INHIBITION OF *IN VITRO* PROTEIN SYNTHESIS BY ppGpp

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1. Introduction

When stringent bacteria (RC^+) are deprived of an essential amino acid, the rate of stable RNA synthesis is reduced. This coupled reaction does not occur during amino acid starvation of relaxed strains (RC^-) (for review see [1]).

Under starvation conditions, two products, MS1 and MS2, have been found to accumulate in the nucleotide pool of RC^+ strains exclusively [2]; MS1 was shown to be a guanosine tetraphosphate: the compound ppGpp [3]. Different laboratories have succeeded in establishing a close relationship between ppGpp accumulation and the reduction in the rate of stable RNA synthesis [4,5]. Using *E.coli* DNA as template and a purified transcription system, Travers et al. have obtained data which they interpreted as showing a specific inhibition of ribosomal RNA synthesis by MS1 product [6]. Recent experiments suggest that the accumulation of the nucleoside tetraphosphate only occurs when the blocking up of protein synthesis is caused by agents leaving intact the ability of ribosomes to catalyze elongation reactions [7–9]. Haseltine [10] has obtained *in vitro* ppGpp synthesis in a reaction involving EF-G factor, ribosomes plus a fraction extracted from RC^+ ribosomes by ammonium chloride. Finally, Blumenthal et al. [11] have shown strong binding of ppGpp to EF-T_u factor or to subunits III + IV of the Qβ replicase which have been identified to EF-T_u and EF-T_s factors, respectively.

These later results did suggest that accumulation of ppGpp could cause inhibition of certain translational steps. The present work gives support to this hypothesis by showing that the EF-T_u and IF_2-catalysed splitting of GTP by ribosomes is strongly inhibited by the nucleoside tetraphosphate, *in vitro*, whereas the EF-G dependent step is insensitive to its effect.

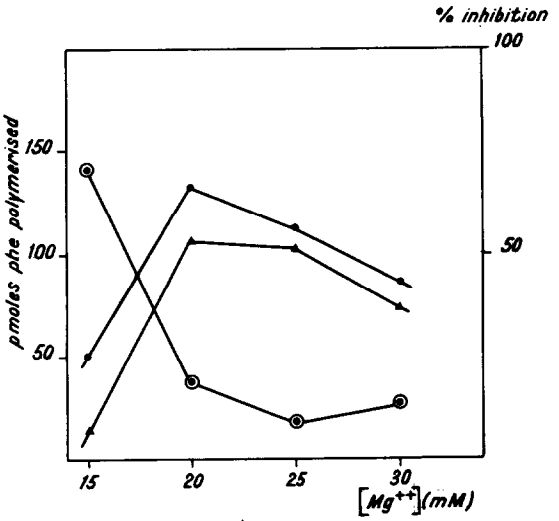

Fig. 1. Influence of Mg^{2+} concentration on the ppGpp effect in a poly U-directed polyphenylalanine synthesizing system. Polyphenylalanine synthesis without (●—●—●) and with 10^{-3} M of ppGpp (▲—▲—▲); (◉—◉—◉) Percentage of inhibition by ppGpp. Poly U-dependent [^{14}C] phenylalanine incorporation was measured by the method of Scheps et al. [21].

2. Material and methods

The characteristics of the products used throughout this work as well as the techniques involved in measuring the different translational steps will be specified in the legends to the figure or tables.

Table 1
Effect of ppGpp on the translation of natural messengers.

mRNA	Valine incorporated		Inhibition (%)
	Without ppGpp (pmoles)	1 mM ppGpp (pmoles)	
E.coli	16.8	4.8	68
R 17	49.0	15.0	69
T_4 late (25 min)	66.0	23.0	65

Conditions for incorporation are according to Salser et al. [15].

3. Results

3.1. *Effect of ppGpp on in vitro protein synthesis using artificial or naturally occurring messengers*

ppGpp, at a concentration close to that at which it is accumulated by amino acid starved RC^+ bacteria markedly reduces the rate of *in vitro* protein synthesis whether directed by artificial or naturally occurring messengers.

For instance, poly U-directed polyphenylalanine synthesis is severely inhibited (more than 80%) at 10^{-3} M ppGpp providing the Mg^{2+} concentration is lower than 20 mM (fig. 1). At higher Mg^{2+} concentrations, when EF-T_u is not involved and the polymerisation process is only dependent on EF-G and peptidyl transferase activities [12], the nucleoside tetraphosphate has little activity. With *E.coli*, R_{17} or T_4 late specific RNA's as messengers, an inhibition of the overall translation rate, which was in the three cases close to 65%, was observed in the presence of ppGpp (table 1). Thus, the extent of inhibition is independent of the kind of messenger used and also remains the same in a range of Mg^{2+} concentration comprised between 8 and 15 mM.

