

Inhibition by Elongation Factor EF G of Aminoacyl-tRNA Binding to Ribosomes

(Millipore filter/EF Tu/GTPase/fusidic acid/ GMPPCP)

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ABSTRACT Elongation factor G (EF G), bound to ribosomes either with GMPPCP or with fusidic acid and GDP, inhibits elongation factor Tu (EF Tu)-dependent binding of Phe-tRNA on the ribosome-poly(U) complex and binding of Ala-tRNA on the initiation complex formed with RNA from bacteriophage R17; GTP hydrolysis associated with Phe-tRNA binding is also inhibited. Moreover, nonenzymic binding of Phe-tRNA at high Mg^{++} concentration is completely blocked by EF G. Thus, EF G appears to bind at a site that overlaps or interacts with the ribosomal A-site.

Studies on the interaction of elongation factor G (EF G) with the ribosome have shown that there is a binding site for this factor (the G-site) on the 50S subunit (1, 2). The G-site is located in a position distinct from the peptidyl-transferase center (2) and it is inactivated by the peptide antibiotics siomycin and thiostrepton, which bind to the 50S subunit and prevent the attachment of EF G (2-8). Recent work by Tanaka and ourselves (9, 10) has shown that siomycin or thiostrepton treatment of ribosomes impairs not only their binding of EF G, but also their binding of aminoacyl-tRNA (enzymic or nonenzymic) and the elongation factor Tu (EF Tu)-dependent hydrolysis of GTP. Since these effects appear to be the consequence of a single action of the antibiotics on the 50S subunit (10), a relationship between the binding site of EF G (the G-site) and that of the aminoacyl-tRNA·EF Tu·GTP complex (which includes the ribosomal A-site) has been suggested (9, 10).

We have now found that with ribosomes of *Escherichia coli* bound EF G, stabilized either by GMPPCP (11-13) or by fusidic acid plus GDP (1, 12, 14-16), acts much like siomycin or thiostrepton treatment in its effects on either enzymic or nonenzymic binding of aminoacyl-tRNA, and on the EF Tu-dependent hydrolysis of GTP. EF G can thus block the ribosomal A-site, a finding that reinforces the earlier suggestion that the G-site may be located near enough to influence, or may even overlap with, the portion of the A-site on the 50S ribosomal surface.

MATERIALS AND METHODS

Ribosomes (washed with 1 M NH_4Cl), EF G, EF T (EF Ts + EF Tu), and crude initiation factors were prepared (17-19) from *E. coli* MRE 600. $[\gamma-^{32}P]GTP$, prepared

according to Glynn and Chappell (20), was purified by DEAE-Sephadex chromatography. Deacylated $tRNA_{Phe}$, a gift from Dr. G. D. Novelli, Oak Ridge National Laboratory, was charged with $[^{14}C]$ phenylalanine (930 cpm/pmol) to 60% of its theoretical acceptor activity. f- $[^3H]$ Met-tRNA and $[^{14}C]$ Ala-tRNA (7300 and 280 cpm/pmol, respectively) were prepared as described (10).

Poly(U), EF G, and Phe-tRNA were bound to 70S ribosomes in three consecutive incubations. Poly(U) (60-110 $\mu g/ml$) was first incubated at 30° for 10 min with 33-38 A_{260} unit/ml of ribosomes in the presence of Tris·HCl (pH 7.8), $Mg(acetate)_2$, NH_4Cl , and dithiothreitol, at concentrations about twice those in the reaction mixture for the binding of Phe-tRNA (specified in the legends). The mixture was chilled and EF G was added, together with either GMPPCP or fusidic acid plus GDP or GTP. Incubation was at 30° for 3 min (with GMPPCP), or at 0° for 5 or 10 min (with fusidic acid plus GDP or GTP).

