Role of the spoT Gene Product and Manganese Ion in the Metabolism of Guanosine 5′-Diphosphate 3′-Diphosphate in Escherichia coli

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Addition of divalent ion chelating agents picolinic acid, 1,10-phenanthroline, or quinoline-2-carboxylic acid to wild type, relA, or relX, but not spoT strains of Escherichia coli increases the levels of guanosine 5′-diphosphate 3′-diphosphate (ppGpp). Poorly chelating analogs of these agents and a larger and more highly charged chelating agent, ethylene glycol bis(β-aminoethyl ether) N,N,N′,N″-tetraacetic acid are ineffective. Mn2+ reverses the increase in ppGpp.

The increase in ppGpp in wild type cells can be explained by an inhibition of degradation. In spoT cells the response is more complex: ppGpp does not increase although degradation is completely inhibited. The lack of increase in spoT cells suggests a role for spoT in synthesis of ppGpp in addition to its known role in degradation.

Growth of both spoT+ and spoT cells is inhibited following chelator addition. This suggests that growth inhibition is through a mechanism not directly involving ppGpp.

The results of this study provide evidence in intact cells for a role for Mn2+ and the spoT gene product in ppGpp degradation, and provide further evidence for an involvement of spoT and possibly divalent ions in ppGpp synthesis.

Exponentially dividing Escherichia coli maintain low levels of the nucleotides ppGpp1 and ppGpp. However, when wild type cells are deprived of a source of an amino acid (1, 2), energy (3–6), sulfur (3), or nitrogen (3), ppGpp or both nucleotides increase. The biochemical mechanisms that regulate metabolism of these nucleotides are complex and have not been clearly defined. At least five gene products are required. relA (7), relB (8), and relC (9) gene products are involved in synthesis in a complex consisting of ribosomes, mRNA, and codon-specific uncharged tRNA. The gene product of another locus, spoT, is important in maintaining ppGpp and ppGpp levels (10, 11). Recently, Pao and Gallant (12) isolated mutants that are unable to accumulate ppGpp during a glucose to succinate shiftdown. They have named the locus for this function relX.

Recent results suggest a role for the spoT gene product and Mn2+ in the metabolism of ppGpp: 1) Breakdown in “cold-shocked” spoT but not in spoT+ cells requires added Mn2+ (13). 2) ppGpp decay in spoT cells is inhibited by tetracycline (11); this inhibition is overcome by Mn2+ (14). 3) Under conditions “in vitro” in which extracts from wild type cells degrade ppGpp, essentially no degradation is detectable with spoT cell extracts (15, 16), this function is partially restored by Mn2+ (15). 4) The rate of synthesis is altered by the spoT mutation (17–19).

To further understand regulation of ppGpp metabolism and the role of spoT, we have added divalent ion chelating agents to E. coli. We found that after addition of certain hydrophobic chelating agents ppGpp increases in wild type, relA, and relX, but not spoT cells, and the increase is overcome by Mn2+. A preliminary report of these results has been published.

EXPERIMENTAL PROCEDURES

Cell Lines—The cell lines used in this study and their sources are listed in Table I. An A500 of 1.0 is equivalent to 5 × 106 cells/ml and 200 μg dry weight.

Radioactive Labeling—Cells were grown overnight in MOPS medium (20) containing 1.3 mM KH2PO4, 0.2% glucose, plus the nutritional requirements listed in Table I. MOPS medium also contains a variety of divalent ions: MgCl2, 0.52 mM; FeSO4, 10 μM; CaCl2, 0.5 mM; CoCl2, 30 mM; CuSO4, 10 mM; MnCl2, 80 mM; and ZnSO4, 10 mM. At the start of the experiment, the overnight culture was diluted 1/100 into the same MOPS medium with 0.4 mM KH2PO4. After 30 min, 11Porthophosphate was added, and at 0.2 A500 (approximately 2 h labeling) aliquots from neutralized stock solutions (in H2O) of picolinic acid (350 mM), nicotinic acid (350 mM), 1,10-phenanthroline (10 mM), 1,5-phenanthroline (10 mM), quinoline-2-carboxylic acid (600 mM), or EGTA (200 mM) were added. At the indicated times, 50 μl aliquots of cells were added to 50 μl of cold 4 M NH4COOH (pH 3.5). The contents were mixed and centrifuged, and 5-μl aliquots of the supernatant were analyzed with chromatography on PEl-cellulose thin layer sheets with 1.5 M K2HPO4 as solvent (1). After drying, the PEI chromogram was exposed to x-ray film overnight. The radioactive spots were removed and counted.