3.2. *Steps in protein synthesis sensitive to ppGpp inhibition*

3.2.1. Initiation

ppGpp caused a 62% inhibition of fMet-tRNA binding to ribosomes in the presence of T_4 late mRNA (table 2). This effect remained of the same amplitude for GTP concentrations varying from 10^{-7} to 2×10^{-3} M and a range of Mg^{2+} concentrations between 6 and 10 mM.

When GMP-PCP, a non splitable GTP analog, was substituted for GTP in the initiation reaction [13], inhibition of fMet-tRNA binding by ppGpp was only 35% (table 2). In the presence of IF_1 factor, fMet-rTNA and poly AUG (coupled activity) or in their absence (uncoupled activity) the IF_2 dependent GTPase activity was totally suppressed by 5×10^{-4} M ppGpp (table 3).

Table 2
Effect of ppGpp on fmet tRNA binding directed by T_4 late messenger RNA in presence of GTP or GMP-PCP.

Experiments	fmet-tRNA bound			Inhibition (%)
	$-T_4$ mRNA (pmoles)	$+T_4$ mRNA (pmoles)		
1)				
GTP	2.5	5.4	2.9	
GTP + ppGpp	1.3	2.4	1.1	62
2)				
GMP-PCP	1.5	2.4	0.9	
GMP-PCP + ppGpp	0.5	1.1	0.6	33

When used, GTP, GMP-PCP and ppGpp were added at concentrations equal to 10^{-4} M, 7×10^{-4} M and 10^{-3} M, respectively. Incubation was performed in the presence of 6 mM $MgCl_2$ with crude initiation factor. General conditions were previously described by J.G.Lelong et al. [16].

Thus, one step in protein synthesis at which the nucleoside triphosphate chiefly appears to interfer is the functioning of translation initiation factor IF_2. Another sensitive step resides in polypeptide elongation as is shown in the next section.

3.2.2. Binding of amino-acyl-tRNA to the 'A' site

ppGpp effect on the EF-T_u dependent binding of amino-acyl-tRNA has been studied. The EF-T_u preparation utilized was almost completely devoid of EF-G contamination and, under the conditions used in our assay system (table 4) more than 87% of the Phe-tRNA binding process was EF-T_u dependent and sensitive to tetracycline. Addition of ppGpp inhibited by 83% this EF-T_u dependent reaction.

We have also analyzed ppGpp effect on the EF-T_u–GTP complex formation. As shown in table 5, the amount of EF-T_u complex is proportional to the EF-T_u concentration. Formation of this complex is clearly sensitive to the addition of ppGpp in accordance with previously described results [11]

Table 3
Effect of ppGpp on IF_2 dependent GTPase activity.

Additions	P_i released (pmoles)	
	Total	Δ
Ribosomes	32.0	-
Ribosomes + Fusidic acid	31.6	< 0
IF_1	1.6	-
IF_2	6.8	-
Ribosomes + IF_1	31.2	< 0
Ribosomes + IF_2	174.0	135.2
Ribosomes + IF_2 + Fusidic acid	172.0	133.6
Ribosomes + IF_2 + ppGpp	38.0	< 0
Ribosomes + IF_1 + IF_2 + pAUG + fMet-tRNA	186.6	146.6
Ribosomes + IF_1 + IF_2 + pAUG + fMet-tRNA + Fusidic acid	178.0	138.6
Ribosomes + IF_1 + IF_2 + fMet-tRNA + ppGpp	28.4	< 0

When used, fusidic acid and ppGpp were added at concentrations equal to 100 μg/ml and 5×10^{-4} M, respectively. IF_1 and IF_2 were obtained according to the procedures of Herzberg et al. [17] and of Donnel and Thach [18], respectively. Ammonium chloride-washed ribosomes were purified according to Zimmerman [14], GTPase activity was measured following the conditions described by Kolakofski et al. [19].

Table 4
Effect of ppGpp on the [^{3}H]Phe-tRNA EF-T_u-dependent binding to ribosomes.

Additions	[^{3}H]Phe-tRNA bound		Δ	Inhibition (%)
	−EF-T_u (pmoles)	+ EF-T_u (pmoles)		
Control	1.10	8.20	7.10	-
+ ppGpp	0.70	1.95	1.25	83
+ Tetracycline	0.80	1.30	0.40	95

Incubation mixture (100 μl) contained: buffer A (Tris, Ph 7.5, 50 mM; $MgCl_2$, 2 mM; KCl 80 mM; dithiothreitol 5 mM), NH_4Cl-washed ribosomes, 4.0 A_{260} units; Phe-tRNA (total tRNA: 55.0 A_{260} units/ml) 20 μl; EF-T_u factor 10 μg; GTP 0.1 mM; when indicated ppGpp or tetracycline were added at concentrations equal to 0.8 mM and 0.4 mM, respectively. The mixture was incubated for 20 min at 25°, then immediately diluted by adding 1 ml of cold buffer A and filtering on a nitrocellulose membrane. Filters were washed several times with cold buffer A, dried and counted in a liquid scintillator. EF-T_u was a gift from Dr. Thang.

albeit to a lower extent than enzymatic Phe-tRNA binding.

