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Role of Guanine Nucleotides in Protein Synthesis. Elongation Factor G and Guanosine 5′-Triphosphate,3′-Diphosphate

(*E. coli*/pppGpp/translocation)

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ABSTRACT **The possible role of guanosine 5′-triphosphate,3′-diphosphate (pppGpp) in protein synthesis by *Escherichia coli* ribosomes and protein factors was examined. Although pppGpp could effectively substitute for GTP in reactions catalyzed by initiation factor 2 (ribosomal binding of fMet-tRNA and formation of *N*-formylmethionylpuromycin) and elongation factor T (ribosomal binding of Phe-tRNA and formation of dipeptidyl-tRNA), pppGpp poorly supported polyphenylalanine synthesis. The interaction of elongation factor G with pppGpp was, therefore, examined in detail. The nucleotide was found to be almost without activity in the translocation reaction, as measured by formation of *N*-acetylphenylalanyl-phenylalanylpuromycin. Nevertheless, the rate of the catalytic hydrolysis of pppGpp to guanosine 5′-diphosphate,3′-diphosphate by elongation factor G and ribosomes was about 30% of the rate of hydrolysis of GTP, a rate of hydrolysis that significantly exceeded the rate of translocation with GTP. Moreover, the rates of the fusidic acid-dependent, elongation factor G-dependent binding of pppGpp and ppGpp to ribosomes were about 75 to 85% the rates of GTP and GDP binding, respectively. We also found that dGTP could substitute for GTP in all reactions examined.**

When stringent strains of *Escherichia coli* are starved for an essential amino acid, they respond by restricting RNA accumulation (1) and producing guanosine 5′-diphosphate,3′-diphosphate (ppGpp) and guanosine 5′-triphosphate,3′-diphosphate (pppGpp) (2–4). These nucleotides have been implicated in control of RNA biosynthesis (3, 5), but their metabolic roles are obscure. A new dimension in the study of ppGpp and pppGpp has been added by the discovery that they can be synthesized in a reaction involving salt-washed ribosomes and a protein from stringent cells isolated from the ribosomal high-salt wash (6).

This *in vitro* synthetic system has made possible the preparation of large quantities of ppGpp and pppGpp (30). Moreover, the ribosome-dependent synthesis of these nucleotides has stimulated investigation of their possible role in protein biosynthesis. Several workers have found ppGpp to be a competitive inhibitor of GTP (7–10) in several partial reactions of protein synthesis involving protein synthetic factors that require GTP, namely, elongation factor G (EF-G), elongation factor T (EF-T), and initiation factor 2 (IF-2) (11).

We, therefore, examined the interaction of pppGpp with these three enzymes, although this report will concentrate on interactions with EF-G. We found that pppGpp completely substitutes for GTP in all functional reactions involving EF-T and IF-2 and partially substitutes for GTP in the guanine nucleotide binding and hydrolysis reactions dependent on EF-G. However, despite the apparent binding of pppGpp to ribosomes and its hydrolysis to ppGpp, pppGpp could not substitute for GTP in the translocation reaction catalyzed by EF-G.

EXPERIMENTAL PROCEDURE

Materials. E. coli B in quarter logarithmic phase were purchased from Grain Processing Corp.; [^{3}H]phenylalanine (50 Ci/mmol) and *E. coli* B tRNA from Schwartz/Mann; [α-^{32}P]GTP, [α-^{32}P]dGTP, and [^{14}C]methionine (212 Ci/mol) from New England Nuclear; [^{14}C]dGDP (472 Ci/mol) and [^{14}C]dGTP (543 Ci/mol) from Amersham/Searle; polyuridylic acid [poly(U)] and 5′-guanylylmethylenediphosphonate (pcppG) from Miles; puromycin dihydrochloride from Nutritional Biochemicals; polyethyleneimine–cellulose (PEI–cellulose) from Brinkman Instruments; and GTP, GDP, dGTP, dGDP, poly(U), and AUG codon from Sigma. GDP and dGDP were repurified by chromatography on DEAE–Sephadex A25. Fusidic acid was generously supplied by Dr. W. O. Godtfredsen of Leo Pharmaceutical Products.