To assay nonenzymic binding of Phe-tRNA to these treated ribosomes, the reaction mixture (20 μl) was supplemented with $[^{14}C]$ Phe-tRNA (5 μl) and incubated as specified in the legends. The reaction was terminated by dilution with 2 ml of buffer with the same ionic composition (and fusidic acid) as the reaction mixture; bound $[^{14}C]$ Phe-tRNA was immediately determined by filtration (21). To assay EF Tu-dependent binding of Phe-tRNA, and to assay its associated GTPase activity, the reaction mixture was supplemented with $[^{14}C]$ Phe-tRNA complexed with EF Tu and $[\gamma-^{32}P]GTP$, and the incubation was continued at 0° for the time indicated. A 15- μl sample was then analyzed for $[^{14}C]$ -Phe-tRNA bound to ribosomes, and 30- μl sample for ^{32}P -labeled inorganic phosphate (19). Owing to a residual endogenous GTPase activity present in the EF T preparations, hydrolysis of GTP was corrected for the values obtained in parallel mixtures without ribosomes and without EF G (components devoid of measurable endogenous GTPase activity). Control mixtures showed that the corrected GTPase activity was completely dependent on the presence of Phe-tRNA. Incubation time for the binding of aminoacyl-tRNA was short (2-3 min) to minimize the effect of the gradual dissociation of the EF G-ribosome complex (8, 14).

Unless otherwise indicated, the Phe-tRNA·EF Tu·GTP complex (see review in ref. 22) was formed by incubation, at 30° for 1 min, of mixtures containing 400 pmol/ml of $[^{14}C]$ -Phe-tRNA, 230 $\mu g/ml$ of EF T, 0.68 μM $[\gamma-^{32}P]GTP$, 1 mM DTT, 10 mM Tris·HCl (pH 7.8), and $Mg(acetate)_2$ and NH_4Cl , at the same concentrations as the reaction mixture

Abbreviations: EF G, elongation factor G; EF Tu, elongation factor Tu; EF Ts, elongation factor Ts; EF T, a mixture of EF Tu and EF Ts; FA, fusidic acid; GMPPCP, 5'-guanylylmethylene diphosphate.

TABLE 1. Effect of EF G plus fusidic acid on EF Tu-dependent binding of Ala-tRNA to preformed fMet-tRNA · 70S ribosome · R17-RNA complex

Additions	pmol bound/A unit	
	f-[³ H]Met-tRNA	[¹⁴ C]Ala-tRNA
None	1.5	0.1
EF G	1.4	<0.1
EF T	1.6	1.6
EF G + EF T	1.4	0.6

Initiation complex was formed by incubation at 34° for 10 min of 33 *A*₂₆₀ units/ml of 70S ribosomes with 1.2 mg/ml of R17 RNA, 0.4 mg/ml of crude initiation factors, 0.4 mM GTP, and 4.7 pmol of f-[³H]Met-tRNA per *A*₂₆₀ unit of ribosomes in buffer containing 5 mM Mg(acetate)₂, 60 mM Tris·HCl (pH 7.8), 50 mM NH₄Cl, and 7 mM 2-mercaptoethanol. The mixture was chilled, and portions were diluted with an equal volume of 3 mM fusidic acid containing 5 mM Mg(acetate)₂ and, where indicated, 0.47 mg/ml of EF G. After 4 min at 0°, 20-μl portions were mixed with 5 μl of 5 mM Mg(acetate)₂ containing 12 pmol of [¹⁴C]Ala-tRNA and, where indicated, 2 μg of EF T. Final ionic concentrations were: 5 mM Mg(acetate)₂, 24 mM Tris·HCl (pH 7.8), and 20 mM NH₄Cl. After incubation at 0° for 3 min, binding of aminoacyl-tRNA was assayed as described in *Methods*. Subtracted values from controls without R17 RNA were: 0.6 pmol fMet-tRNA and 0.2–0.4 pmol of Ala-tRNA.

containing ribosomes and EF G. Longer incubations did not further increase the subsequent binding of Phe-tRNA to ribosomes.

Concentrations of NH₄Cl and Tris·HCl were low in the reaction mixtures (about 20 and 10 mM, respectively), since at higher concentrations these salts impair the stability of the EF G · GDP · ribosome · fusidic acid complex (ref. 14, and our observations).

