INHIBITORY EFFECT OF EF G AND GMPPCP ON PEPTIDYL TRANSFERASE

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1. Introduction

Evidence has been accumulating which suggests that the binding of EF G and GTP takes place at or near the A site of the ribosome which accepts the complex of EF Tu, aminoacyl-tRNA and GTP. Thus, the complex of GMPPCP with EF G as well as fusidic acid and GDP with EF G has been found to inhibit the binding of aminoacyl-tRNA effectively at the A site [1–4]. These results suggested that the site for EF G and GMPPCP binding may be identical to or overlap with that for EF G, GDP, and fusidic acid. On the other hand, recent evidence suggests that these sites are similar but not identical to each other [5]. In this communication, we report that the puromycin reaction of *N*-acetyl[^{14}C]phenylalanyl-tRNA bound nonenzymatically at the D site is significantly inhibited by the presence of EF G and GMPPCP, while it is insensitive to the presence of EF G, fusidic acid and GTP, indicating that these two sites are distinctly different in terms of its effect on the peptidyl transferase activity.

2. Materials and methods

2.1. *Materials*

E. coli B (early log) and *E. coli* Q13 (middle log) were purchased from the Grain Processing Company and General Biochemicals, respectively. Ribosomes from *E. coli* Q13 and EF G from *E. coli* B were prepared as described previously [6]. 5′-Guanylylmethylene diphosphonate (GMPPCP) was obtained from Miles Laboratories. Fusidic acid (Na salt) (Batch # 14) was a gift from E. R. Squibb and Sons Co. through the courtesy of Dr. A. Laskin. The specific activities of [^{14}C]phenylalanine and [^{3}H]methionine were 455 Ci/mole and 3270 Ci/mole, respectively. Preparation of [^{14}C]phenylalanyl-tRNA [7], *N*-acetyl[^{14}C]phenylanyl-tRNA [8] and formyl [^{3}H]-methionyl-tRNA [9] were obtained as described previously. The counting efficiencies of ^{14}C and ^{3}H were 10^6 cpm/μc and 4.9×10^5 cpm/μc, respectively.

2.2. *Preparation of the various ribosomal complexes*

The mixture (0.5 ml) for the formation of the complex having *N*-acetyl[^{14}C]phenylalanyl-tRNA nonenzymatically bound at the D site contained 50 mM Tris–HCl (pH 7.2), 50 mM NH_4Cl, 6 mM Mg acetate, 2.64 mg of ribosomes, 100 μg of polyuridic acid (poly U), and 550 μg of tRNA containing 2.5×10^5 cpm of *N*-acetyl[^{14}C]phenylalanyl-tRNA The mixture was incubated for 20 min at 30°C. The complex was isolated on a Sephadex G-100 column [10].

The procedure for the formation of the complex having non-enzymatically bound [^{14}C]phenylalanyl-tRNA at the D site was similar to that for *N*-acetyl-[^{14}C]phenylalanyl-tRNA except that it (0.5 ml) contained 1 mM dithiothreitol (DTT), 140 μg of poly U, 2.46 mg of tRNA containing 7.5×10^5 cpm of [^{14}C]phenylalanyl-tRNA and 3.7 mg of ribosomes. The complex thus formed was isolated by the sucrose density gradient method [6].

The procedure for the preparation of the ribosomal

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complex having *N*-acetyl[^{14}C]phenylalanyl-tRNA at the D site which was translocated from the A site by the action of EF G and GTP was as follows. In the first step, the ribosomal complex having $tRNA^{phe}$ and *N*-acetyl[^{14}C]phenylalanyl-tRNA at the D and the A site respectively, was prepared as described previously [10]. The reaction mixture (1 ml) contained 50 mM Tris–HCl (pH 7.2), 50 mM NH_4Cl, 14 mM Mg acetate, 1 mM DTT, 805 μg of ribosomes, 2 mg of tRNA containg 9×10^5 cpm of *N*-acetyl-[^{14}C]phenylalanyl–tRNA and 200 μg of poly U. The mixture was incubated for 15 min at 30°C. The complex formed was then mixed with 0.5 mM puromycin and further incubated for 25 min at 30°C to convert *N*-acetyl[^{14}C]phenylalanyl–tRNA at the D site to $tRNA^{phe}$. The complex formed was isolated by the sucrose gradient method. In the second step, the A-site bound *N*-acetyl[^{14}C]phenylalanyl–tRNA was translocated to the D site. The mixture (0.6 ml) for the translocation reaction contained 50 mM Tris–HCl (pH 7.2), 50 mM NH_4Cl, 13 mM Mg acetate, 1 mM DTT, 0.1 mM GTP, 23.6 μg of EF G and 4.65 A_{260nm} units of the ribosomal complex containing 1.22×10^4 cpm of *N*-acetyl[^{14}C]phenylalanyl-tRNA obtained as described above. The mixture was incubated for 30 min at 37°C to complete the translocation reaction. This complex thus obtained was used for the puromycin reaction of the bound *N*-acetyl[^{14}C]-phenylalanyl-tRNA.

