF-Tu + EF-Ts) were prepared from E.coli BT2^r, as reported (10). Crude initiation factors, isolated from the 0.5 M NH_4Cl ribosomal wash by precipitation with $(NH_4)_2SO_4$ between 40 and 85% saturation, and f-[^{3}H]Met-tRNA (spec.act.190 cpm/pmol) were provided by Mr. J. Voigt from this Lab. Purified $tRNA^{Phe}$ (780 pmol of $tRNA^{Phe}/A_{260}$ unit) was prepared and charged as described (11). [^{14}C]Phe-$tRNA^{Phe}$ was 90% charged (705 pmol [^{14}C]Phe bound/A_{260} unit). [^{14}C]Phe-$tRNA^{Phe}$ and Phe-$tRNA^{Phe}$ were acetylated by the method of Haenni and Chapeville (12).

Poly(U)· or poly(A,U,G)·ribosome complexes were obtained by incubating ribosomes for 5 min at 30°C in the presence of saturating amounts of poly(U) or poly(A,U,G)(4 µg/A_{260} unit ribosomes) in standard buffer (SB) with 10 mM $MgCl_2$. SB contained 60 mM Tris-HCl, pH 7.8, 30 mM KCl, 30 mM NH_4Cl, 2 mM dithiothreitol. For further details see legends.

RESULTS

The nonenzymatic binding of Phe-$tRNA^{Phe}$ or of Ac-Phe-$tRNA^{Phe}$ to poly(U)·ribosomes has been observed to take place predominantly at the A-site with high [Mg^{2+}](15-20 mM) and at the P-site with low [Mg^{2+}](5-10 mM)(13-14). In our system, preincubation with EF-G and GMPPCP reduced to the same degree the ability of poly(U)·ribosome complexes to bind [^{14}C]Phe-$tRNA^{Phe}$ (Fig. 1A) or Ac-[^{14}C]Phe-$tRNA^{Phe}$ (Fig. 1B) both at high and low [Mg^{2+}]. GMPPCP alone or EF-G alone had no significant effect on binding of tRNA to ribosomes. Similar inhibitions were also obtained when EF-G was bound to poly(U)·ribosome in the presence of fusidic acid plus GTP or GDP (not shown). This indicates that formation of a stable complex between EF-G and ribosomes prevents nonenzymatic binding of charged $tRNA^{Phe}$ in both conditions required for its binding to either A-site or P-site in the absence of EF-G. To be sure that in our system at low [Mg^{2+}] without EF-G binding was taking place predominantly at the ribosomal P-site we checked puromycin reactivity of Ac-[^{14}C]Phe-$tRNA^{Phe}$ bound to ribosomes in this condition. Fig. 2 shows that puromycin reactivity was only increased 15% by subsequent addition of EF-G plus GTP which causes translocation of tRNA from A- to P-site. Ac-[^{14}C]Phe-$tRNA^{Phe}$ unreactive to puromycin (=36%) in the presence of EF-G and GTP was likely bound to ribosomes inactive in the peptidyl-transferase reaction (see later).

