IN VITRO DEGRADATION OF GUANOSINE TETRAPHOSPHATE (ppGpp) BY AN ENZYME ASSOCIATED WITH THE RIBOSOMAL FRACTION FROM *ESCHERICHIA COLI*

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1. Introduction

It is generally excepted that in *relA*$^+$ strains of *Escherichia coli* the unusual nucleotides, guanosine 5′-diphosphate, 3′-diphosphate (ppGpp) and guanosine 5′-triphosphate, 3′-diphosphate (ppGpp) are essential for stringent control of stable RNA synthesis (reviewed [1]). The two compounds accumulate when stringent (*relA*$^+$) *E. coli* strains are starved for an essential amino acid, a process that leads to a significant curtailment of stable RNA production; studies in vitro have shown that ppGpp specifically blocks synthesis of rRNA. In vitro (p)ppGpp is synthesized by the stringent factor on ribosomes when they, for various reasons, are not actively engaged in protein synthesis [2].

In vivo ppGpp is metabolically labile and rapidly turned over [3–5]; when stringent *E. coli* cells are resupplemented with the essential amino acid, the ppGpp concentration immediately drops to the basal level; the degradation reaction can be inhibited by the morphine analogue levallorphan [6,7]. Except for few genetic data [8–10] nothing is known about the enzyme(s) involved in and the mechanism of the degradation reaction.

Experiments reported here describe the in vitro system for the study of the ppGpp breakdown reaction. The enzyme that degrades ppGpp to 5′-GDP and probably pyrophosphate has been found in stringent and relaxed *E. coli* strains. Although this enzyme is associated with ribosomes, the latter are not essential for ppGpp breakdown, which requires Mg^{2+}- or Mn^{2+}-ions and is stimulated by Na-formiate. Degradation of ppGpp is inhibited by levallorphan and pGpp.

2. Materials and methods

[α-^{32}P]GTP, [γ-^{32}P]ATP and [^{3}H]GDP were purchased from New England Nuclear. For other materials and methods of protein determination see [11]. Levallorphan tartrate was a gift from Hoffman-La Roche, Basel, Switzerland. [^{3}H]Guanosine 5′-diphosphate, 3′-diphosphate (pp[^{3}H]Gpp) was prepared from [^{3}H]GDP using stringent factor and the ribosomal system from *E. coli* [11]; similarly ^{32}P-labeled ppGpp was prepared from [α-^{32}P]GTP yielding p$\overset{*}{p}$Gpp or with [γ-^{32}P]ATP yielding ppGp$\overset{*}{p}$; an asterisk over the 'p' indicates the ^{32}P-label in this position. The compounds were purified by DEAE-cellulose chromatography [12] and analyzed by two-dimensional polyethyleneimine thin-layer chromatography [13] using cold ppGpp as marker. As judged by autoradiography the compounds were 94–98% pure.

E. coli strains CGSC 2834/a (*relA*$^+$), CP78 (*relA*$^+$, CCA$^+$, *his*$^-$, *leu*$^-$, *arg*$^-$, *thr*$^-$, *B1*$^-$), CP79 (*relA*$^-$, CCA$^+$, *his*$^-$, *leu*$^-$, *arg*$^-$, *thr*$^-$, *B1*$^-$; CP78 and CP79 were kindly provided by M. Deutscher, Farmington, CO) were grown in 1% trypton, 0.5% yeast extract, 0.2% glucose and 0.5% NaCl and harvested at a density of 0.8–1.0 A_{578} units/ml. Cells were washed once and suspended in 3 vol. buffer (10 mM Tris–HCl, pH 7.7, 6 mM 2-mercaptoethanol, 10 mM Mg-acetate, 2 μg/ml DNAase), and disrupted in a French press cell at 16 000 psi and then centrifuged at

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8000 × *g* for 15 min. The supernatant fraction is referred to as crude cell extract. The latter as well as the more purified fraction (see fig.3) are rather labile; about 70% of the ppGpp degrading activity of the crude cell extract is lost, when pre-incubated for 10 min at 37°C without ppGpp. The ppGpp degrading activity is also present in crude cell extract from the relaxed strain CP79, however, it contains only one-third to one-fifth of the activity present in the CP78 strain.