In these experiments, no extracellular ppGpp was detectable. This was determined after centrifugation of a culture aliquot for 2 min at room temperature in a Beckman Microfuge B and analysis of the supernatant by the method described above.

Identification of ppGpp—The 32P-containing material which increased during chelator treatment and migrated with ppGpp in the one-dimensional chromatography system was shown to be ppGpp by the following criteria. The radioactive material migrated with unlabelled authentic ppGpp in two types of two-dimensional chromatography systems on PEl-cellulose sheets (step-formate followed by 1.5 M KH2PO4). The radioactive material was degraded by alkaline phosphatase and was absorbed by charcoal. The alkaline phosphatase digestion was done by neutralizing an aliquot of the supernatant with an equal volume of 2 N Tris. Alkaline phosphatase

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The abbreviations used are: ppGpp, guanosine 5′-triphosphate 3′-diphosphate; ppGpp, guanosine 5′-diphosphate 3′-diphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether) N,N,N″,N‴-tetraacetic acid; PEI, polyethyleneimine; A500, absorbance at 650 nm.
were obtained from M. Cashel, National Institutes of Health. The ppGpp after chelator addition is at least 8-fold over basal in all cells. 

Increase is dependent upon experimental conditions. The increase in marked with a +. No significant change (less than 2-fold) from basal level is indicated by a -.

J. Gallant, University of Washington, Seattle, Wash. The other strains Pastan, National Institutes of Health. NFR59X and NF1035 are from Adhya, National Institutes of Health.

amino acid starvation column is a composite of our results and results from numerous published investigations. The absolute value for the increase is dependent upon experimental conditions. The increase in ppGpp after chelator addition is at least 5-fold over basal in all cells marked with a +. No significant change (less than 2-fold) from basal level is indicated by a -.

Mn²⁺, spoT, and ppGpp Metabolism

in ppGpp were observed, both ATP and GTP decreased. These results show that increases in ppGpp are not necessarily correlated with alterations in ATP.

Effect in Mutant Cells—To understand the mechanism for the increase in ppGpp, we tested the effects of picolinic acid in cells with mutations in genes which influence ppGpp metabolism. We first tested the requirement for a functional relA product and found increases in ppGpp in relA strains treated with picolinic acid (Table I). The ppGpp responses in one strain and in its isogenic parent are shown in Fig. 1 (inset).

A relA independent increase in ppGpp is characteristic of an energy shiftdown (4). We therefore tested the effect of relX, a gene whose product is required for ppGpp synthesis during a glucose-to-succinate shiftdown (12). ppGpp increases within 15 min after addition of picolinic acid to relX or relX cells grown on glucose (Fig. 2). Thus, the increase in ppGpp is independent of both relA and relX.

We then tested the effect of spoT (10, 11). Surprisingly, spoT cells show no increase in ppGpp after chelator addition (Fig. 2). This is true for all spoT mutant strains tested, regardless of accompanying genotypes (Table I).

Effect on Degradation and Synthesis—The increase in ppGpp could be due to changes in synthesis, or degradation, or both. Chloramphenicol addition, to block synthesis of ppGpp (1, 5), was used to examine degradation. In wild type cells, picolinic acid treatment increases the half-time of degradation from less than 20 sec to about 3 min. Essentially identical results are found with AT3 (Fig. 3) and N720 (data not shown) when similar experiments are performed. In all strains tested, the increase in ppGpp is observed at least 5 min after the addition of picolinic acid (Fig. 2).