Table I shows that the EF-G bound to ribosomes in the presence of GTP

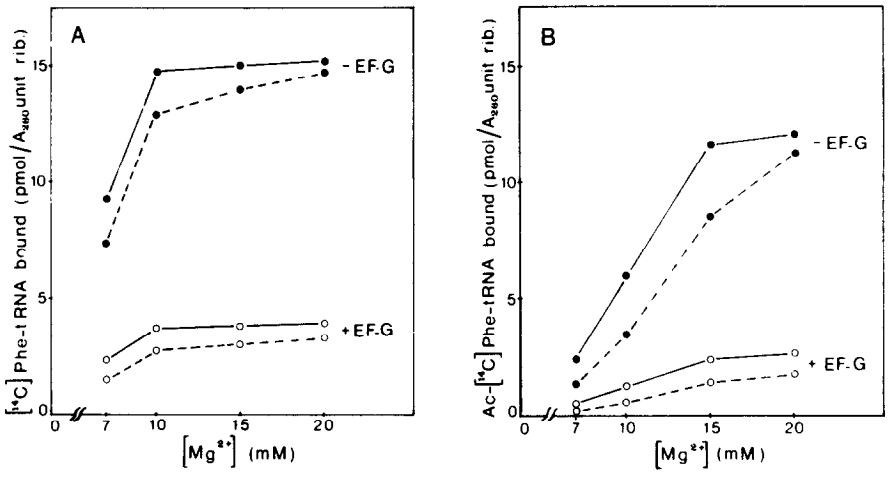

Fig. 1. EF-G-mediated inhibition of the nonenzymatic binding of Phe-tRNA^{Phe} and Ac-Phe-tRNA^{Phe} to ribosomes. 2.5 A_{260} units of ribosomes precharged with poly(U), 10 nmol GMPPCP and 27 µg EF-G were incubated for 5 min at 30°C in a volume of 95 µl SB in the presence of various $MgCl_2$ concentrations. Then 108 pmol of [^{14}C]Phe-tRNA (A) or 85 pmol of Ac-[^{14}C]Phe-tRNA (B) in 5 µl SB were added to the reaction mixtures. After 2 (---) and 5 min (——) of further incubation at 30°C 40 µl aliquots were withdrawn and immediately analyzed by Millipore filtration. Plus EF-G (o), controls minus EF-G (•). Values are corrected for charged tRNA^{Phe} retained on filter in the absence of ribosomes (0.4 - 0.6 pmol).

and fusidic acid inhibited initiation factor-mediated binding of both Ac-Phe--tRNA and f-Met-tRNA which takes place at the P-site (14-15). Grunberg-Manago et al. already observed that EF-G bound to ribosomes in similar conditions inhibited IF-2-dependent GTPase (16). From these results we conclude that EF-G prevents the enzymatic or nonenzymatic binding of tRNA to the P-site as already shown for the A-site (5-8).

Table II illustrates a series of experiments showing the effect of bound tRNA^{Phe} on the formation of EF-G·ribosome complex. Ac-Phe-tRNA^{Phe} bound nonenzymatically to the P-site (line b) inhibited formation of EF-G·[^{3}H]GMPPCP·ribosome complex to the same extent as the EF-T-mediated binding of Phe-tRNA^{Phe} to the A-site (line c). Uncharged tRNA^{Phe} bound under equivalent conditions showed a similar inhibitory effect (not shown). EF-G GTPase activity did not restore the ability of the Ac-Phe-tRNA^{Phe}·poly(U)·ribosome complex to bind EF-G (line d). Nonenzymatic binding of Ac-Phe-tRNA^{Phe} to the P-site followed

Table I. EF-G-mediated inhibition of the enzymatic binding of Ac-Phe-tRNA^{Phe} and f-Met-tRNA to ribosomal P-site

1st incubation	2nd incubation (plus initiation factors)	
	Experiment 1	Experiment 2
addition to reaction mixture	Ac-[^{14}C]Phe-tRNA bound to ribosomes	f-[^{3}H]Met-tRNA bound to ribosomes
	pmol	pmol
none	4.0	2.0
GTP	10.5	6.1
GTP + EF-G	10.8	6.2
GTP + fusidic acid	7.1*	4.1*
GTP + fusidic acid + EF-G	3.0	1.4

* Inhibition due to EF-G present in crude initiation factors preparation

1.3 A_{260} units of ribosomes precharged with poly(U)(Exp.1) or with poly(A,U,G) (Exp.2) were incubated for 5 min at 30°C in 90 µl SB - 6 mM $MgCl_2$ in the presence, as indicated of 60 nmol GTP, 100 nmol fusidic acid, 8 µg EF-G. Incubation at 30°C was continued for 10 min after addition of 10 µl SB containing 20 µg crude initiation factors and either 39 pmol of Ac-[^{14}C]Phe-tRNA^{Phe} (Exp.1) or 20 pmol f-[^{3}H]Met-tRNA (Exp.2). Samples were analyzed by Millipore filtration. Values are corrected for charged tRNA, retained on filter in the absence of ribosomes (0.8 - 1.1 pmol). The values of the first line with no addition represent the nonenzymatic binding of Ac-[^{14}C]Phe-tRNA or f-[^{3}H]Met-tRNA to ribosomes.