Ribosomes and elongation factors were stored in buffer

TABLE 2. Effect of EF G plus GMPPCP on EF Tu-dependent Phe-tRNA binding to 70S ribosomes and associated GTP hydrolysis

Additions	pmol/A unit	
	[¹⁴ C]Phe-tRNA bound	[³² P]GTP hydrolyzed
GMPPCP	1.4	0.1
GMPPCP + EF G	0.7	0.8
GMPPCP + EF T	5.2	8.1
GMPPCP + EF G + EF T	1.0	1.9

A mixture containing ribosomes complexed with poly(U) was made 30 μM in GMPPCP, and further incubated for 3 min at 30° with or without EF G. Both mixtures were further divided into 30-μl portions and supplemented with 15 μl of an incubated mixture of [¹⁴C]Phe-tRNA and [³²P]GTP, with or without EF T. The final composition was: 8 *A*₂₆₀ units/ml of 70S ribosomes, 10 mM Tris·HCl (pH 7.8), 50 mM NH₄Cl, 6 mM Mg(acetate)₂, 2 mM dithiothreitol, 15 μg/ml of poly(U), 20 μM GMPPCP, 0.22 μM [³²P]GTP (500 cpm/pmol), 18 pmol of [¹⁴C]Phe-tRNA per *A*₂₆₀ unit of 70S ribosomes, and, when present, 70 μg/ml of EF T and 200 μg/ml of EF G. After incubation at 0° for 2 min, the reaction mixtures were analyzed for Phe-tRNA bound and GTP hydrolyzed, as described in *Methods*.

containing 50% glycerol. Consequently, 3–4% glycerol was present in the reaction mixtures for the binding of aminoacyl-tRNA.

RNA from phage R17 was prepared as described (17). Poly(U) was from Schwartz and from Sigma. [*Methyl*-³H]-methionine (11.1 Ci/mmol), [³H]GDP (11.4 Ci/mmol), [³H]GTP (9.9 Ci/mmol), [¹⁴C]phenylalanine (0.513 Ci/mmol) and [¹⁴C]alanine (0.152 Ci/mmol) were from the Radiochemical Center, Amersham.

RESULTS

Inhibition by bound factor G of enzymic binding of Phe-tRNA

In the presence of fusidic acid and either GTP or GDP, EF G can form a stable complex with ribosomes (12, 14–16). Fig. 1*a* shows that under these conditions, prior incubation of ribosomes with increasing concentrations of EF G led to the formation of increasing amounts of EF G · [³H]GDP · ribosome · fusidic acid complex, and to a parallel inhibition of subsequent EF Tu-dependent, poly(U)-coded, binding of [¹⁴C]Phe-tRNA. Maximal inhibition rarely exceeded 60–70%

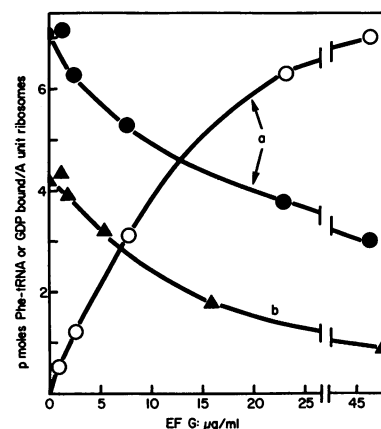


Fig. 1. (a) Binding of [¹⁴C]Phe-tRNA to ribosomes complexed to different degrees with EF G · [³H]GDP · fusidic acid. After binding of EF G in the presence of [³H]GDP, portions of the reaction mixtures were diluted with buffer and assayed by filtration for [³H]GDP bound to ribosomes (○—○). The remaining part of the reaction mixtures [containing poly(U)] was supplemented with an equal volume of [¹⁴C]Phe-tRNA (that had been incubated with EF T and GTP). Final concentrations were: 13 *A*₂₆₀ units/ml of 70S ribosomes, 40 μg/ml of poly(U), 25 mM NH₄Cl, 30 mM Tris·HCl (pH 7.8), 9 mM Mg(acetate)₂, 5 mM dithiothreitol, 1.8 mM fusidic acid, 2.5 μM GTP, 0.45 μM [³H]-GDP (1300 cpm/pmol), 30 μg/ml of EF T, 18 pmol of [¹⁴C]-Phe-tRNA per *A* unit of 70S ribosomes, and EF G as indicated. Bound Phe-tRNA (●—●) was determined after 2 min of incubation at 0°.

