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Mechanism of the *in vitro* breakdown of guanosine 5′-diphosphate 3′-diphosphate in *Escherichia coli*

(guanosine 5′-diphosphate/ATP-PP_i exchange/*spoT*$^+$/*spoT*$^-$)

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ABSTRACT **Degradation of guanosine tetraphosphate (ppGpp) involves an enzyme associated with the ribosomal fraction from *spoT*$^+$ strains of *Escherichia coli*. Double-label experiments with $p\overset{*}{p}[^3H]Gpp$, $pp[^3H]G\overset{*}{p}p$, or $pp[^3H]Gp\overset{*}{p}$ as substrate strongly suggest that ppG is the degradation product and that the enzyme releases two phosphates coordinately from the 3′ position of ppGpp. In the absence of pppA this reaction proceeds in an uncoupled fashion, yielding ppG and PP_i, but in the presence of pppA the decay is considerably enhanced and a pppA-PPi exchange reaction occurs in which the 3′-pyrophosphoryl group of ppGpp displaces the γ and β phosphates of pppA. Sodium PP_i at 4 mM inhibits decay of ppGpp regardless of whether or not pppA is present.**

Auxotrophic strains of *Escherichia coli* are known to accumulate rapidly the unusual nucleotides guanosine 5′-diphosphate 3′-diphosphate (ppGpp) and guanosine 5′-triphosphate 3′-diphosphate (pppGpp) when deprived of an essential amino acid (for reviews, see refs. 1 and 2). It has been proposed that (p)ppGpp functions as a pluripotent regulator molecule that controls a number of biosynthetic and catabolic pathways; it restricts synthesis of stable RNA (3), of purine nucleotides (4), of glycolytic esters (5), and of phospholipids (6).

In order to elucidate the function of (p)ppGpp as a major regulator molecule in bacterial cells, it is essential to understand the precise mechanism of its synthesis and degradation as well. The mode of synthesis of (p)ppGpp has been discovered by Haseltine *et al.* (7) who showed that these compounds are synthesized on ribosomes by an enzyme named "stringent factor." Despite attempts by a number of groups, the mechanism of ppGpp breakdown has remained unresolved until recently (8, 9). Although, *in vivo*, ppGpp is rapidly degraded when *E. coli* cells are resupplemented with the lacking amino acid (10), attempts to demonstrate degradation *in vitro* have failed (11–15). Genetic studies have shown that *in vivo* breakdown of ppGpp is associated with the *spoT*$^+$ allele; in *spoT*$^-$ mutants, ppGpp breakdown is significantly slower than in wild-type strains (16, 17). This may indicate that *spoT*$^-$ mutants either have a similar but less active degradation device or utilize an alternative pathway. The enzyme involved and the mechanism of ppGpp breakdown are the subject of this communication.

MATERIALS AND METHODS

The ppGpp degrading enzyme was prepared from the stringent *E. coli* strains CGSC 2834/a (*relA*$^+$) and CP78 (*relA*$^+$, CCA$^+$, *his*$^-$, *leu*$^-$, *arg*$^-$, *thr*$^-$, *B1*$^-$) or from the relaxed strain CP79 (*relA*$^-$, CCA$^-$, *his*$^-$, *leu*$^-$, *arg*$^-$, *thr*$^-$, *B1*$^-$); CP78 and CP79 were kindly provided by M. Deutscher (Farmington, CT); K10 (*spoT*$^-$, *relA*$^-$, *tonA*22) were from A. Böck (Regensburg, Germany). $[8-^3H]GTP$ (New England Nuclear, Boston, MA) was used for preparation of $pp[^3H]Gpp$ (specific activity, 2 Ci/mmol). The ^{32}P-labeled $p\overset{*}{p}Gpp$, $ppG\overset{*}{p}p$ and $ppGp\overset{*}{p}$ (specific activity, 1–15 Ci/mmol) were prepared by using crude stringent factor (kindly provided by S. Fehr) and ribosomes from *E. coli* (CP78) as described (18). The compounds were analyzed by two-dimensional polyethyleneimine thin-layer chromatography using three different systems. System 1: first dimension, 0.5 M LiCl/4 M Na formate, pH 3.4; second dimension, 1.5 M KH_2PO_4, pH 3.5. System 2: first dimension, 1.5 M LiCl/2 M Na formate, pH 3.4; second dimension, 1.5 M KH_2PO_4, pH 3.5. System 3: first dimension, three steps with LiCl; second dimension, three steps with Na formate (19). Unlabeled ppGpp obtained from Sanruku-Ocean Co. (Tokyo) was used as marker. Radioautography revealed a purity of the compounds of at least 94%. Protein was estimated by the method of Lowry *et al.* (20) with bovine serum albumin as standard. Yeast inorganic pyrophosphatase was obtained from Sigma.

