INTERACTION OF GUANOSINE 5'-DIPHOSPHATE, 2'-(OR 3'-) DIPHOSPHATE(ppGpp) WITH ELONGATION FACTORS FROM E. COLI

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Summary: The interactions of guanosine 5'-diphosphate, 2'-(or 3'-) diphosphate(ppGpp) with the polypeptide elongation factors Tu(EF Tu) and G(EF G) have been studied. The data indicate that ppGpp binds with EF Tu to form an EF Tu-ppGpp complex, and inhibits, in a competitive manner, the exchange reaction of Tu-GDP and ^{3}H-GDP. The ribosome-dependent GTPase reaction catalyzed by EF G is also depressed by ppGpp.

In the course of the studies on the stringent control in Escherichia coli, Cashel and Gallant(1) observed the accumulation of two unusual phosphorylated compounds, MS I and MS II, in extracts of rel^{+} cells[1)] cultivated in a medium deprived of required amino acids. Later, the structure of MS I was assigned as guanosine 5'-diphosphate, 2'(or 3'-) diphosphate(ppGpp) by Cashel and Kalbacher(2).

In a previous report from our laboratory on the function of rel gene in E. coli, Sokawa, Sokawa, and Kaziro(3) have suggested that rel^{-} strains of E. coli may possess an abnormal protein synthesizing machinery which can not carry out protein synthesis under the limited supply of an amino acid(or an aminoacyl-tRNA). Several recent studies(1, 4, 5) suggest that ppGpp is formed as the results of the idling of translational process but rapidly di-

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1) The rel^{+} and rel^{-} are used to represent the genotypes of the strains having stringent and relaxed controls, respectively.

minishes when the protein synthesis is fully blocked by addition of chloramphenicol. These findings prompted us to investigate the interaction of ppGpp with soluble factors in the protein synthesizing system.

This paper describes the studies on the interaction of ppGpp with the polypeptide elongation factors Tu(EF Tu) and G(EF G). The evidence to be presented here indicate that ppGpp binds with EF Tu to form a complex EF Tu-ppGpp. It inhibits exchange of Tu-GDP and ^{3}H-GDP in the presence and in the absence of EF Ts. It also depresses the EF G catalyzed GTPase reaction although less strongly than GDP.

Materials and Methods: ppGpp was a kind gift from Dr. M. Cashel, the National Institute of Health. EF Tu, EF Ts, and EF G were prepared as described elsewhere(6, 7). EF Tu purified from CP 79 (rel$^-$) cells was supplied by Miss M. Takasugi. The details of the incubations and assay conditions are given in the appropriate legends.

Results: Inhibition of the exchange reaction. EF Tu, when isolated in the presence of Mg^{2+} ions and GDP, contains one mole of tightly bound GDP per mole of protein(8). This bound GDP exchanges with ^{3}H-GDP at 0°C in the presence of a catalytic amount of EF Ts, or at 37°C in the absence of EF Ts.

$$\text{Tu-GDP} + \text{Ts} \rightleftharpoons \text{Tu-Ts} + \text{GDP} \text{---------(1)}$$

$$\text{Tu-Ts} + {}^3\text{H-GDP} \rightleftharpoons \text{Tu-}{}^3\text{H-GDP} + \text{Ts} \text{------(2)}$$

$$\text{Sum: Tu-GDP} + {}^3\text{H-GDP} \rightleftharpoons \text{Tu-}{}^3\text{H-GDP} + \text{GDP} \text{-----(3)}$$

In the experiments shown in Table I, it was demonstrated that the exchange reaction in the presence of Ts was inhibited by 0.3

Table I. Effect of ppGpp on the exchange reaction

System	Incubation	Formation of Tu-^{3}H-GDP
		cpm
1. Complete	0°C, 1 min	31,000
+ ppGpp	0°C, 1 min	9,800
2. Complete - Ts	37°C, 1 min	19,310
- Ts + ppGpp	37°C, 1 min	3,216

The reaction mixture contained in 0.08 ml; 50 mM Tris-HCl buffer, pH 7.5, 150 mM NH_4Cl, 10 mM magnesium acetate, 10 mM 2-mercaptoethanol, 7.8 μM ^{3}H-GDP(specific activity 308), 0.3 mM ppGpp when indicated, 200 pmoles of Tu-GDP, and 500 units of Ts. One unit of Ts is the amount of Ts which catalyzes the exchange of one pmole of Tu-GDP with ^{3}H-GDP in 1 min at 0°C. The time and temperature of incubation are as specified. The reaction was terminated by the addition of 3 ml of a cold buffer containing 0.01 M Tris-HCl buffer, pH 7.5 and 0.01 M magnesium acetate. The diluted reaction mixture was filtered through a nitrocellulose filter(Sartorius, MF50 25 mm, 0.45 μ) and the filter was washed three times with 5 ml of the above buffer solution. The filter was dried under infrared lamp and the radioactivity was determined in a toluene based scintillation fluid. The counting efficiency for ^{3}H-GDP was about 25 %.

mM ppGpp.[2] The exchange reaction at 37°C in the absence of Ts was also found to be inhibited by ppGpp(experiment 2 of Table I).