(b) Effect of EF G concentration, in the presence of GMPPCP, on EF Tu-dependent binding of Phe-tRNA to ribosomes. Binding of [¹⁴C]Phe-tRNA (▲—▲) after prior incubation with GMPPCP and various concentrations of EF G was conducted as described in *Methods*. Final concentrations were: 17 *A*₂₆₀ units/ml 70S ribosomes, 50 μg/ml of poly(U), 10 mM Tris·HCl (pH 7.8), 6 mM Mg(acetate)₂, 50 mM NH₄Cl, 0.2 mM GMPPCP, 2 mM dithiothreitol, 16 pmol of [¹⁴C]Phe-tRNA per *A*₂₆₀ unit of ribosomes (not incubated with EF T), 40 μg/ml of EF T, and EF G as indicated. 30 μg/ml of deacylated tRNA_{Phe} was present to reduce nonenzymic binding of Phe-tRNA, which, under these conditions, was less than 10% of the enzymic binding.

(Fig. 1), even with as much as 200 $\mu\text{g}/\text{ml}$ of EF G in the reaction mixture (see Fig. 2 below).

EF G can also bind strongly to ribosomes in the absence of fusidic acid if GTP is replaced by its nonhydrolyzable analog GMPPCP (2, 11–13). Under these conditions, prior incubation of the ribosomes with increasing amounts of EF G also led to decreased subsequent binding of Phe-tRNA (as an EF Tu·GMPPCP·Phe-tRNA complex). Maximal inhibition was about 80% (Fig. 1*b*; see also Table 2 below).

Inhibition of binding of Ala-tRNA to initiation complex

To rule out the possibility that the effects observed above might be peculiar to the poly(U) system, we studied the effect of EF G on the binding of Ala-tRNA to initiation complexes formed with phage R17 RNA as messenger (23). In this system, most Ala-tRNA binds to the A-site of ribosomes carrying fMet-tRNA (10). Table 1 shows that incubation of the initiation complex with fusidic acid, GTP, and EF G inhibited 60% the subsequent EF Tu-dependent binding of Ala-tRNA, though it did not destabilize the initiation complex, as measured by the retention of fMet-tRNA. Fusidic acid alone did not affect the binding of Ala-tRNA (controls not shown, and ref. 10).

Inhibition of factor Tu-dependent GTP hydrolysis

Fig. 2 shows the effect of EF G plus fusidic acid on the GTP hydrolysis associated with the enzymic binding of Phe-tRNA