by EF-T-directed binding of Phe-tRNA^{Phe} to the A-site which causes formation of the Ac-Phe-Phe-tRNA^{Phe} in the A-site and of uncharged tRNA in the P-site inhibited even more strongly binding of EF-G and GMPPCP to ribosomes (line e). The presence of ribosomes in a functional pretranslocative state in this last experiment was confirmed by running a control in which Phe-tRNA^{Phe} was substituted by [^{14}C]Phe-tRNA^{Phe}. Alkaline hydrolysates of the isolated complexes were analyzed by descending paper chromatography as described by Felicetti and Lipmann (17). This showed that 66% of bound [^{14}C]Phe-tRNA^{Phe} had reacted with Ac-Phe-tRNA^{Phe} to form the corresponding dipeptide. The portion of enzymatically bound [^{14}C]Phe-tRNA which did not react with Ac-Phe-tRNA^{Phe} corresponded therefore to the amount of Ac-[^{14}C]Phe-tRNA^{Phe} which was puromycin unreactive in the presence of EF-G and GTP (cf. Fig. 2).

Table II. Effect of $tRNA^{Phe}$ on the formation of EF-G·GMPPCP·ribosome complexes

Addition to reaction mixture		$[^3H]$GMPPCP bound to ribosomes
1st incubation	2nd incubation	
		pmol/A_{260} unit ribosomes
a) none	none	10.7
b) Ac-Phe-$tRNA^{Phe}$	none	3.9
c) none	Phe-$tRNA^{Phe}$ + EF-T + GTP	4.0
d) Ac-Phe-$tRNA^{Phe}$	EF-G + GTP	3.9
e) Ac-Phe-$tRNA^{Phe}$	Phe-$tRNA^{Phe}$ + EF-T + GTP	2.1

6.5 A_{260} units of ribosomes precharged with poly(U) were incubated for 10 min at 30°C with or without 280 pmol Ac-Phe-$tRNA^{Phe}$ in 100 µl of SB - 10 mM $MgCl_2$ (first incubation). Then, 50 µl of SB - 10 mM $MgCl_2$ with or without 7 µg EF-G and 15 nmol GTP, or 15 µg EF-T, 280 pmol Phe-$tRNA^{Phe}$ and 3 nmol GTP were added and incubation was continued for 10 min at 30°C (second incubation). The ribosomal complexes were isolated on sepharose 4B. 0.7 - 0.8 A_{260} units of the isolated complexes were incubated with 4 µg EF-G and 125 pmol $[^3H]$GMPPCP for 5 min at 30°C in a final volume of 125 µl SB - 10 mM $MgCl_2$. 100 µl aliquots were withdrawn and analyzed for bound $[^3H]$GMPPCP by Millipore filtration. Values are corrected for $[^3H]$GMPPCP retained on filter when EF-G was omitted (0.6 - 0.8 pmol).

Other experiments not illustrated showed, moreover, that about 65-70% of the ribosomes were able to bind charged $tRNA^{Phe}$ suggesting that the $[^3H]$GMPPCP and EF-G bound in the presence of charged $tRNA^{Phe}$ (see Table II) had reacted with the portion of ribosomes inactive in the Phe-$tRNA^{Phe}$ binding reaction.