Displacement of ^{3}H-GDP from Tu-^{3}H-GDP: When Tu-^{3}H-GDP was incubated at 0°C with both ppGpp and Ts, the radioactivity bound to Tu was rapidly displaced. Either Ts or ppGpp alone did not cause the decrease in the radioactivity from Tu-^{3}H-GDP(Table II). At 37°C, where the exchange reaction does not require the presence of Ts, the displacement of ^{3}H-GDP from Tu-^{3}H-GDP was observed without the addition of Ts(line 5 of Table II).

From the above experiments, it appears that ppGpp binds with Tu

2) The thin layer chromatography of ppGpp on polyethyleneimine cellulose plate revealed the existence of a faster running spot of impurity which was located between guanosine 5'-diphosphate and guanosine 5'-triphosphate. No correction was made on the concentration of ppGpp for this contaminating nucleotide.

Table II. Displacement of ^{3}H-GDP by ppGpp

System	Incubation	Tu-^{3}H-GDP remaining
		cpm
Complete	0°C, 0 min	13,150
" + Ts	0°C, 5 min	11,942
" + ppGpp	0°C, 5 min	11,576
" + Ts + ppGpp	0°C, 5 min	1,854
" + ppGpp	37°C, 5 min	2,550

Tu-^{3}H-GDP was prepared in the incubation containing the following components in a final volume of 0.54 ml: 0.05 M Tris-HCl buffer, pH 7.5, 10 mM magnesium acetate, 10 mM 2-mercapto-ethanol, 4000 units of Tu-GDP, and 4 mμmoles of ^{3}H-GDP(specific activity 680). The mixture was incubated for 5 min at 37°C to ensure the complete equilibration of ^{3}H-GDP and Tu-GDP, and cooled to 0°C. Ten-μl aliquots of this mixture containing 74 units of Tu-^{3}H-GDP and 74 pmoles ^{3}H-GDP both having a specific activity of 340, were used for the subsequent incubation. The reaction mixture for the displacement experiment contained in a final volume of 0.08 ml: 0.05 M Tris-HCl buffer, pH 7.5, 10 mM magnesium acetate, 0.15 M NH_4Cl, 10 mM 2-mercaptoethanol, 10 μl of the above solution containing Tu-^{3}H-GDP and ^{3}H-GDP, and 150 units of Ts and/or 24 mμmoles of ppGpp as specified. The incubation was carried out as given in the table and the amount of Tu-^{3}H-GDP remaining after the incubation was assayed as described in Table I.

to form Tu-ppGpp complex with the concomitant displacement of GDP.

$$\text{Tu-GDP} + \text{Ts} \rightleftharpoons \text{Tu-Ts} + \text{GDP} \text{--------(4)}$$

$$\text{Tu-Ts} + \text{ppGpp} \rightleftharpoons \text{Tu-ppGpp} + \text{Ts} \text{------(5)}$$

$$\text{Sum: Tu-GDP} + \text{ppGpp} \rightleftharpoons \text{Tu-ppGpp} + \text{GDP} \text{-----(6)}$$

Fig. 1 shows the displacement of ^{3}H-GDP from Tu-^{3}H-GDP in the presence of Ts as the function of the concentration of ppGpp. From the data in Fig. 1, the equilibrium constant of Reaction 6 was calculated approximately as 0.1. A similar curve of displacement was obtained when Tu purified from CP 79(*rel*$^-$) was used instead of Tu from Q 13(*rel*$^+$) cells(Fig. 1).

Stabilization of free Tu: In order to confirm further the formation of Tu-ppGpp complex, the heat-stability of Tu was measured in the presence and absence of ppGpp. As expected, ppGpp dis-

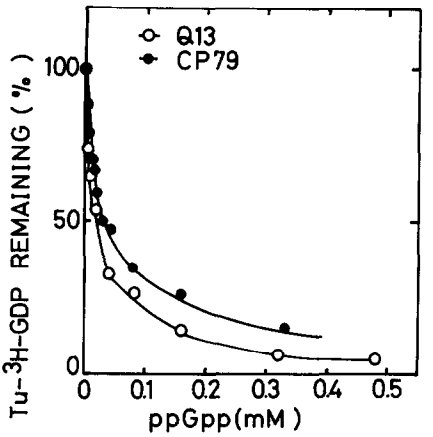

Fig. 1.