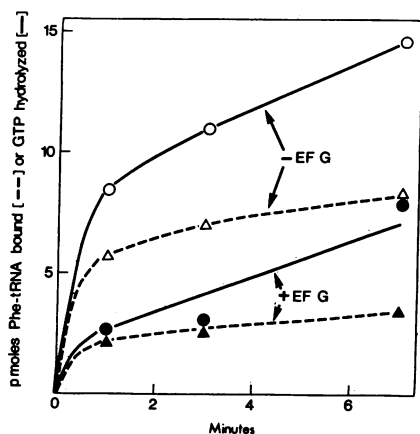


Fig. 2. Effect of EF G plus fusidic acid on (EF Tu)-dependent Phe-tRNA binding to 70S ribosomes, and associated GTP hydrolysis. Ribosomes complexed with poly(U) were incubated for 5 min at 0° with 300 $\mu\text{g}/\text{ml}$ of EF G, 3.7 mM fusidic acid, and 0.68 μM [γ - ^{32}P]GTP. This mixture, and a parallel mixture without EF G, were supplemented with [^{14}C]Phe-tRNA·EF Tu·[γ - ^{32}P]GTP complex (prepared as in *Methods*) and were further incubated at 0°; at intervals, samples were analyzed for Phe-tRNA bound and GTP hydrolyzed. The final composition was: 8 A_{260} units/ml of 70S ribosomes, 10 mM Tris·HCl (pH 7.8), 20 mM NH_4Cl , 10 mM $\text{Mg}(\text{acetate})_2$, 2 mM dithiothreitol, 15 $\mu\text{g}/\text{ml}$ of poly(U), 0.68 μM [γ - ^{32}P]GTP (460 cpm/pmol), 2.5 mM fusidic acid, 70 $\mu\text{g}/\text{ml}$ of EF T, 18 pmol of [^{14}C]Phe-tRNA per A_{260} unit of 70S ribosomes, and, when present, 200 $\mu\text{g}/\text{ml}$ of EF G. The background GTP hydrolysis without Phe-tRNA and EF Tu (15, 16) was determined in parallel reaction mixtures. This hydrolysis was completely dependent on EF G; most of it took place in the first 5 min after the addition of EF G, and increased from 12 to 14 pmol per A_{260} units of ribosomes during the subsequent 7 min. Results (in pmol per A_{260} unit of ribosomes) have been corrected for this hydrolysis.

(reviewed in ref. 22); the results are corrected for the GTP hydrolysis that took place during the formation of the EF G·GDP·ribosome·fusidic acid complex. It is seen that EF G inhibited EF Tu-dependent binding and GTP hydrolysis to a similar extent, especially at the short time intervals. Under the conditions of these experiments, we have repeatedly observed an excess of GTP hydrolysis over binding (Fig. 2). As reported by other authors (24), this effect might be due to the low concentration of NH_4^+ and Tris·HCl in the reaction mixtures.

A similar experiment, but with EF G bound with GMPPCP instead of fusidic acid, is shown in Table 2. To minimize the possibility of exchange between free and bound [γ - ^{32}P]GTP and GMPPCP, the concentration of GMPPCP was lowered 10 times compared with Fig. 1, and [γ - ^{32}P]GTP was only in a 50% molar excess over Phe-tRNA. It is seen that EF G strongly inhibited both the binding of Phe-tRNA and its associated GTP hydrolysis. In the absence of EF G (not shown), GMPPCP did not inhibit binding or GTPase activity.

Inhibition of nonenzymic binding of Phe-tRNA

In the absence of EF T (and GTP), efficient binding of Phe-tRNA to ribosomes complexed with poly(U) requires high Mg^{++} concentrations (21). Table 3 shows that at 20 mM Mg^{++} , prior incubation of the ribosomes with either EF G, GMPPCP (Exp. 1), fusidic acid, or fusidic acid plus EF G (Exp. 2), did not affect the binding of Phe-tRNA. However, prior incubation with EF G plus GMPPCP (Exp. 1), or with EF G plus fusidic acid and GTP (Exp. 2), completely abolished the poly(U)-stimulated binding.

TABLE 3. Effect of EF G on nonenzymic binding of Phe-tRNA to ribosomes

Exp.	Additions	pmol Phe-tRNA bound/ A unit		
		-Poly(U)	+Poly(U)	Stimulated by poly(U)
1	None	3.5	9.1	5.6
	GMPPCP	3.5	8.9	5.4
	EF G	3.7	8.9	6.2
	EF G + GMPPCP	2.0	1.6	-0.4
2	None	1.0	4.3	3.3
	FA	0.9	4.2	3.3
	FA + GTP	1.0	4.0	3.0
	FA + EF G	1.0	4.5	3.5
	FA + EF G + GTP	0.8	1.0	0.2