The mixture (0.5 ml) for the formation of the complex having non enzymatically bound *N*-acetyl[^{14}]-phenylalanyl-tRNA both at the A and the D site contained 50 mM Tris–HCl (pM 7.2), 50 mM NH_4Cl, 13 mM Mg acetate, 2.64 mg of ribosomes, 100 μg of poly U and 1.1 mg of tRNA containing 5×10^5 cpm of *N*-acetyl[^{14}C]phenylalanyl-tRNA. The incubation was carried out for 20 min at 30°C and the complex thus formed was isolated on a Sephadex G-100 column.

The mixture (0.51 ml) for the formation of the initiation complex with phage MS2 RNA contained 50 mM Tris–HCl (pH 7.2), 60 mM NH_4Cl, 10 mM Mg acetate, 1 mM DTT, 0.2 mM GTP, 625 μg of crude initiation factors (ribosomal wash) [11], 500 μg of MS2RNA [11], 4.6 mg of ribosomes and 2.4 mg of tRNA containing 1.8×10^5 cpm of formyl[^{3}H]-methionyl-tRNA. The incubation was carried out for 20 min at 37°C and the initiation complex formed was isolated by the sucrose density gradient.

3. Results

3.1. *Inhibition of peptide bond formation by EF G and GMPPCP*

It has been found that in the presence of 6 mM Mg^{2+} *N*-acetylphenylalanyl-tRNA as well as phenylalanyl-tRNA bind almost exclusively to the D site [12]. In the experiment shown in fig. 1, the complex of *N*-acetyl[^{14}C]phenylalanyl–tRNA with ribosome and poly U was prepared in this fashion, and the puromycin reaction with the bound *N*-acetyl[^{14}C]phenylalanyl-tRNA was studied in the presence of GMPPCP and various amounts of EF G. As indicated in this figure, the progressive inhibitory effect of GMPPCP and EF G was observed as the amount of EF G in the reaction mixture was increased. It should be noted in this figure that in a similar experiment fusidic acid, GTP and EF G did not exert any significant effect on the *N*-acetyl[^{14}C]phenylalanyl puromycin formation. The inhibitory effect of EF G is dependent on the presence

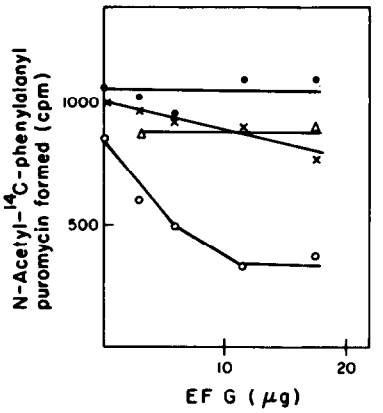

Fig. 1. Effect of various amounts of EF G on; the puromycin reaction with the D site nonenzymatically bound *N*-acetyl-[^{14}C]phenylalanyl-tRNA. The mixture (0.1 ml) for the puromycin reaction contained 50 mM Tris–HCl (pH 7.2), 50 mM NH_4Cl, 6 mM Mg acetate, 1 mM DTT, 0.6 mM puromycin and 1 A_{260nm} unit of the complex containing 2.8×10^3 cpm of *N*-acetyl[^{14}C]phenylalanyl-tRNA obtained as described in 'Materials and methods'. Where indicated, none (●), 1 mM GMPPCP (○), 1 mM fusidic acid and 0.1 mM GTP (×), 0.1 mM GTP (△) and the indicated amounts of EF G were added. The mixture was incubated for 10 min at 30°C. 0.8 ml of 10 mM Tris–HCl (pH 7.8) and 2 ml of ethylacetate were added to this mixture and after shaking vigorously, 1.5 ml of the ethylacetate fraction were counted in 5 ml Bray's solution [22].