Preparation of ppGpp Degrading Protein Fraction. The enzyme catalyzing the degradation reaction was prepared as follows. The various strains were grown as reported (8) and harvested at 0.8 A_{578} unit/ml; growth was stopped by pouring the cell suspension onto crushed ice. Cells were washed once and resuspended in 3 vol of buffer A (10 mM Tris·HCl, pH 7.7/10 mM Mg acetate/6 mM 2-mercaptoethanol/1 mM $MnCl_2$/2 μg of DNase per ml). Cells were broken in a French pressure cell at a pressure of 16,000 psi (110 MPa). Intact cells were removed by centrifugation at 8000 × *g* for 15 min. Eight to 9 ml of the lysate, referred to as crude cell extract, was layered on top of a 3-ml cushion of 2 M sucrose in buffer B (20 mM Tris·HCl, pH 7.7/5 mM Mg acetate/2 mM $MnCl_2$/2 mM dithiothreitol/0.1 mM ppGpp) and centrifuged at 200,000 × *g* for 17 hr in a Beckman SW 40 rotor. From each gradient, five fractions were collected from the top (8). Fractions 1 (6 ml) and 2 (1.3 ml) contained the soluble enzymes, tRNA, etc.; ppGpp degrading activity was found in fraction 3 (1.5 ml), which consisted of membranes and ribosomes, and fraction 4 (2 ml), which contained the majority of the ribosomes; fraction 5 (0.5 ml) was discarded.

For further purification, fraction 3 or 4 was diluted 1:3 with 20 mM Tris·HCl, pH 7.7/5% (wt/vol) glycerol/1 mM $MnCl_2$/5/mM Mg acetate/2 mM dithiothreitol; it was spun at 60,000 × *g* for 30 min; the pellet was almost free of ppGpp degrading activity, indicating that the enzyme is not part of the cell envelope. The supernatant fraction was centrifuged at

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Abbreviations: ppGpp, guanosine 5′-diphosphate 3′-diphosphate; pppGpp, guanosine 5′-triphosphate 3′-diphosphate; $p\overset{*}{p}Gpp$, $ppG\overset{*}{p}p$, $ppGp\overset{*}{p}$, ^{32}P-labeled compounds in which the asterisk indicates the position of the label.

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Table 1. Decay of p$\overset{*}{p}$Gpp by a ribosomal fraction isolated from *spoT*$^-$ (K10) or *spoT*$^+$ (CP78) strains of *E. coli*

Assay condition	p$\overset{*}{p}$Gpp degraded to pppG, nmol	ppG, nmol	pppG + ppG, nmol
spoT$^+$:			
−pppA	0.02	4.91	4.93
+pppA	3.83	3.95	7.78
spoT$^-$:			
−pppA	0.08	0.04	0.12
+pppA	1.04	0.05	1.09

The ribosomal fractions prepared from strain CP78 or K10 were used as source for the ppGpp degrading enzyme. Where indicated, 4 mM pppA was present in the assay mixture which was incubated for 10 min at 37°. The degradation products were analyzed by two-dimensional thin-layer chromatography (system 3).

200,000 × *g* for 12 hr; the ribosomal pellet contained the ppGpp degrading activity and is referred to as ribosomal fraction.