Preparation of Ribosomes, Elongation and Initiation Factors, Charged tRNA, and Nucleotides. Preparation (12) of *E. coli* B EF-G, EF-T, and NH_4Cl-washed ribosomes was modified as follows: The ribosomes were stored at −10° after dialysis against 50% (v/v) glycerol containing 10 mM imidazole-HCl (pH 7.4), 10 mM $MgCl_2$, 40 mM KCl, and 1 mM 2-mercaptoethanol; EF-T was not purified beyond the DEAE–Sephadex A50 stage; and EF-G and EF-T were both stored at −20° after dialysis against 60% glycerol containing 10 mM $MgCl_2$, 10 mM imidazole-HCl (pH 7.4), and 1 mM dithiothreitol. A mixture of partially purified IF-1 and IF-2 was prepared as described (13) and stored at −20° after dialysis against 60% glycerol containing 0.5 M NH_4Cl (pH 7.4), 20 mM imidazole-HCl (pH 7.4), and 1 mM dithiothreitol. Phe-tRNA and fMet-tRNA were prepared by standard methods, including passage

Abbreviations: EF-G and EF-T, elongation factors G and T (a mixture of Tu and Ts), respectively; IF-1 and IF-2, initiation factors 1 and 2, respectively; PEI–cellulose, polyethyleneimine–cellulose; pcppG, 5′-guanylylmethylenediphosphonate; ppGpp, guanosine 5′-diphosphate,3′-diphosphate; pppGpp, guanosine 5′-triphosphate,3′-diphosphate. The assignment of pyrophosphate to the 3′ position in ppGpp and pppGpp is based on the enzymatic studies of Sy and Lipmann (28) and the carbon natural abundance nuclear magnetic resonance studies of Que *et al.* (29) with ppGpp.

through Sephadex G-25, from unfractionated *E. coli* B tRNA. Phe-tRNA was acetylated as described (14).

Preparation and purification of ppGpp, pppGpp, [5′-α-^{32}P]ppGpp, and [5′-α-^{32}P]pppGpp has been described (30). The source of [5′-α-^{32}P]guanine nucleotides of identical specific activity (150 cpm/pmol) for the EF-G-dependent binding of guanine nucleotides to ribosomes was a single synthetic reaction stopped when the added [α-^{32}P]GTP had been converted to 15% GDP, 25% GTP, 25% pppGpp, and 35% ppGpp. The nucleotides were purified as lithium salts, dissolved in 50 mM imidazole-HCl (pH 7.4), adjusted to 30 μM, and used in the binding studies.

Standard Assay Conditions. Initiation assays contained 5 mM $MgCl_2$, 100 mM NH_4Cl (pH 7.4), 50 mM imidazole-HCl (pH 7.4), and 12 mM 2-mercaptoethanol. Guanine nucleotide binding assays contained 10 mM $MgCl_2$, 10 mM NH_4Cl (pH 7.4), 50 mM imidazole-HCl (pH 7.4), and 12 mM 2-mercaptoethanol. All other assays contained 10 mM $MgCl_2$, 80 mM NH_4Cl (pH 7.4), 50 mM imidazole-HCl (pH 7.4), and 12 mM 2-mercaptoethanol.

Initiation Assays. Binding of f-[^{14}C]Met-tRNA to ribosomes was measured after the reaction was stopped with 3 ml of a cold solution (4°) containing 5 mM $MgCl_2$, 100 mM NH_4Cl (pH 7.4), and 10 mM imidazole-HCl (pH 7.4). The sample was passed through a Millipore filter, which was washed with 8 ml of the above solution, dried, and counted. Formation of *N*-formyl[^{14}C]methionylpuromycin was measured after the reaction was stopped with 1.25 ml of 100 mM sodium acetate (pH 5.5). The sample was extracted with 1.5 ml of ethyl acetate; 1.0 ml of the ethyl acetate phase was counted (15).