Assay for ppGpp degradation. The assay system contained in final vol. 50 μl: 50 mM Tris·HCl, pH 7.7, 15 mM Mg-acetate, 4 mM $MnCl_2$, 2 mM dithiothreitol, 90 mM Na-formiate, 25 mM NH_4-acetate, 0.2 mM radioactively-labeled ppGpp (spec. act. p$\overset{*}{p}$Gpp 3–15 Ci/mmol, ppGp$\overset{*}{p}$ 0.1–0.3 Ci/mmol, pp[^{3}H]Gpp Ci/mmol) and the enzyme fractions as indicated in the legends to tables and figures. Where indicated 5 mM ATP was present in the assay mixture. When decay of ppGpp was studied in crude cell extracts best results were obtained with 15 mM Mg-acetate and 4 mM $MnCl_2$. With the more purified enzyme preparations 3–6 mM Mg-acetate or 0.5–1.0 mM $MnCl_2$ were sufficient. For most of the experiments we routinely used 15 mM Mg-acetate and 4 mM $MnCl_2$. In the presence of 90 mM Na-formiate the breakdown of ppGpp was stimulated about 10–20% in crude cell extract. Incubation of the assay mixture was carried out at 37°C for 10 min; the reaction was stopped by adding 1 μl 88% formic acid; a protein precipitate was removed by centrifugation and the supernatant fraction analyzed for ppGpp breakdown products by one-dimensional (1.5 M KH_2PO_4, pH 3.5) or two-dimensional chromatography (first dimension 0.5 M LiCl/4 M formic acid, pH 3.5; second dimension 0.75 M KH_2PO_4, pH 3.5). Guanosine nucleotides were used as markers and identified under ultraviolet light.

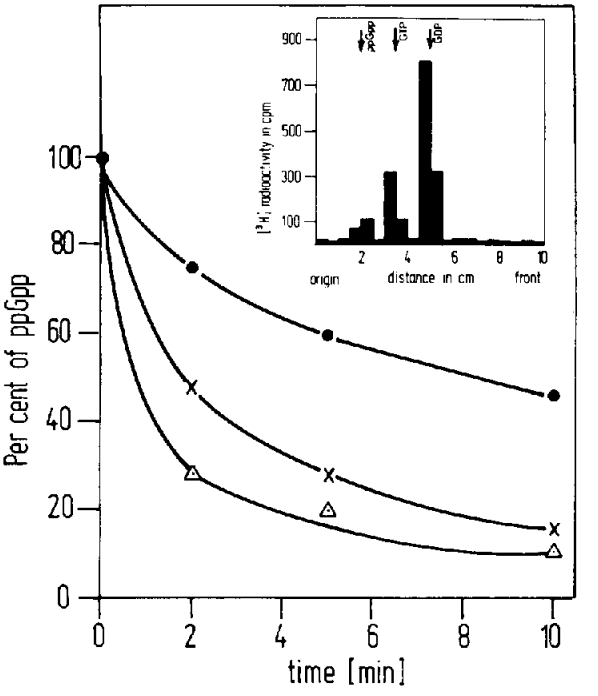

Fig.1. Time curve of the decay of pp[^{3}H]Gpp in the presence of various concentrations of crude cell extract. Crude cell extract was prepared from strain CP78 as outlined in the Materials and methods section. The following concentrations of crude cell extract were assayed in the standard ppGpp degradation assay (with 4 mM ATP): (•——•) 100 μg protein; (x——x) 200 μg protein; (△- - - -△) 300 μg protein. At the time indicated 5 μl assay mixture were withdrawn, mixed with 2 μl 44% formic acid and analyzed by one-dimensional PEI thin-layer chromatography (see insertion); the chromatogramm was cut in 0.5 cm strips and counted in Bray's solution in a liquid scintillation counter. The main degradation product was GDP. The insertion shows a PEI thin layer plate of an assay which contained 300 μg protein of crude cell extract and was incubated at 37°C for 10 min.