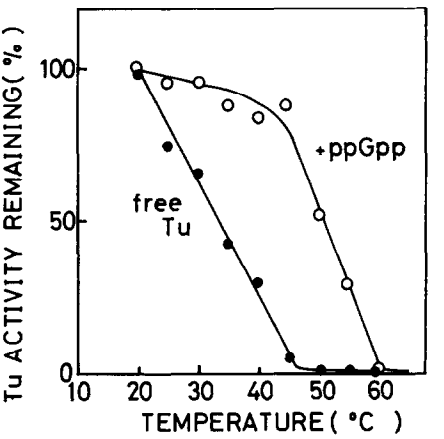

Fig. 2.

Fig. 1. The displacement of ^{3}H-GDP from Tu-^{3}H-GDP by ppGpp. Tu-^{3}H-GDP was prepared as described in the legend to Table II. The composition of the reaction mixture for displacement experiments were also as in Table II except for the concentration of ppGpp as indicated. The incubation was carried out for 5 min at 30°C in the absence of Ts, after which time the amount of Tu-^{3}H-GDP remaining was assayed as described in Table I. -o-, with Tu from E. coli Q13; and -●-, with Tu from E. coli CP 79 (rel$^-$).

Fig. 2. Heat stability of Tu in the presence and absence of ppGpp. Free Tu was prepared from Tu-^{3}H-GDP by extensive dialysis against 0.02 M Tris-HCl buffer, pH 7.5, 10 mM 2-mercaptoethanol, and 5 mM EDTA. It was then incubated in a solution containing 0.05 M Tris-HCl buffer, pH 7.5, 0.15 M NH_4Cl, 10 mM magnesium acetate, 10 mM 2-mercaptoethanol, and with or without 40 µM ppGpp for 5 min at the temperature specified. After heat treatment, ^{3}H-GDP(specific activity, 308) was added to 13 µM to each incubation mixture and the Tu activity remaining was assayed as described in Table I. -o-, in the presence of ppGpp; -●-, in the absence of ppGpp.

played a marked stabilizing effect on free Tu which is otherwise highly unstable; being denatured completely by incubation at 45°C for 5 min(Fig. 2). In the presence of 40 µM ppGpp, Tu was found to be much more stable, and retained its full activity after heating for 5 min at 45°C. This indicates that ppGpp binds with Tu and stabilizes it.

Inhibition of ribosome-dependent GTPase: The effect of ppGpp on

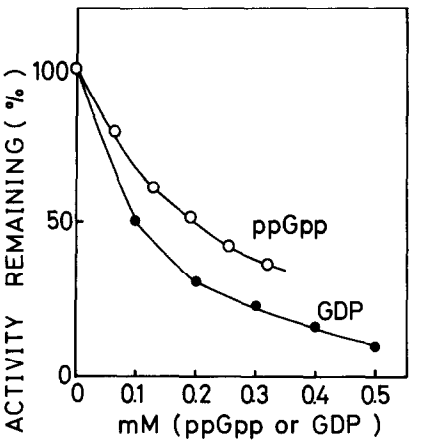

Fig. 3. Inhibition of EF G-catalyzed GTPase reaction by ppGpp. The GTPase reaction was assayed as described elsewhere(6) with 10 OD_{260} units of ribosomes, 2.5 milliunits of EF G, 0.1 mM GTP, and GDP or ppGpp as indicated. The inorganic phosphate liberated from GTP in the absence of inhibitor was 2.8 mμmoles. -o-, with ppGpp; and -●-, with GDP.

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the ribosome-dependent GTPase reaction catalyzed by EF G was studied. As illustrated in Fig. 3, the reaction was inhibited by ppGpp although the extent of inhibition was less than that by GDP. When 0.1 mM GTP was used as substrate, about 50 % inhibition of the GTPase reaction was attained with 0.2 mM and 0.1 mM of ppGpp and GDP, respectively.

Discussion: The foregoing evidence suggests that ppGpp can interact with Tu to form Tu-ppGpp complex. Probably ppGpp interacts with Tu at the site where normally GDP attaches. The fact that the free Tu prepared from Tu-GDP by prolonged dialysis against a buffer containing EDTA was stabilized by ppGpp indicates the formation of the Tu-ppGpp complex. Since the equilibrium constant of reaction 6 is approximately 0.1, the affinity of ppGpp to Tu is lower than that of GDP(4.9×10^{-9} M) by one order of magnitude.

We do not know at present whether these inhibitory effects of ppGpp are simply due to its structural similarity with GDP, or the

inhibition of polypeptide elongation factors by ppGpp has a specific physiological significance. Since the accumulation of ppGpp was observed during the slow-down of translational process, we could interpret its correlation with EF Tu and EF G in several ways. However we would like to refrain from any conclusions as to the physiological significance of this interactions until the formation and mechanism of action of ppGpp are clearly understood.

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