Elongation Assays. Assays for measuring polyphenylalanine synthesis, EF-T-dependent binding of Phe-tRNA to a ribosome-poly(U)-*N*-acetyl-Phe-tRNA complex, and EF-T-dependent formation of *N*-acetyl-Phe-Phe-tRNA were as described (12, 16), except that 20% glycerol was included in the latter two assays. A modification of earlier translocation assays was used (12, 16), involving the initial preparation of a ribosomal complex bearing poly(U) and *N*-acetyl-Phe-[^{3}H]Phe-tRNA in the A site (17, 18). Ribosomes (2 mg/ml), poly(U) (0.2 mg/ml), and unlabeled *N*-acetyl-Phe-tRNA (0.4 mg/ml) were incubated at 37° for 1 hr, then chilled in ice, and finally added to an equal volume of a mixture containing [^{3}H]Phe-tRNA (0.4 mg/ml), EF-T (155 μg/ml), and GTP (20 μM). After 1 hr at 0°, the reaction mixture was layered onto 2 volumes of 5% (w/v) sucrose containing Buffer A [20 mM $MgCl_2$–80 mM NH_4Cl (pH 7.4)–50 mM imidazole-HCl (pH 7.4)–12 mM 2-mercaptoethanol] and centrifuged at 40,000 rpm for 3.5 hr in a Beckman SW41 rotor. The ribosomal pellets were quickly rinsed with, then dissolved in, Buffer A (final concentration: 14–20 A_{260} units/ml) to yield the "pre-translocation complex." Since puromycin only reacts with peptidyl-tRNA bound in the ribosomal P site, the translocation reaction was measured by detecting formation of puromycin derivatives of [^{3}H]phenylalanine, presumably largely *N*-acetylphenylalanyl-[^{3}H]phenylalanylpuromycin, as most of the ribosome-bound [^{3}H]phenylalanine had been incorporated into *N*-acetyl-Phe-[^{3}H]Phe-tRNA (12, 16–18). The reaction was stopped with 0.5 ml of 100 mM sodium acetate (pH 5.5). The puromycin derivatives were extracted with 1.5 ml of ethyl acetate, and 1.0 ml of the ethyl acetate phase was counted (15).

EF-G-Dependent Reactions Uncoupled from Protein Synthesis. The fusidic acid-dependent, EF-G-dependent binding of guanine nucleotides to ribosomes was measured by Millipore filtration (12). Hydrolysis of [5′-α-^{32}P]guanine 5′-triphosphate nucleosides to 5′-diphosphate nucleosides was measured by thin-layer chromatography on PEI–cellulose (3). The data presented here are based on the amount of [α-^{32}P]GDP, [α-^{32}P]dGDP, and [5′-α-^{32}P]ppGpp generated.

TABLE 1. *Functions of IF-2 supported by pppGpp*

Nucleotide added	Exp. I: Binding of fMet-tRNA to ribosomes (pmol of Met bound)	Exp. II: Formation of *N*-formylmethionyl puromycin (pmol of Met in ethyl acetate phase)
None	4.6	9.2
pppGpp	30.8	60.9
GTP	28.0	45.9
dGTP	30.3	48.4
pcppG	11.9	9.2

In Exp. I each assay contained 1 mg/ml of ribosomes, 0.004 A_{260} units/ml of AUG codon, 0.4 mg/ml of f-[^{14}C]Met-tRNA, 34.6 μg/ml of IF-1/IF-2, and 0.2 mM guanine nucleotide, as indicated. Incubation was for 20 min at 20°. In Exp. II each assay contained 1 mM puromycin and all the components in Exp. I. Incubation was for 40 min at 20°. Data are expressed as pmol/ml of reaction.

TABLE 2. *Functions of EF-T supported by pppGpp*

Nucleotide added	Exp. I: Enzymatic binding of Phe-tRNA to ribosomes (pmol of Phe bound)	Exp. II: Formation of *N*-acetyl-Phe-Phe-tRNA (pmol of Phe in ethyl acetate phase)
None	1.0	1.4
pppGpp	16.6	12.8
GTP	18.8	12.1
dGTP	17.3	11.4
pcppG	13.8	1.4

A mixture of ribosomes (2 mg/ml), poly(U) (0.2 mg/ml), and unlabeled *N*-acetyl-Phe-tRNA (0.4 mg/ml) was incubated for 1 hr at 37° and then chilled in ice. Each assay contained, in a final volume of 0.050 ml, 0.025 ml of this mixture, as well as 0.4 mM guanine nucleotide, as indicated, 50 μg of [^{3}H]Phe-tRNA, 3.9 μg of EF-T, and 20% (v/v) glycerol. Incubation was for 20 min at 0°. In Exp. I the reaction was stopped by Millipore filtration. In Exp. II the reaction was stopped with 0.1 M KOH, with subsequent acidification with HCl and extraction with ethyl acetate (16). Data are expressed as pmol/ml of reaction.