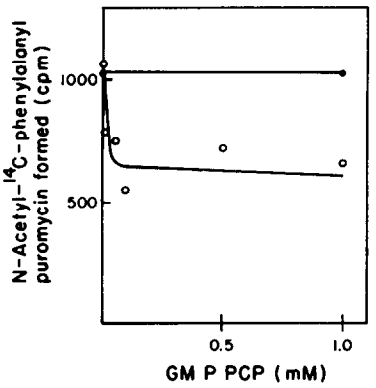

Fig. 2. Inhibitory effect of various amounts of GMPPCP on the puromycin reaction with the D site nonenzymatically bound *N*-acetyl[^{14}C]phenylalanyl-tRNA. The procedure for the puromycin reaction was essentially the same as that of fig. 1. Where indicated, 11.7 μg of EF G (○) and the various amounts of GMPPCP were added. (●) No EF G was added.

Table 1
Inhibitory effect of EF G and GMPPCP on the puromycin reaction with the nonenzymatically bound [^{14}C]phenylalanyl-tRNA at the D site

EF G	GMPPCP	[^{14}C]phenylalanyl puromycin formed (cpm)
–	–	300
–	+	291
+	–	289
+	+	147

The mixture (0.2 ml) for the puromycin reaction contained 50 mM Tris–HCl (pH 7.2), 80 mM NH_4Cl, 13 mM Mg acetate, 1 mM DTT, 0.5 mM puromycin and 2 $A_{260\ nm}$ units of the complex containing 1.8×10^3 cpm of [^{14}C]phenylalanyl–tRNA obtained as described in 'Materials and methods'. Where indicated, 23.6 μg of EF G and 0.2 mM GMPPCP were added. The incubation was carried out for 30 min at 30°C, 0.8 ml of 10 mM Tris–HCl (pH 7.8) and 2.5 ml of ethylacetate were added to the mixture, and after shaking vigorously, 2 ml of ethylacetate fraction were counted in 10 ml of Bray's solution.

of GMPPCP and GTP did not substitute for GMPPCP for this effect. The maximum inhibition on the puromycin reaction was observed when approximately two molecules of EF G were present per one ribosomal complex. When the concentration of GMPPCP was varied, the inhibition was apparent at 10^{-4} M (fig. 2). The K_i value for GMPPCP obtained from the competitive inhibitory effect on the release tRNA was 6×10^{-4} M [13]. Thus, the maximum effect of GMPPCP was obtained well below the K_i value of GMPPCP on translocation. It is noted in this figure also that the complete inhibition could not be obtained even with ten-fold higher concentration of GMPPCP. A similar inhibitory effect of EF G and GMPPCP on peptide bond formation was observed with the D site bound phenylalanyl-tRNA (table 1). One can therefore conclude that the blocking of free amino group is not necessary for the inhibitory effect of GMPPCP and EF G on the puromycin reaction.

It has been found previously that the presence of tRNA or *N*-acetyl phenylalanyl-tRNA at the A site may retard the rate of the puromycin reaction with the D site bound *N*-acetyl[^{14}C]phenylalanyl–tRNA [14]. The hindrance due to the presence of *N*-acetyl-[^{14}C]phenylalanyl-tRNA at the A site was not observed, however, on the final level of the puromycin derivative formed [14]. Thus it is possible to react the D site bound *N*-acetyl[^{14}C]phenylalanyl-tRNA with puromycin even in the presence of the other *N*-acetyl[^{14}C]phenylalanyl-tRNA on the A site. It has also been reported that the presence of aminoacyl-tRNA at the A site may [15] or may not [4] inhibit the binding of EF G to the ribosomes. It was therefore of interest to examine the effect of EF G and GMPPCP on the puromycin reaction with the complex which has *N*-acetyl[^{14}C]phenylalanyl-tRNA both at the A and the D sites. Such complexes can be prepared in the presence of 13 mM Mg^{2+} because *N*-acetyl[^{14}C]-phenylalanyl-tRNA binds to both the A and D sites of the ribosomes in the absence of any added factors [12] In the experiment shown in table 2, it is clear that EF G and GMPPCP exert an inhibitory effect on the D site bound *N*-acetyl[^{14}C]phenylalanyl-tRNA even if the A site was occupied, suggesting that the presence of nonenzymatically bound *N*-acetyl[^{14}C]-phenylalanyl-tRNA at the A site may not interfere with the binding of EF G and GMPPCP to the ribosome. It is noted in this table that the puromycin reaction was more than doubled by the addition of EF G and GTP, indicating that the A site is occupied under the experimental conditions used.