To release the ppGpp degrading enzyme, the ribosomal pellet was extracted with 0.6–0.8 ml of buffer C (5 mM Tris·HCl, pH 7.7/1 mM EDTA/2 mM $MnCl_2$/2 mM dithiothreitol/10% glycerol). The enzyme could also be released by extraction of the ribosomal fraction with 1 M NH_4Cl; however, it seemed to be less stable under these conditions. The ribosomal extract obtained by low-salt extraction was centrifuged at 150,000 × *g* for 1 hr to remove disintegrated ribosomes and then concentrated by precipitation with 45 g of $(NH_4)_2SO_4$ per 100 ml. The precipitate was centrifuged and dissolved in buffer C in a final volume of 2.0 ml. When the $(NH_4)_2SO_4$ fraction was passed through a Sephadex G-100 column and eluted with buffer C, the main ppGpp degrading activity peak was well retarded, suggesting a molecular weight <150,000. It is not yet possible to get an exact estimate of the molecular weight because of the lability of the enzyme. Attempts to rechromatograph the main peak on longer Sephadex G-100 columns resulted in complete loss of degrading activity.

That the ppGpp degrading enzyme is the *spoT* gene product was demonstrated when the *spoT*$^-$ mutant strain K10 was assayed; only in the presence of pppA were low levels of ppGpp degrading activity detectable (Table 1).

Composition of the ppGpp Degrading System. The 50-µl assay mixture consisted of 2.5 µmol of Tris·HCl (pH 7.7), 0.2 µmol of $MnCl_2$, 0.1 µmol of dithiothreitol, 4.5 µmol of Na formate, 1.25 µmol of NH_4 acetate, 50–100 µg of degrading enzyme, and 0.01 µmol of p$\overset{*}{p}$Gpp (specific activity 2 Ci/mmol). If not otherwise indicated, the ribosomal fraction was used as source of the degrading enzyme. Where indicated, 4 mM pppA was present; the pppA or Na PP_i solutions were titrated with the appropriate magnesium concentration. The components were added in the order listed and the reaction was initiated by adding labeled ppGpp. After incubation at 37° for 20 min, the reaction was terminated by addition of 1 µl of 88% formic acid, and the resulting precipitate was removed by centrifugation. Aliquots (2–5 µl) were spotted on polyethyleneimine cellulose sheets which, if not otherwise indicated, were developed one-dimensionally in 1.5 M KH_2PO_4 (pH 3.5). Unlabeled guanosine nucleotides identified under UV light, were used as markers; the spots were cut out and assayed for radioactivity in a liquid scintillation counter.

Table 2. Decay of double-labeled ppGpp in the absence and presence of pppA

Substrate	Assayed	pppG ^3H	pppG ^{32}P	ppG ^3H	ppG ^{32}P	pG ^3H	pG ^{32}P
pp[^{3}H]-Gp$\overset{*}{p}$	−pppA	0.65	0.31	3.76	0.07	0.09	0
	+pppA	2.43	0.18	3.59	0.05	0.69	0.04
pp[^{3}H]-G$\overset{*}{p}$p	−pppA	0.36	0	2.93	0.02	0.06	0
	+pppA	2.17	0.13	3.18	0	0.5	0.06
$\overset{*}{p}$p[^{3}H]-Gpp	−pppA	0.30	0.41	5.56	5.86	0.52	0.55
	+pppA	3.50	3.51	4.61	4.74	0.30	0.32

Products formed,† nmol/50-µl assay

† The products were identified by polyethyleneimine thin-layer chromatography (system 2 or 3).

Table 3. ^{32}P-Labeled products obtained by degradation of ppGp$\overset{*}{p}$ or ppG$\overset{*}{p}$p in the absence of pppA

Assay	^{32}P, % In PP_i	In P_i
ppG$\overset{*}{p}$p	76.2	23.8
ppGp$\overset{*}{p}$	75.1	24.9
ppGp$\overset{*}{p}$ and inorg. pyrophosphatase	3.6	96.4

Fraction 4 of the SW 40 centrifugation step was used as source of the ppGpp degrading enzyme. Assay conditions were as described in *Materials and Methods* except, where indicated, 2 units of yeast inorganic pyrophosphatase and 4 mM Mg acetate were present. The ^{32}P-labeled products were analyzed by thin-layer chromatography using either system 2 or 3.