RESULTS

Our initial investigation into the possible substrate role of pppGpp in reactions catalyzed by IF-2 (Table 1) and EF-T (Table 2) indicated that the nucleotide could substitute completely for GTP. The IF-2-dependent binding of fMet-tRNA to ribosomes and formation of *N*-formylmethionylpuromycin (a presumptive measure of a functional binding reaction) were both supported by pppGpp, as well as GTP and dGTP,

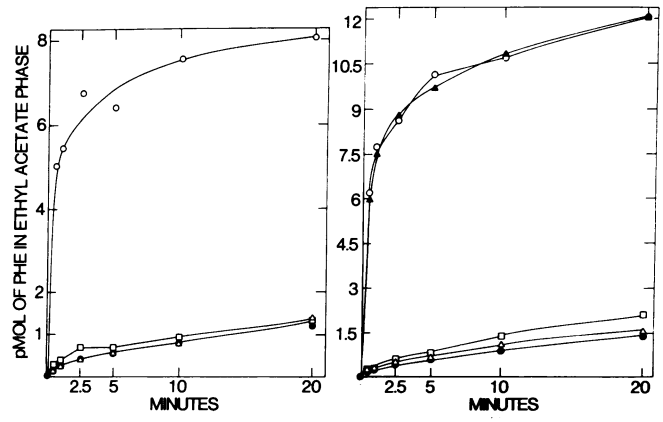

FIG. 1 (*Left*). Requirement for EF-G and GTP in translocation. The translocation assay contained 7.35 A_{260} units/ml of pretranslocation complex, 1 mM puromycin, and, as indicated, 0.2 mM GTP and 43.2 μg/ml of EF-G. Incubation was at 8° for the indicated times. Reaction volume was 0.1 ml. Data are expressed as pmol/ml of reaction. (○) EF-G + GTP, (□) EF-G only, (●, △) no nucleotide or GTP only.

FIG. 2 (*Right*). Failure of pppGpp in translocation. The translocation reaction contained 9.9 A_{260} units/ml of pretranslocation complex, 1 mM puromycin, 43.2 μg/ml of EF-G, and 0.2 mM guanine nucleotide, as indicated. Other reaction conditions were as in Fig. 1. (○) dGTP, (▲) GTP, (□) pppGpp, (△) pcppG, (●) no nucleotide.

while pcppG partially supported binding but not formation of the puromycin derivative. Similarly, EF-T-dependent binding of Phe-tRNA to ribosomes and formation of *N*-acetyl-Phe-Phe-tRNA (again, a presumptive measure of a functional binding reaction), were supported by pppGpp, as well as GTP and dGTP, while pcppG supported only the binding reaction.

Polyphenylalanine synthesis, however, was very poorly supported at high concentrations of pppGpp (Table 3) and not at all at lower concentrations. This low rate of synthesis is probably caused by either residual GTP or degradation products of pppGpp which contaminate our preparations of pppGpp.

Since pppGpp was an effective substrate in the reactions catalyzed by EF-T, this failure of polyphenylalanine synthesis suggested that the nucleotide would not support the translocation catalyzed by EF-G. The assay we used was the puromycin sensitivity of ribosome-bound *N*-acetyl-Phe-Phe-tRNA, which reacts with the antibiotic only when bound in the ribosomal P site (12, 16–18). This assay for translocation, as well as others directed by poly(U) (19, 20), possess a little understood translocational activity independent of EF-G and GTP that has been called nonenzymatic translocation. We found that this nonenzymatic activity can be minimized at low temperatures (less than 10°); Fig. 1 demonstrates the strong requirement for both EF-G and GTP at 8°.

The participation of various guanine nucleotides in translocation is presented in Fig. 2. GTP and dGTP, but not pppGpp and pcppG, effectively supported the reaction.

The failure of pppGpp in translocation suggested that it would also be unable to substitute for GTP in the EF-G-dependent catalytic hydrolysis of GTP (21) and binding of guanine nucleotides to ribosomes (22–24). We found, however, that pppGpp could participate in these uncoupled reactions, for it was both hydrolyzed to ppGpp and bound by ribosomes.

