THE EFFECT OF ppGpp ON *IN VITRO* PROTEIN SYNTHESIS BY A WHEAT EMBRYO SYSTEM

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1. Introduction

Cashel and co-workers [1–3] have isolated from *E. coli* extracts a compound, originally designated as magic spot 1, which was identified as guanosine 5′ diphosphate, 3′ (or 2′) diphosphate (ppGpp).This compound is specially interesting since it seems to be involved in the inhibition of mRNA and tRNA synthesis in stringent bacteria starved for a required amino acid. More recently [4] ppGpp has been found to bind elongation factor Tu(EF Tu), a key factor in bacterial protein synthesis and a host subunit of Qβ replicase.

It seemed interesting, therefore, to determine whether ppGpp also binds to eukaryotic elongation factor 1(EF 1), the functional equivalent of EF Tu, and to establish whether this GTP analog has any effect on an *in vitro* protein synthesis from higher cells.

In this communication, we report experiments that demonstrate that ppGpp inhibits *in vitro* protein synthesis in a wheat embryo system. It is also shown to interact strongly with wheat embryo EF 1 with the consequent blocking of the EF 1 action on the binding of aminoacyl-tRNA to wheat ribosomes. ppGpp also inhibits the wheat translocation reaction as measured by the assay of *N*-acetyl-phenylalanyl-puromycin synthesis.

2. Materials and methods

ppGpp was a generous gift of Dr. M. Cashel of the National Institutes of Health, USA.[^{3}H]GTP, [^{3}H]GDP and [^{14}C]phenylalanine were purchased from New England Nuclear Corporation. The analog 5′-guanylylmethylenediphosphonate was obtained from Miles Laboratories.

Ribosomes, aminoacyl-tRNA and crude supernatant factors from wheat embryos were prepared as described by Allende [5]. The same reference gives the details of the assays for the *in vitro* synthesis of polyphenylalanine by wheat ribosomes directed by poly U. The assay for the EF 1-dependent retention of [^{3}H]GTP on nitrocellulose membranes was as published by Jerez et al. [6]. The binding of aminoacyl-tRNA to wheat ribosomes in the presence of EF 1 was assayed by the classical procedure of Nirenberg and Leder [7] except that the Mg^{2+} ion concentration was 5 mM and 0.5 mM GTP was added.

The assay of *N*-acetylphenylalanyl-puromycin synthesis by wheat ribosomes has been reported by Gatica and Allende [8]. The isolation of the ternary complex: wheat EF 1·GTP·aminoacyl-tRNA by gel filtration has been reported previously [9].

Partially purified wheat EF 1 was obtained by a modification of the procedure of Legocki and Marcus [10].

3. Results and discussion

Fig. 1 shows that ppGpp is a potent inhibitor of the binding of [^{3}H]GTP to wheat EF 1 (open circles). It is apparent from this graph that the analog can compete with GTP and that the affinity of the factor for this compound is of a similar order of magnitude as for GTP. In the same graph (filled circles) the effect

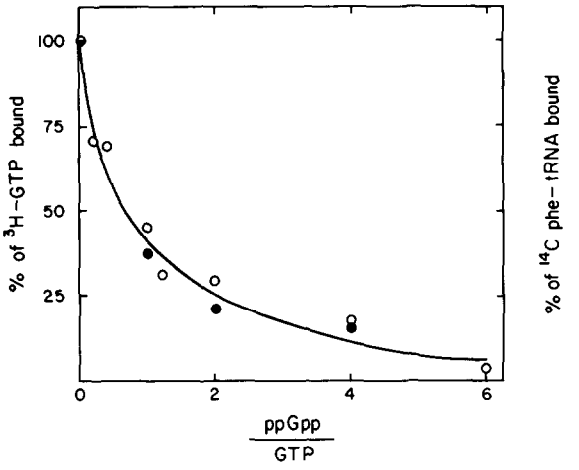

Fig. 1. Inhibition of the binding of wheat EF 1 to [^{3}H]GTP and of [^{14}C]phe-tRNA binding to ribosomes by ppGpp. The binding of [^{3}H]GTP to EF 1 was assayed by the nitrocellulose filter retention method described by Jerez et al. [6]. In this experiment (○—○—○), 90 μg of partially purified wheat EF 1 and 2.5 μM of [^{3}H]GTP (specific activity 1000 μCi/μmole) were used. The 100% value (no ppGpp present) corresponded to 18 pmoles of [^{3}H]GTP retained on the filter. The assay of [^{14}C]phe-tRNA binding to ribosomes was as described by Nirenberg and Leder [7] except that 5 mM $MgCl_2$ was used. In this experiment (●—●—●) 2.25 A_{260} units of washed wheat ribosomes; 20 pmoles of [^{14}C]phe-tRNA (specific activity 100 μCi/μmole); 40 μg of poly U; 90 μg of partially purified wheat EF 1 and 0.5 mM GTP were employed. The 100% value (no ppGpp present) corresponded to 3.25 pmoles of [^{14}C]phe-tRNA bound to ribosomes.