Preliminary binding of poly(U) and EF G to ribosomes was done as described in *Methods*. Phe-tRNA was bound in mixtures containing: 13 A_{260} units/ml of 70S ribosomes, 25 $\mu\text{g}/\text{ml}$ of poly(U) (when present), 10 mM Tris·HCl (pH 7.8), 20 mM $\text{Mg}(\text{acetate})_2$, 2 mM dithiothreitol, and 16 pmol of [^{14}C]Phe-tRNA per A_{260} unit of ribosomes. In addition, reaction mixtures of *Exp. 1* contained 50 mM NH_4Cl and, where indicated, 0.2 mM GMPPCP and 240 $\mu\text{g}/\text{ml}$ of EF G; those of *Exp. 2* contained 10 mM NH_4Cl and, when present, 2 mM fusidic acid, 0.93 μM [^3H]GTP, and 200 $\mu\text{g}/\text{ml}$ of EF G. Incubations at 25° for 2 min (*Exp. 1*) and at 0° for 3 min (*Exp. 2*) were immediately followed by filtration analysis. Retention of tritium label by the Millipore filters indicated that more than 70% of the ribosomes were complexed with EF G·GDP.

DISCUSSION

EF G, bound to ribosomes with GMPPCP or with fusidic acid plus GDP (11–16), strongly inhibits (60–80%) EF Tu-dependent binding of aminoacyl-tRNA to the ribosomal A-site, including both Phe-tRNA on the ribosomal complex with poly(U) and Ala-tRNA on the initiation complex formed with R17 RNA. Since the same effect was observed with an EF G·ribosome complex stabilized by two inhibitors that act very differently, the effect is evidently due to the EF G and not to the inhibitor complexed to the ribosome in the presence of EF G. Moreover, with either inhibitor the GTPase activity associated with the enzymic binding of Phe-tRNA is also inhibited, and to a similar extent; hence, EF G can apparently prevent all detectable interaction between the GTP·EF Tu·aminoacyl-tRNA complex and the ribosome. The residual interaction observed might be due either to ribosomes unable to firmly bind EF G, or to a displacement of EF G from ribosomes by the GTP·EF Tu·aminoacyl-tRNA complex.

Nonenzymic binding of Phe-tRNA at 20 mM Mg⁺⁺, which also takes place mainly in the ribosomal A-site (25), is even more sensitive to inhibition by bound EF G (Table 3). Thus, the binding of EF G on the 50S subunit (1, 2) evidently blocks *any* binding of aminoacyl-tRNA, a finding that suggests that the binding site of this factor (the G-site) and that of aminoacyl-tRNA (the A-site) overlap on the 50S ribosomal surface. Alternatively, EF G and aminoacyl-tRNA might bind at separate sites that influence each other's conformation. Similar conclusions have been independently reached by Richman and Bodley (this issue, 686–689), by Miller (this issue, 752–755), by Richter (manuscript submitted to *Nature*), and by Baliga and Munro (with rat-liver ribosomes, ref. 26).

Proximity or overlapping of G- and A-sites was first inferred from the inhibition by siomycin and thiostrepton of both aminoacyl-tRNA and EF G binding to ribosomes (9, 10). Moreover, our earlier suggestion that a common region on the 50S subunit may activate the GTPase of both EF G and EF Tu (10) is now made more plausible by the demonstrated inhibition of the EF Tu-dependent GTPase by EF G (Fig. 2, Table 2). Overlapping or interaction of G- and A-sites might also make obligatory, as first suggested by Moldave (27), the release of EF G (or at least a "pushing aside" of this factor) after translocation, before binding of the GTP·EF Tu·aminoacyl-tRNA complex can take place, and also make obligatory the release of EF Tu before binding of EF G.

Inhibition of aminoacyl-tRNA binding was equally observed when the ribosome·EF G complex was formed with either GMPPCP or fusidic acid plus GDP. Thus, GTP hydrolysis does not seem to induce a drastic change in the interaction between EF G and the A-site.

Fusidic acid, hitherto considered an inhibitor only of translocation (28, 29), clearly can also inhibit aminoacyl-tRNA binding by stabilizing EF G on the ribosome and blocking the A-site. Moreover, the latter action may be more relevant to its inhibitory action on protein synthesis, and may explain the unexpected finding that polysomes inhibited by fusidic acid *in vitro* carry their peptidyl-tRNA in the puromycin-reactive position (30, 31), and the finding that in protoplasts inhibited by fusidic acid, puromycin can release, albeit incompletely, nascent peptidyl chains from the ribosomes (32).

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