When the catalytic hydrolysis of guanine nucleotides was measured at 37° (Fig. 3), hydrolysis of pppGpp to ppGpp occurred at about 1/3 the rate of hydrolysis of GTP to GDP or dGTP to dGDP. Similar relative rates were also obtained with the pretranslocation complex and at 0°, 8°, and 25°, as well as 37°. Nevertheless, the rate of hydrolysis of pppGpp to ppGpp was substantially greater than the rate of translocation observed with GTP. In an experiment at 8°, initial rates of translocation and hydrolysis were directly compared. While there was little or no translocation with pppGpp, its hydrolysis occurred about 25-times faster than GTP-driven translocation. GTP hydrolysis was about 70-times faster. The requirement for both ribosomes and EF-G in the hydrolysis of pppGpp, as well as inhibition by fusidic acid, is demonstrated in Table 4.

The EF-G-dependent binding of GDP and GTP to ribosomes occurs optimally at low monovalent cation concentrations and requires the presence of fusidic acid, but only GDP is actually found bound to ribosomes (22–24). At relatively high nucleotide concentrations, the binding reaction occurs rapidly at 0° (22–24), but at lower concentrations the rate of GTP binding was much slower than that of GDP (ref. 16 and unpublished data). We, therefore, chose low nucleotide concentrations (2.4×10^{-7} M) for comparing the rates of binding of pppGpp and ppGpp to those of GTP and GDP. When pppGpp is compared to GTP, and ppGpp to GDP, the rates are about 75–85% of those seen with GTP and GDP (Fig. 4). Similar data were obtained when the pretranslocation complex was used instead of free ribosomes.

When the nucleotide concentrations were increased 10-fold, all the rates increased substantially, and there was little apparent difference in the rates of binding. An equally dramatic increase in reaction rates has been observed in the presence of methanol at low nucleotide concentrations (16). After 2 min in 10% methanol there was little difference in the quantity of nucleotides bound, compared to substantial differences without methanol (Table 5). The requirement for both ribosomes and EF-G in the methanol-stimulated binding of

TABLE 3. *Failure of pppGpp to support polyphenylalanine synthesis*

	pmol of Phe polymerized				
Nucleotide	0 mM nucleotide	2 mM nucleotide	0.2 mM nucleotide	0.02 mM nucleotide	0.002 mM nucleotide
None	0.1	—	—	—	—
GTP	—	14.9	13.1	8.7	1.5
dGTP	—	13.4	11.4	8.4	3.1
pppGpp	—	0.5	0.5	0.1	0
pcppG	—	0.2	—	—	—
GDP	—	0.1	—	—	—
dGDP	—	0.2	—	—	—
ppGpp	—	0.1	—	—	—

Each assay contained 40 μg/ml of ribosomes, 100 μg/ml of poly(U), 200 μg/ml of [^{3}H]Phe-tRNA, 77.6 μg/ml of EF-T, 43.2 μg/ml of EF-G, and guanine nucleotides as indicated. Reaction volume was 0.05 ml. Incubation was for 1 min at 37°. Data are expressed as pmol/ml of reaction.

guanine nucleotides demonstrated in Table 5 (16) was also observed without methanol. dGTP is bound much faster than GTP, although the two nucleotides appeared to be equivalent in all other experimental systems.

DISCUSSION

We have examined the ability of pppGpp to substitute for GTP in protein synthesis in two well-studied model systems, AUG-dependent initiation and poly(U)-dependent chain elongation (11). We found that pppGpp was at least as effective as GTP in catalyzing IF-2-dependent ribosomal binding of fMet-tRNA and formation of *N*-formylmethionylpuromycin, and in catalyzing EF-T-dependent ribosomal binding of Phe-tRNA and formation of *N*-acetyl-Phe-Phe-tRNA. However, pppGpp had little activity in the EF-G-dependent translocation reaction, as well as in polyphenylalanine synthesis. This result indicates that a significant difference must exist in the catalytic sites formed by the ribosome with EF-G, on the one hand, and with EF-T and IF-2, on the other. Whether this difference resides in the ribosomal component or the supernatant factor component of the catalytic sites is unknown.