3.2.3. Polymerization reaction

In order to test the ppGpp effect on the (poly U-dependent) polymerization step proper, a [^{3}H]-Phe-tRNA–EF-T_u–ribosome complex was performed [12] using ammonium chloride-treated ribosomes, washed free of EF-G factor [14]. EF-G factor was then added and the polymerization reaction was followed by measuring the amount of [^{3}H] phenylalanine incorporated in a TCA-precipitable form. Adding ppGpp at a concentration as high as 8×10^{-4} M caused no significant inhibition of this incorporation (table 6).

This result is consonant with that derived from the experiment described above which shows that the poly U-dependent synthesis of polyphenylalanine at high Mg^{2+}, an EF-T_u independent, EF-G and peptidyl transferase mediated process, is not affected by ppGpp.

4. Discussion

The ppGpp effects on *in vitro* protein synthesis which are reported in this paper, require relatively high concentrations of the nucleoside-tetraphosphate, usually ranged between 10^{-4} and 10^{-3} M. It must, however, be recalled that ppGpp accumulation by amino acid starved RC^+ bacteria, precisely reaches this order of magnitude [9].

It is interesting to notice that two out of the three

Table 5
Effect of ppGpp on the formation of EF-T_u–GTP complex.

EF-T_u factor added (μg)	GTP bound (pmoles)		Inhibition (%)
	Control	+ ppGpp	
8	2.9	2.0	32
20	6.5	3.2	52

The incubation mixture (100 μl) contained: buffer 'A' (see table 4), 10 mM $MgCl_2$; 4.4 μM [^{32}P]GTP; and 0.4 mM ppGpp when indicated. Formation of the EF-T_u complex was obtained under conditions described by Thang et al. [20].

GTP splitting reactions normally occurring during polypeptide synthesis, namely the IF_2-mediated binding of initiator tRNA and the EF-T_u catalyzed positioning of amino acid tRNA's to the A site are strongly inhibited by ppGpp whereas another GTP involving step, the EF-G mediated translocation is not. It might be relevant to recall in this respect that EF-G factor has precisely been implicated in ribosomal dependent ppGpp formation by some kind of idling reaction which would occur during amino acid starvation of RC^+ bacteria [10].

The shut-off of two GTP splitting processes by ppGpp (namely the IF_2 and EF-T_u mediated steps) could perhaps contribute to maintain a high GTP pool for the continued production of the nucleoside tetraphosphate under the influence of the EF-G factor.

Whatever the exact physiological significance of this effect, it must be a rapidly reversible one since prestarved RC^+ bacteria immediately resume protein synthesis upon readdition of the requisite amino acid.

As to the mechanism involved in ppGpp inhibition of protein synthesis, the present results indicate that this compound does not simply act as a competitive analog for GTP since varying the GTP concentration to a very large extent does not appreciably modify the inhibitory index (see in particular results from table 2). Furthermore, and as already stated, the EF-G mediated splitting of GTP is probably insensitive to the nucleoside tetraphosphate since the translocation reactions normally evolve in its presence.

That ppGpp modifies the conformational state of ribosomes in addition to being capable of interacting with IF_2 and EF-T_u factors is a possibility which we are presently investigating.

Table 6
Effect of ppGpp on polyphenylalanine synthesis from [^{3}H]-Phe-tRNA prebound to ribosomes

Additions	[^{3}H] phenylalanine incorporated			
	–EF-T_u factor (pmoles)	+EF-T_u factor (pmoles)	Δ	Inhibition (%)
Control	0.75	8.50	7.90	-
+ ppGpp	0.80	7.40	6.60	16

For [^{3}H] Phe-tRNA binding to ribosomes we have used the conditions described in table 4. After the [^{3}H] Phe-tRNA–EF-T_u–ribosome complex was performed, according to Thang et al. [12], EF-G factor (4 μg for 100 μl of incubation mixture) was added. Incubation was for 15 min at 37°. Samples were precipitated by adding cold 5% TCA, then boiled for 10 min. After cooling, they were filtered, washed with 5% TCA, dried and counted. When indicated ppGpp was used at 0.8 mM. EF-G was a gift from Dr. Thang.

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