Table 2
Inhibitory effect of EF G and GMPPCP on the puromycin reaction with the complex having *N*-acetyl[^{14}C]-phenylalanyl-tRNA both at the A and the D site

EF G (μg)	GMPPCP	GTP	*N*-acetyl[^{14}C]phenylalanyl puromycin formed (cpm)
–	+	–	992
3.8	–	–	1093
3.8	+	–	737
19.0	–	–	1193
19.0	+	–	693
19.0	–	+	2416

The procedure for the puromycin was essentially the same as that of fig. 1 except that it contained 13 mM Mg acetate and 1.24 $A_{260\ nm}$ units of the complex containing 5.6 × 10^3 cpm of *N*-acetyl[^{14}C]phenylalanyl-tRNA obtained as described in 'Materials and methods'. Where indicated, 0.2 mM GMPPCP, 0.1 mM GTP, and the various amounts of EF G were added. The incubation was carried out for 15 min at 30°C.

Table 3
Reversal of the inhibitory effect of EF G and GMPPCP on the peptide bond formation by GDP

GMPPCP (mM)	GDP (mM)	*N*-acetyl[^{14}C]phenyl-alanyl puromycin formed (cpm)	% inhibition
–	–	1130	
0.2	–	597	47
–	0.2	1041	
0.2	0.2	790	24
–	0.5	1172	
0.2	0.5	964	18

The procedure for the puromycin reaction was essentially the same as that of fig. 1 except that it contained 14 μg of EF G and 1.17 $A_{260\ nm}$ units of the complex containing 2.9 × 10^3 cpm of *N*-acetyl[^{14}C]phenylalanyl-tRNA obtained as described in 'Materials and methods'. Where indicated, 0.2 mM GMPPCP and the various amounts of GDP were added.

3.2. *The reversal of the effect of GMPPCP by GDP*

It has been reported that the binding of EF G to ribosomes in the presence of GMPPCP is competitively inhibited by the increasing concentration of GDP [16]. It was therefore expected that the addition of GDP may release the inhibitory effect of GMPPCP on the puromycin reaction. The data in table 3 show that this is indeed the case. GMPPCP (0.2 mM) inhibited the puromycin reaction approximately 50% and this inhibitory effect was diminished by 50% in the presence of the equal concentration of GDP. Higher reduction of GMPPCP inhibitory effect was observed with a higher concentration of GDP, suggesting the competitive nature of GDP and GMPPCP.

3.3. *The effect of EF G and GMPPCP on N-blocked aminoacyl-tRNA which was placed on the D site by the initiation factors or EF G*

During polypeptide synthesis, under the physiological condition, the ribosome will go through a stage where it has peptidyl-tRNA on the D site and aminoacyl-tRNA at the A site. If EF G and GTP bind to the ribosome at this stage in a similar fashion to the binding of EF G and GMPPCP, one might expect this binding may interfere with the physiological process of the peptide bond formation. This would then result in retardation of peptide bond formation, because it will not take place until EF G is released from the ribosome. To examine this possibility, *N*-blocked aminoacyl-tRNA was placed on the D site by the physiological means and the puromycin reaction with this *N*-blocked aminoacyl-tRNA was carried out and the effect of EF G and GMPPCP was tested on this reaction. In this experiment, *N*-acetyl-[^{14}C]phenylalanyl-tRNA, poly U, and ribosomal complex was prepared at 13 mM Mg^{2+} and puromycin was allowed to react with the complex, converting *N*-acetyl[^{14}C]phenylalanyl-tRNA bound at the D site to $tRNA^{phe}$. To this complex were added EF G and GTP to translocate *N*-acetyl[^{14}C]phenylalanyl-tRNA at the A site to the D site and to release the D site bound $tRNA^{phe}$. Thus the complex obtained here represents one which contains *N*-acetyl[^{14}C]-phenylalanyl–tRNA at the D site by the action of EF G and GTP. The puromycin reaction with the bound *N*-acetyl[^{14}C]phenylalanyl-tRNA on this complex was examined. As shown in table 4, no appreciable effect of EF G and GMPPCP was observed on the puromycin reaction of *N*-acetyl[^{14}C]phenylalanyl-tRNA placed on the D site by the action of EF G. That this was not due to the possible presence of residual GDP in the system was shown by the observation that even ten fold higher concentration of GMPPCP did not cause appreciable decrease of peptide bond formation reaction.