DISCUSSION

In this report we have shown that ribosome complexes with EF-G and tRNA are mutually inhibitory under conditions which induce binding of tRNA mainly to the P-site. Such mutual exclusion had previously been shown for the A-site (5-8). Formation of stable EF-G·ribosome complexes requires, therefore, the interaction of EF-G with a ribosomal region involving both A- and P-site. The fact that tRNA plus poly(U) stimulates the EF-G GTPase activity, as first shown by Conway and Lipman (1) and recently confirmed in this Lab (unpublished

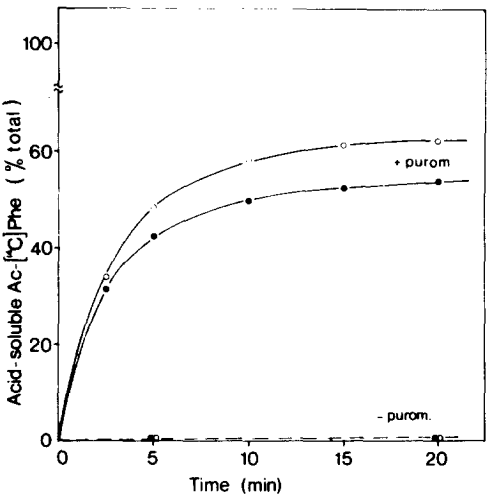

Fig. 2. Puromycin reactivity of Ac-Phe-tRNA nonenzymatically bound to ribosomes in the presence of 10 mM $MgCl_2$. 17 A_{260} units ribosomes precharged with poly(U) and 750 pmol of Ac-[^{14}C]Phe-tRNA were incubated for 10 min at 30°C in 150 µl of SB - 10 mM $MgCl_2$ and then chilled to 0°C. Ac-[^{14}C]Phe-tRNA·poly(U)·ribosome complexes were isolated by gel filtration on Sepharose 4B (9) and carried 16.5 pmol of Ac-[^{14}C]Phe-tRNA bound per A_{260} unit ribosomes. 3.2 A_{260} units of ribosomal complexes were thereafter incubated at 30°C in the presence of 1.5 mM puromycin in 260 µl of SB - 10 mM $MgCl_2$. At intervals 40 µl aliquots were withdrawn. Formation of acid-soluble Ac-[^{14}C]Phe-puromycin was determined as described (11). Ribosomal complexes assayed without (•—•) and with (o—o) preincubation for 5 min at 30°C in the presence of 6 µg EF-G and 75 nmol of GTP. No liberation of acid-soluble radioactive material was observed in the absence of puromycin (---).

results), and that at the same time it inhibits formation of the EF-G·ribosome complex, suggests an important regulatory role for tRNA in the interaction of EF-G with ribosomes. EF-G leaves the ribosome during polypeptide synthesis after each round of GTP hydrolysis in order to allow interaction of EF-Tu with ribosomes (11,5-8); any stabilization of the EF-G·ribosome complex would negatively affect the dynamics of elongation. In the absence of tRNA, EF-G·GDP·ribosome complex - the product of the GTPase reaction - tends to some extent to remain associated (2-4) while in its presence, as shown in this report, even GMPPCP is not able to stabilize EF-G·ribosome association. The functional meaning of tRNA inhibition on the EF-G binding to ribosomes is illustrated by the observation that stable binding of EF-G is inhibited also with ribosomes carrying peptidyl-tRNA in the A-site and uncharged tRNA in the P-site, i.e.

in a state corresponding to the pretranslocative complex on which EF-G acts during elongation. From these results it can be postulated that tRNA plays a role in facilitating the release of EF-G from ribosomes during the cyclic process of elongation. It is interesting to observe that unlike EF-G, EF-2 from rat liver seems to be able to complex with 80S ribosomes also when peptidyl-tRNA is bound to the P-site (18). Preliminary experiments performed in this Lab with E.coli polysomes carrying peptidyl-tRNA in the P-site confirmed the inhibition of EF-G binding to ribosomes caused by Ac-Phe-tRNA as described in this communication. This suggests that considerable differences exist in this respect between bacterial and mammalian system.

How the presence of tRNA in the A- and P-site influences the interaction of EF-G with the ribosomal region responsible for expression of GTPase activity is presently under investigation.

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