RESULTS AND DISCUSSION

In a preliminary report we presented evidence that decay of ppGpp is catalyzed by a ribosome-associated enzyme that rapidly degrades ppGpp to ppG (8). Because of the lack of purity of the system, it was not possible to distinguish whether the two phosphates in the 3′ position of ppGpp were released one by one or as a pyrophosphoryl group; moreover, it was not clear whether the high-energy-containing 3′-pyrophosphoryl group of ppGpp is conserved and turned over to an appropriate acceptor. In order to elucidate the mechanism of the phosphate release from ppGpp, double-labeling experiments with either pp[^{3}H]Gp$\overset{*}{p}$, pp[^{3}H]G$\overset{*}{p}$p, or $\overset{*}{p}$p[^{3}H]Gpp were carried out. The conversion of the substrate to the various degradation products

Table 4. Inhibition of the ppGpp degrading reaction by sodium pyrophosphate

Na PP_i	Decay of ppGpp, nmol	Formation of $\overset{*}{p}$ppA from ppGp$\overset{*}{p}$, nmol
Exp. 1:		
Control	8.41	4.62
+0.1 mM	8.27	3.24
+0.2 mM	7.41	3.23
+1.0 mM	6.45	2.34
+4.0 mM	4.16	0.37
Exp. 2:		
Control	6.01	
+0.1 mM	6.10	
+0.2 mM	5.76	
+1.0 mM	4.83	
+4.0 mM	3.69	

In Exp. 1 the assay mixture contained 0.01 µmol of ppGp$\overset{*}{p}$ and unlabeled pppA (4 mM). Incubation was at 37° for 15 min; products were analyzed one-dimensionally by thin-layer chromatography in 1.5 M KH_2PO_4 (pH 3.5). In Exp. 2, pppA was omitted and 0.01 µmol of p$\overset{*}{p}$Gpp was used as substrate. In the control experiment, no PP_i was present.

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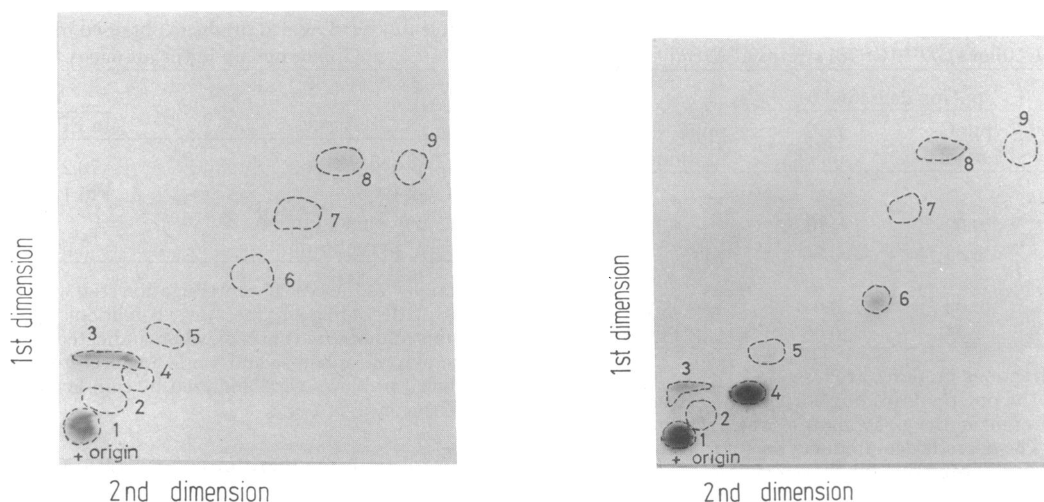

FIG. 1. Degradation of ppGpp̊ in the presence (*Right*) and absence (*Left*) of pppA (4 mM) and autoradiography of the products obtained by two-dimensional thin-layer chromatography. The decay reaction was carried out with 0.01 μmol of ppGpp̊. Incubation was at 37° for 20 min; 5-μl aliquots of the mixture were applied to the polyethyleneimine sheets together with unlabeled marker nucleotides. Chromatography was carried out in system 3. Spots: 1, ppGpp; 2, pppG; 3, PP_i; 4, pppA; 5, ppG; 6, ppA; 7, pG; 8, P_i; 9, pA.