Table 4
Effect of EF G and GMPPCP on the puromycin reaction with the D site bound *N*-acetyl[^{14}C]phenylalanyl-tRNA which was translocated from the A site by the action of EF G

EF G	GMPPCP	*N*-acetyl[^{14}C]phenylalanyl puromycin formed (cpm)
+	–	1878
–	+	1784
+	+	1668

The mixture (0.2 ml) for the puromycin reaction contained 170 μl of the mixture, which contained the complex having *N*-acetyl[^{14}C]phenylalanyl-tRNA at the D site, obtained as described in 'Materials and methods'. Where indicated, 11.8 μg of EF G and 1 mM GMPPCP were added. The incubation was carried out for 10 min at 30°C. The rest of the procedure was essentially the same as that of table 1.

Table 5
Effect of EF G and GMPPCP on the puromycin reaction with the formyl[^{3}H]methionyl–tRNA of the initiation complex

EF G	GMPPCP	Formyl[^{3}H]methionyl puromycin formed (cpm)
–	–	507
–	+	513
+	–	532
+	+	437

The mixture (0.2 ml) for the puromycin reaction contained 50 mM Tris–HCl (pH 7.2), 80 mM NH_4Cl, 10 mM Mg acetate, 1 mM DTT, 1.7 $A_{260\ nm}$ unit of the initiation complex containing 8.6 × 10^2 cpm of formyl[^{3}H]methionyl-tRNA obtained as described in 'Materials and methods', and 0.5 mM puromycin. Where indicated, 23.6 μg of EF G and 0.2 mM GMPPCP were added. After incubating for 3 min at 30°C in the absence of puromycin, the complete reaction mixture was further incubated for 10 min at 30°C. The rest of the procedure was essentially the same as that of table 1 except that 10 ml of Triton X-100-toluene scintillators were used.

Another naturally occurring substrate for the puromycin reaction is the initiation complex of ribosomes. It has been established that formylmethionyl-tRNA [17] (like *N*-acetyl phenylalanyl-tRNA [18]) would bind to 30 S ribosomal subunit at the site corresponding to the D site directly. Thus, formyl[^{3}H]methionyl-tRNA bound in the presence of initiation factors and GTP is placed at the D site in a physiological way. As shown in table 5, the reaction of this complex with puromycin was insensitive to the presence of EF G and GMPPCP. One can therefore conclude from these results that the presence of EF G and GTP or GDP on a ribosome which may occur temporarily during polypeptide synthesis may not interfere with naturally occurring polypeptide bond formation.

4. Discussion

It has been shown that the puromycin reaction with the ribosomal bound formylmethionyl-tRNA is inhibited by the presence of IF 2 and GMPPCP on ribosomes [19]. From this observation it has been suggested that the terminal phosphate energy of GTP is used to release IF 2 from a ribosome. Since it was known that EF G and GMPPCP will bind to a ribosome, it was of interest to examine if the binding of GMPPCP and EF G to ribosome would exert a similar effect as the binding of IF 2 and GMPPCP. The data presented in this communication indicate that the presence of EF G and GMPPCP on a ribosome may not interfere with the puromycin reaction of the D site bound *N*-acetyl[^{14}C]phenylalanyl-tRNA provided that the complex was prepared non-enzymatically. The inhibition was never complete and it varied from 30% to 60%. The reason for this incomplete inhibition is not known at the present moment. It is possible that a part of *N*-acetyl[^{14}C]phenylalanyl-tRNA bound to the D site in the presence of Mg^{2+} alone may be bound in an irregular fashion so that it is susceptible to the action of EF G and GMPPCP. It should be pointed out that under an identical condition, the presence of EF G, fusidic acid and GDP on a ribosome did not interfere with the puromycin reaction. These observations suggest that the binding site of EF G and GMPPCP and that of EF G, fusidic acid and GDP on a ribosome may not be identical. It is of interest to note the recent report that two molecules of EF G bind to a ribosome in the presence of GMPPCP, while only one molecule of EF G binds to a ribosome with fusidic acid [20]. The additional molecule of EF G on a ribosome may exert steric hindrance to the puromycin reaction with the aminoacyl-tRNA which is loosely bound to the D site.

The fact that the observed inhibitory effect of EF G and GMPPCP on peptide bond formation does

not operate in physiological protein synthesis was suggested by the finding that naturally placed *N*-blocked aminoacyl-tRNA reacted with puromycin even in the presence of relatively high concentration of EF g and GMPPCP. Although it has been established that non-enzymatically bound aminoacyl-tRNA is physiologically active in that it can form a peptide bond [21], the present observation suggest that at least a part of non-enzymatically bound aminoacyl-tRNA may not be identical to the naturally placed aminoacyl-tRNA on the D site.

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