3. Results and discussion

As shown in fig.1 crude cell extract from stringent *E. coli* strains (CP78 or CGSC 2834/a) rapidly degrades tritium-labeled ppGpp to GDP; only little GTP is formed. Almost 80% of the ppGpp are split during the first 3 min incubation. Further analysis of the degradation products by two dimensional thin-layer chromatography shows that 5′-[α-^{32}P]GDP is formed when ^{32}P-labeled p$\overset{*}{p}$Gpp is used as substrate (fig.2). That the product is pGp rather than ppG can be excluded for the following reason: When the degradation product p$\overset{*}{p}$G is isolated and complemented with the stringent factor system [11], ppGpp is formed which would not be the case when pGp were the degradation product. When degradation of ppGpp is carried out with double-labeled p$\overset{*}{p}$[^{3}H]Gpp the ^{32}P:^{3}H ratio in the GDP region is 1 : 1.

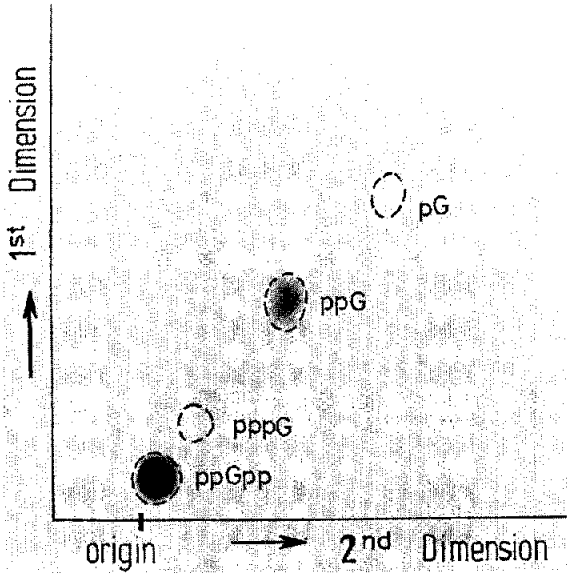

Fig.2. Two-dimensional polyethyleneimine thin-layer chromatography of ^{32}P-labeled pp*Gpp and its decay product. Degradation of ^{32}P-labeled pp*Gpp was carried out with fraction 3 (fig.3) in the 50 μl assay mixture as described in the Materials and methods section. Aliquots, 5 μl, were applied to a PEI sheet and chromatographed two-dimensionally (see Materials and methods section). The dotted lines indicate the position of cold nucleotides visualized by ultraviolet light. Exposure time of the X-ray film was 12–15 h.

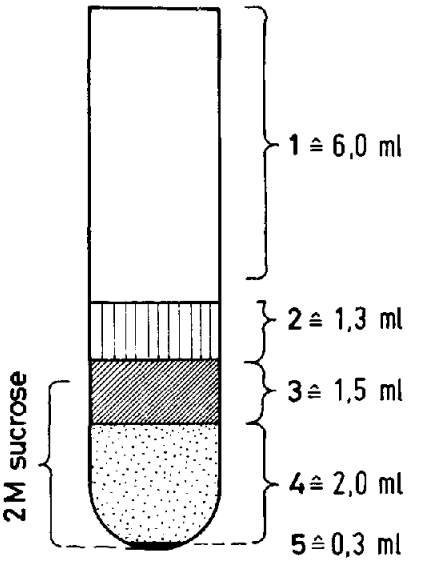

Fig.3. Fractionation of crude cell extract by centrifugation in a Beckman SW40 rotor. Crude cell extract, 8–9 ml, were layered on top of 3 ml 2 M sucrose in 20 mM Tris–HCl, pH 7.7, 5 mM Mg-acetate, 2 mM dithiothreitol, centrifuged in a Beckman SW40 rotor for 17 h at 200 000 × *g*. The centrifugate was fractionated from the top as indicated; the various fractions were analyzed for ppGpp degrading activity as shown in table 1. For further purification fraction 3 and 4 were diluted with 4.5 ml and 6 ml buffer, respectively (20 mM Tris–HCl, pH 7.7, 2 mM dithiothreitol, 5 mM Mg-acetate).