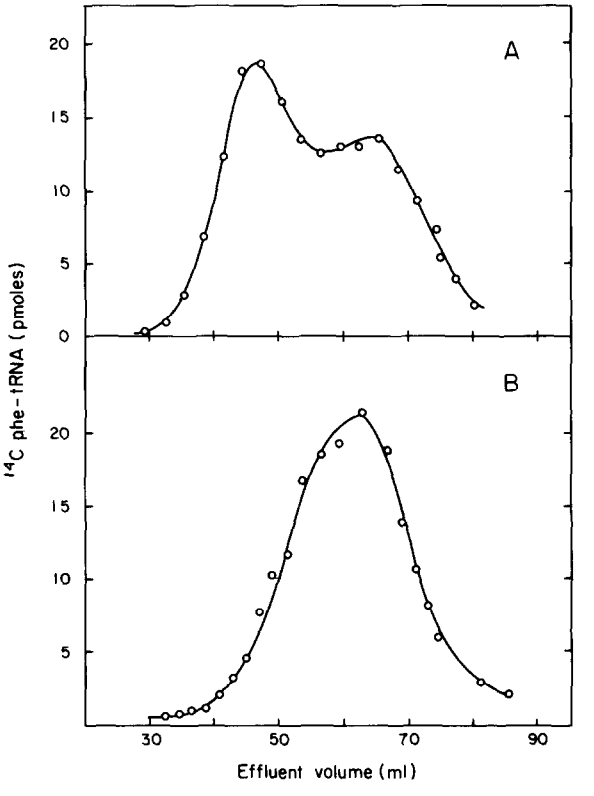

Fig. 2. The isolation of ternary complex wheat EF 1·GTP·[^{14}C]-phe-tRNA by gel filtration. The incubation mixture of 2 mg of partially purified wheat EF 1, 1120 pmoles of [^{14}C]phe-tRNA and 0.2 mM nucleotide in 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NH_4Cl, 1 mM DTT was passed through a Biogel P-300 column (2 × 40 cm) equilibrated and eluted with the same buffer containing 2.5 μM nucleotide. Aliquots of the fractions were precipitated with cold 5% trichloroacetic acid, filtered through glass fiber and counted in a scintillation counter. In A, GTP was used while in B the analog ppGpp was employed.

Table 1
Specificity of nucleotide requirements for the EF 1-dependent binding of aminoacyl-tRNA to wheat ribosomes.

Nucleotide added	[^{14}C]phe-tRNA bound(pmoles)
–	1.42
GTP	3.26
GDP	0.73
ppGpp	0.84
GMP-PCP	4.26

The binding of [^{14}C]phe-tRNA to wheat ribosomes in the presence of poly U was assayed as in fig. 1. The incubations were carried out using 2.25 A_{260} units of washed wheat ribosomes, 20 pmoles of [^{14}C]phe-tRNA, 90 μg of partially purified wheat EF 1 and 0.5 mM of the different nucleotides.

of the analog on the EF 1-dependent binding of aminoacyl-tRNA to wheat ribosomes is demonstrated. The coincidence of the inhibition effects indicates that the blocking of aminoacyl-tRNA binding to ribosomes is due to the interaction of ppGpp with EF 1. In this respect, ppGpp differs from another GTP analog, 5′ guanylmethylenediphosphonate (GMP-PCP) which also competes with GTP for EF 1 [6] but which can effectively replace GTP in the binding of aminoacyl-tRNA to ribosomes (table 1). The effects of ppGpp in these experiments are similar to those of GDP which

Table 2
The effect of ppGpp on translocation as measured by the synthesis of *N*-acetylphenylalanyl-puromycin.

Nucleotides (mM)	*N*-acetyl phe-puromycin (pmoles)	Activity (%)
None	0.18	4.8
GTP (0.5)	3.7	100.0
GDP (0.5)	0.23	6.2
ppGpp (2)	0.18	4.8
GTP (0.5) + ppGpp (0.5)	2.52	68.0
GTP (0.5) + ppGpp (2)	1.47	39.7
GTP (0.5) + GDP (2)	1.59	43.0

The assay for the synthesis of *N*-acetylphenylalanyl puromycin from [^{14}C]*N*-acetylphenylalanyl-tRNA and puromycin by wheat ribosomes was as described by Gatica and Allende [8]. The system contained 2.25 A_{260} units of ribosomes; 240 μg of a crude preparations of wheat EF 2, 34 pmoles of [^{14}C]*N*-acetyl phe-tRNA, 1 mM puromycin, 20 mM $MgCl_2$, 50 mM Tris-HCl pH 7.5, 25 mM NH_4Cl and the specified nucleotide in a volume of 100 μl. The incubation was for 15 min at 37°. The *N*-acetyl-phe-puromycin formed was detected by the amount of counts extractable into ethyl acetate. A control tube without added EF 2 had less than 0.1 pmoles of product.