The failure of pppGpp to support translocation was not reflected in EF-G-dependent reactions uncoupled from translocation. Catalytic hydrolysis of pppGpp to ppGpp occurred at about 30–40% of the rate of the comparable hydrolysis of GTP to GDP, with either free ribosomes or the pretranslocation complex, and this hydrolysis of pppGpp exceeded the amount of translocation catalyzed by EF-G and GTP.

In addition, pppGpp and ppGpp were bound to ribosomes in the presence of EF-G and fusidic acid at 75–85% of the rates of GTP and GDP, respectively. This was observed with both free ribosomes and the pretranslocation complex. The binding rates of pppGpp and ppGpp, like those of GTP and GDP, could be substantially accelerated in 10% methanol.

In the GTP binding assay the actual nucleotide bound is GDP (22–24). Similarly, when pppGpp was bound to ribosomes and the bound nucleotide eluted from the Millipore filter, the nucleotide recovered was ppGpp (unpublished data).

TABLE 4. *Requirement for ribosomes and EF-G for catalytic hydrolysis of pppGpp to ppGpp*

	Exp. I		Exp. II
	nmol of GTP hydrolyzed	nmol of dGTP hydrolyzed	nmol of pppGpp hydrolyzed
+ EF-G	20	10	7
+ Ribosomes	8	13	3
+ EF-G + ribosomes	491	472	582
No addition	7	10	0
EF-G + ribosomes + fusidic acid	32	—	98

Each assay contained 1 mg/ml of ribosomes, 86.4 μg/ml of EF-G, and 1 mM [5′-α-^{32}P]guanine nucleoside 5′-triphosphate and 0.1 mM fusidic acid, as indicated. Reaction volume was 0.025 ml. In Exp. I incubation was for 30 min at 37°. In Exp. II incubation was for 60 min at 37°. Generation of [5′-α-^{32}P]guanine nucleoside 5′-diphosphates was determined by PEI–cellulose chromatography (3). Data are expressed as nmol/ml of reaction.

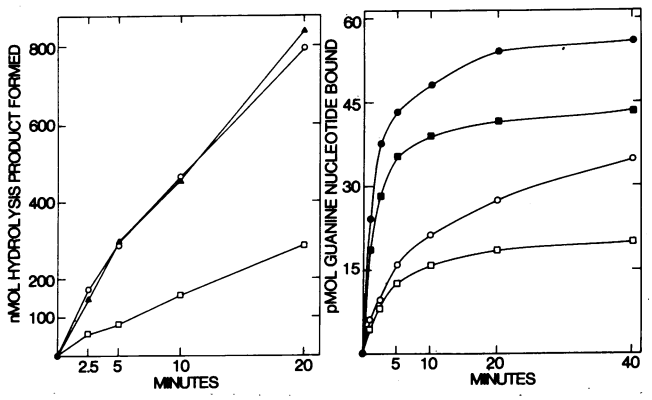

FIG. 3 (*Left*). EF-G-dependent catalytic hydrolysis of pppGpp. Each reaction contained 1 mg/ml of ribosomes, 86.4 μg/ml of EF-G, and 2 mM [5′-α-^{32}P]guanine nucleoside 5′-triphosphate, as indicated. Reaction volume was 0.05 ml, and incubation was at 37°. At the indicated times 0.005 ml of the reaction mixtures was spotted on PEI–cellulose and processed (3). Generation of [5′-α-^{32}P]guanine nucleoside 5′-diphosphates was followed. Data are expressed as nmol/ml of reaction. (▲) GTP, (○) dGTP, (□) pppGpp.

FIG. 4 (*Right*). EF-G-dependent binding of pppGpp and ppGpp to ribosomes. Each reaction contained 0.4 mg/ml of ribosomes, 28.7 μg/ml of EF-G, 1 mM fusidic acid, and 2.4 × 10^{-7} M [5′-α-^{32}P]guanine nucleotide, as indicated. Incubation was at 0° for the indicated times. Reaction volume was 0.25 ml. Data are expressed as pmol/ml of reaction. (●) GDP, (■) ppGpp, (○) GTP, (□) pppGpp.

These findings in the uncoupled EF-G-dependent reactions were based on studies with [5′-α-^{32}P]pppGpp and [5′-α-^{32}P]-ppGpp. They have been confirmed with less extensive studies with [5′-γ-^{32}P]pppGpp and [3′-β-^{32}P]pppGpp to exclude the possibility of EF-G-mediated hydrolysis of the 3′-β phosphate of pppGpp (unpublished data).