With double-labeled pp[^{3}H]Gp$\overset{*}{p}$ the product GDP contains only tritium but no ^{32}P-label. Most of the ^{32}P-radioactivity released from pp[^{3}H]Gp$\overset{*}{p}$ co-migrates with inorganic phosphate, little is found in the pyrophosphate region.

Since our enzyme preparations are rather crude, the ppGpp degrading enzyme may release pyrophosphate from the 3′-position of ppGpp which is then rapidly split into inorganic phosphate by a pyrophosphatase. Alternatively the two phosphates in the 3′-position of ppGpp are step-wise removed, a reaction that seems to be unlikely since no p$\overset{*}{p}$Gp has been found. Whether with a more purified enzyme the high energy containing 3′-pyrophosphate group can be rescued by transfer to an appropriate acceptor is at present under study.

In an attempt to relate the degrading activity to a subcellular fraction of the *E. coli* cell, the crude cell extract was fractionated by centrifugation (fig.3).

Table 1 shows that fraction 3 and 4 contain significant amounts of degrading activity. While fraction 4 has the majority of the ribosomes present in the cell, fraction 3 consists of ribosomes and membranes. Since fraction 3 (table 1) had most of the ppGpp degrading activity, it was further purified by step-wise centrifugation starting with a spin at 60 000 × *g* for 30 min in a Beckman 50Ti rotor. The ppGpp degrading activity remains in the supernatant fraction indicating that this enzyme is not part of the membrane fraction. The 60 000 × *g* supernatant fraction is then centrifuged at 150 000 × *g* for 4 h, the resulting pellet consisting of ribosomes contains the degrading activity, indicating that this enzyme is associated with ribosomes. That decay of ppGpp, however, does not depend on ribosomes is shown in table 2. Neither RNAase, nor the antibiotics chloramphenicol, puro-

Table 1
Analysis of the fractionated cell extract

Fractions[a]	mg protein/ ml	total protein (mg)	nmol degraded p*ppGpp/ mg protein × 10 min
1	4.0	24.0	0
2	17.0	22.1	0
3	67.2	100.8	88.7
4	32.0	64.0	28.2
5	93.3	28.0	20.7

[a] Fractions were isolated as described in the legend to fig.3

Decay of p*ppGpp and analysis of the products were carried out as described in the Materials and methods section. The degradation activities of the various fractions were obtained from experiments with a linear dependence on the protein concentrations

mycin or streptomycin significantly inhibit this reaction. Of the substances tested levallorphan and pGpp were inhibitory; inhibition by the former agrees with the in vivo results that this drug prevents decay of ppGpp [6,7].

Table 2 also shows that ATP, not, however, GTP or GDP slightly stimulates ppGpp breakdown, a stimulation which is even more significant when crude cell extract is assayed (unpublished). In the presence of ATP the degradation products are GTP and GDP; probably the latter is phosphorylated by a kinase present in our protein fraction.

Table 2
Effect of various antibiotics and nucleotides on degradation of ^{32}P-labeled p*ppGpp.

Conditions	nmol p*ppGpp degraded
Control	4.5
+ RNAase (5 μg)	4.0
+ chloramphenicol (5 μg)	4.4
+ puromycin (5 μg)	4.2
+ levallorphan (6 mM)	2.4
+ streptomycin (5 μg)	4.3
Control	4.5
+ pGpp (5 mM)	1.2
+ 2′dGDP (5 mM)	4.5
+ 3′dGDP (5 mM)	4.4
+ ATP (5 mM)	4.8–5.0
+ GTP (5 mM)	4.7
+ GDP (5 mM)	4.0

Assay mixture for ppGpp decay in the control was the same as described in the Materials and methods section, using 70 μg protein of fraction 3 (see fig.3 and table 1). Numbers in parentheses represent either μg antibiotic/50 μl assay mixture or the concentration of the nucleotide or drug in the assay. No antibiotics or nucleotides listed were added in the control

In conclusion, the data reported here show that degradation of guanosine polyphosphates starts with ppGpp; the guanosine pentaphosphate is not a prerequisite for the decay reaction. The products of the degradation pathway are 5′-GDP and probably pyrophosphate. The ppGpp degrading enzyme associated with the ribosomal fraction, however, can be released by a 1 M NH_4Cl wash (unpublished).

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