Table 3
The effect of ppGpp on the synthesis of poly phenylalanine by a wheat embryo system.

Nucleotides added (mM)	[^{14}C]phe polymerized (pmoles)	Activity (%)
None	0.07	0.60
GTP (0.5)	12.00	100.00
ppGpp (0.5)	0.18	1.50
GDP (0.5)	1.30	11.00
GMP-PCP (0.5)	0.04	0.33
GTP (0.5) + ppGpp (4)	0.70	6.00
GTP (0.5) + GDP (4)	1.70	14.00
GTP (0.5) + GMP-PCP (4)	0.01	0.08

The assay for polyphenylalanine synthesis from [^{14}C]phe-tRNA has been described [5]. In this experiment 2.25 A_{260} units of washed wheat ribosomes, 160 μg of crude elongation factor preparation containing both EF 1 and EF 2, 20 pmoles of [^{14}C]phe-tRNA and 40 μg of poly U were used under standard assay conditions.

can bind to EF 1 but which cannot replace GTP in the binding of aminoacyl-tRNA to ribosomes due to the fact that the complex EF 1–GDP cannot interact with aminoacyl-tRNA to form a ternary complex. The capacity of GTP and ppGpp to form ternary complexes with wheat EF 1 and aminoacyl-tRNA can be directly assayed by gel filtration analysis as shown in fig. 2. The formation of ternary complex is detected by the appearance of a fraction of aminoacyl-tRNA eluting earlier than free aminoacyl-tRNA on a Biogel P-300 column.

In fig. 2A, it can be observed that in the presence of EF 1 and GTP, [^{14}C]phe-tRNA measured as ^{14}C radioactivity precipitable by cold 5% trichloroacetic acid, emerges from the column as two components. The larger component appears between 40 and 50 ml of the elution volume while the minor component between 55 and 70 ml. Separate experiments, measuring direct binding of [^{14}C]phe-tRNA to wheat ribosomes in similar column fractions have established that the early eluting [^{14}C]phe-tRNA is in the form of the ternary complex: EF 1·GTP·phe-tRNA.

In fig. 2B, where ppGpp has replaced GTP in the incubation and column buffer, it is clear that the complexed form of [^{14}C]phe-tRNA has disappeared and only the slower peak corresponding to free aminoacyl-tRNA is evident.

The interaction of ppGpp with wheat EF 1 can also be observed by the fact that it converts a heavy form of the factor (M.W. 200,000) into a lighter species (M.W. 50,000–80,000) as GTP and GDP also do [11].

The effect of ppGpp on the translocation reaction carried out by the wheat system was also determined. As shown by two laboratories [8, 10], the synthesis of *N*-acetyl-phenylalanyl-puromycin by washed wheat embryo ribosomes requires the action of elongation factor 2 (EF 2), or translocase and GTP.

This reaction is also strongly inhibited by ppGpp (table 2). GDP which is a product of this translocation reaction is also an efficient inhibitor.

As expected from its effect on the binding of aminoacyl-tRNA to ribosomes and on the translocation reaction, ppGpp is an inhibitor of the overall amino acid polymerization process. The effect of the analog on the synthesis of polyphenylalanine from phe-tRNA by wheat ribosomes directed by poly U is shown in table 3. It resembles GMP-PCP and GDP in that it cannot replace GTP in the polymerization requirement. However, ppGpp is a stronger inhibitor than GDP and a less potent one than GMP-PCP.

Presently the findings reported in this communication may be of some use in the study of the mechanism of binding of aminoacyl-tRNA to eukaryotic ribosomes but have little physiological meaning since ppGpp has not been detected in higher organisms. However, the conclusion that the presence of a phosphoester bond in the 3′ (or 2′) position of GDP, and presumably of GTP, does not affect the great affinity of EF 1 for these nucleotides may have some physiological importance since 3′ substituted guanosine 5′ polyphosphates are known to occur at the 5′ terminus of some RNA's [12]. It seems possible, therefore, that EF 1 of eukaryotes or EF Tu of bacteria may bind specifically to pppGpXp...5′ termini of completed or nascent RNA chains. This possibility is rather interesting in the light of the findings that point to the participation of EF Tu in the synthesis of some RNA's [4] and to the interaction of wheat EF 1 with the aminoacylated tRNA-like structures of the 3′ ends of plant viral RNA's [13].

Acknowledgements

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