was followed by two-dimensional thin-layer chromatography. As indicated in Table 2, in the absence of pppA the main degradation product formed is ppG. The molar ratio of ^{32}P- to ^{3}H-labeled ppG was 1:1 with pp̊[^{3}H]Gpp as substrate but not with pp[^{3}H]Gp̊p or with pp[^{3}H]Gpp̊, strongly suggesting that the two phosphates have been removed from the 3′ position of ppGpp. In the presence of pppA, the overall degradation of ppGpp was significantly stimulated and ppG most likely was converted to pppG by a nucleoside diphosphate kinase contaminating our ribosomal fraction (21).

The release of the two phosphates from the 3′ position of ppGpp could occur either step-by-step or by a pyrophosphoryl group transfer. As pointed out recently (8), the first possibility seems to be less likely because one would expect ppGp as an intermediate, a product we could not detect. In order to investigate the alternative possibility, the availability of a ppGpp degrading enzyme relatively free of pyrophosphatase activity was essential. This was achieved by using fraction 4 from the SW 40 centrifugation step, after further purification as described for fraction 3, as the enzyme source. Table 3 shows that, with ppGpp̊ or ppGp̊p as substrate, PP_i was the main product of the decay reaction; this was confirmed when, with addition of yeast inorganic pyrophosphatase, the ^{32}P label appeared as inorganic phosphate.

In an attempt to find out whether an appropriate molecule would accept the high-energy containing 3′ pyrophosphoryl group of ppGpp, various nucleotides were assayed; however, none of them (pA, ppA, pppG, ppG, or pG) turned out to be the acceptor. Only in the presence of pppA, ^{32}P-labeled phosphate released from ppGpp̊ or ppGp̊p comigrated with pppA (Fig. 1), suggesting a pppA–PP_i exchange reaction. This assumption was supported when the ppGpp degrading reaction was assayed in the presence either of ^{32}P-labeled p̊ppA and unlabeled PP_i or of P^{32}Pi and unlabeled pppA. In the former experiment, the ^{32}P label was found in the pyrophosphoryl group but not in the ppGpp spot; in the latter experiment, ^{32}P-labeled pppA was obtained (data not shown). The ppGpp degrading enzyme apparently did not catalyze a significant pppA–p[^{3}H]A exchange reaction as is known for the CoA-dependent step catalyzed by acetyl-CoA synthetase (22) or by the phenylalanine-dependent exchange mediated by gramicidin S synthetase (23); whether this lack of exchange was due to use of the less-purified enzyme system remains to be seen. The data shown in Table 4 indicate that decay of ppGpp and the pppA–PPi exchange reaction are closely coupled. Increasing concentrations of PP_i not only displaced the ^{32}P-labeled p̊ppA obtained by the decay of ppGpp̊ but also inhibited the overall ppGpp degrading reaction. This PP_i-mediated inhibition was also observed in the absence of pppA.

In conclusion, the experimental data reported here show that the enzyme present in the ribosomal fraction of *spoT*$^{+}$ strains of *E. coli* degrades ppGpp to ppG and PP_i. In the presence of pppA, the decay reaction is significantly stimulated and the 3′-pyrophosphoryl group of ppGpp exchanges, displacing the γ and β phosphates of pppA. The former reaction is due to a nucleoside diphosphate kinase-dependent reaction that phosphorylates ppG to pppG (21). This finding is in agreement with *in vivo* data that the rate constant of ppGpp degradation is decreased by an order of magnitude when production of high-energy-containing compounds is blocked (24, 25). It is intriguing to speculate that the pppA–PP_i exchange reaction is related to the amino acylation process of tRNA which consequently would link the decay reaction to the protein-synthesizing machinery. Alternatively the pppA–PP_i exchange reaction is distinguished from the tRNA charging reaction and a step in the ppGpp degrading process. Further purification of the ppGpp degrading enzyme should help to solve this problem.

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