The ability of pppGpp to substitute rather extensively for GTP in the uncoupled reactions while failing to support translocation has various interpretations. Most interestingly, it can be argued that pppGpp fails in some translocation step that occurs after its hydrolysis. On the other hand, it is possible

TABLE 5. *Methanol-stimulated binding of guanine nucleotides to ribosomes*

	pmol of nucleotide bound			
Nucleotide added	+ Ribosomes + EF-G + methanol	+ Ribosomes + EF-G	+ Ribosomes + methanol	+ EF-G + methanol
GDP	64.3	33.2	0	0
ppGpp	56.8	24.5	0	0
dGDP	65.8	37.6	0.2	0.6
GTP	48.5	10.0	0	0
pppGpp	46.9	8.2	0	0
dGTP	51.6	22.3	0.1	0.4

Reaction components were as described in Fig. 4, except that assays also contained 10% (v/v) methanol, as indicated. [^{14}C]-dGDP and [^{14}C]dGTP were also used, as indicated. Reaction volume was 0.50 ml. Incubation was for 2 min at 0°. Data are expressed as pmol/ml of reaction.

that the uncoupled reactions are not directly related to the translocation reaction. This could explain recent paradoxical findings from other laboratories which demonstrate a reduced affinity of EF-G in the uncoupled reactions for ribosomes with peptidyl-tRNA in the A site relative to free ribosomes (25–27). It is possible, but we think unlikely, that the reduced hydrolysis of pppGpp relative to GTP is in itself sufficient to explain the failure of translocation. Finally, the uncoupled reactions that occur with the pretranslocation complex might, in fact, occur on free ribosomes in the preparations; that is, that ribosomes capable of translocation are unable to hydrolyze pppGpp.

The chemical basis for the failure of pppGpp in translocation is of interest. We find that dGTP can substitute for GTP with EF-G, as has been previously observed (19, 23). We further find that dGTP can replace GTP with EF-T and IF-2, indicating that the presence of the 2′-hydroxyl group in GTP is not essential in any of these reactions. We do note, however, a significant difference in the effect of the 3′-pyrophosphate group in the EF-G reaction as compared to the EF-T and IF-2 reactions. Clearly a free 3′-hydroxyl, as in GTP and dGTP, is not essential in the EF-T and IF-2 reactions, but may well be required in the translocation reaction. On the other hand, the inhibitory effect of the 3′-pyrophosphate of pppGpp in the translocation reaction may be entirely steric.

The experiments presented here demonstrate slight activity of pppGpp in both polyphenylalanine synthesis and the stoichiometric translocation assay. If the latter assay is accelerated by performing it at 37° with high pppGpp and EF-G concentrations, there is a significant, but still substantially reduced, translocation reaction. Both this reaction and the low polyphenylalanine synthetic activity observed with pppGpp have been successively reduced, but not entirely abolished, by repeated repurification of pppGpp. Furthermore, assay of column fractions demonstrated that the pppGpp peak did not coincide with the peak of residual synthetic activity. The activity of our purest preparation is consistent with residual contamination by GTP of about 0.1%. We cannot, however, eliminate the possibilities that pppGpp is slightly active in translocation or that the low activity is due to formation of an active degradation product of pppGpp.

It is unlikely that conversion of pppGpp to GTP can account for the apparent pppGpp activity in the EF-T- and IF-2-dependent reactions. First, if pppGpp were degraded to GTP by some component in the EF-T reaction, the GTP so generated should drive the EF-G-dependent translocation reaction. Nevertheless, pppGpp is almost inactive in polyphenylalanine synthetic reactions containing EF-T, although the GTP concentrations giving half-maximal rates in translocation, enzymatic binding, and dipeptide formation are all about 2–6 μM. Furthermore, studies over a broad concentration range indicate that the rate-limiting amounts of pppGpp, GTP, and dGTP are similar in both the IF-2- and EF-T-dependent reactions; nor do time-course measurements indicate a lag in pppGpp activity, as compared to GTP, in either the IF-2- or EF-T-dependent reactions (manuscript in preparation). Finally, preliminary pppGpp hydrolysis studies with IF-2 give no indication of significant conversion of labeled pppGpp (present at 10 μM) to either GTP or GDP.

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