TABLE I

Bacterial strains and response to chelator

| Strain | Relevant alleles | Comments | Amino acid starvation | Chelation |
|--------|-----------------|----------|-----------------------|-----------|
| N720   | relA⁺ spoT⁺ mel supF | +        | +                     |           |
| CP78   | relA⁺ spoT⁺ arg leu his thr thi | +        | -                     | +         |
| CF169  | relA⁺ spoT⁺ valSts | +        | -                     | +         |
| CF170  | relA⁺ spoT⁺ isogenic to CF169 | +        | -                     | +         |
| NF589X | relA⁺ spoT⁺ metB | +        | +                     |           |
| NF1035 | relA⁺ spoT⁺ isogenic to NF58 | +        | +                     |           |
| NF58   | relA⁺ spoT⁺ isogenic to NF58 | +        | +                     |           |
| NF59   | relA⁺ spoT⁺ isogenic to NF58 | +        | +                     |           |
| NF161  | relA⁺ spoT⁺ argA metB | +        | +                     |           |
| NF162  | relA⁺ spoT⁺ argA metB | +        | +                     |           |
| SA500  | relA⁺ spoT⁺ his | +        | +                     |           |

(0.3 unit) was added, and the solution was incubated at room temperature for 5 min.

Materials—Cellulose MN300 PEI thin layer sheets were obtained from Brinkmann; picolinic acid, chloramphenicol, EGTA, 1,10-phenanthroline, nicotinic acid, and alkaline phosphatase from Sigma; quinoline-2-carboxylic acid from Aldrich; ppGpp, 1,5-phenanthroline, and [32P]orthophosphate from ICN; MnCl₂ from Mallinckrodt, ZnCl₂, CoCl₂, FeCl₃, and KH₂PO₄ from Baker; and sodium formate from Fisher.

RESULTS

Increase in ppGpp after Chelator Addition—Incubation of E. coli (Table I) with the ion chelator picolinic acid (22) increases ppGpp. With strain N720, a 15-fold increase is maintained for at least 3½ h (Fig. 1). No ppGpp is detectable before or after addition of picolinic acid. Other chelating agents, 1,10-phenanthroline and quinoline-2-carboxylic acid (22), also increase ppGpp (Table II). Nicotinic acid and 1,5-phenanthroline, analogs of picolinic acid and 1,10-phenanthroline, respectively, which are poor ion chelators (22), do not. Picolinic acid is ineffective at 0.7 mM. Since the chelating agents are relatively hydrophobic and may be able to enter the cell, chelation leading to the increase in ppGpp could be either from medium or within the cell. To distinguish between these two possibilities, a larger and more highly charged chelating agent, EGTA, was tested. We found that it is effective in elevating ppGpp levels (Table II), even though it chelates transition metal ions and other divalent ions (23) more effectively than do the other agents that increase ppGpp. This suggests that chelation within the cell is important.

The effect of chelating agents on ATP and GTP levels was also measured. In the experiment shown in Fig. 1, ATP levels remained constant during the 3½-h incubation. GTP levels decreased about 20% within 5 to 10 min and then remained constant. In some other experiments in which similar increases

TABLE II

Effect of divalent ion chelating agents on ppGpp

| Agent              | Concentration | 10 min | 15 min |
|--------------------|---------------|--------|--------|
| Picolinic acid     | 3.5           | 18.9   | 20.8   |
| Nicotinic acid     | 3.5           | 1.6    | 0.2    |
| Quinoline-2-carboxylic acid | 6             | 4.4    | 4.4    |
| 1,10-Phenanthroline| 0.15          | 7.9    | 7.7    |
| 1,5-Phenanthroline | 0.15          | 0.8    | 0.7    |
| EGTA               | 2             | 2.2    | 1.7    |

FIG. 1. Increase in ppGpp following treatment with picolinic acid. N720 cells were grown as described under "Experimental Procedures." At zero time (0.2 A₅₆₀), 3.5 mM picolinic acid was added, and aliquots were taken at indicated times. Inset, effect in relA cells. Cells were grown to 0.2 A₅₆₀ at 32°C as described above. At zero time, picolinic acid was added and samples were taken as indicated. ○—○, relA⁺; ●—○, relA⁻ (CF189); ●—●, relA⁻ spoT⁻ (CF170).
picolinic acid-treated, H.
with picolinic acid. AT3
A-A;
ppGpp about 3-fold above that with valine treatment; thus the 70%
Mn⁺⁺ (10
decrease corresnonds to the increase in uoGnn after chelator addition.
NF161: control (e); 3.5 mM picolinic acid, 10
min (f), and 15 min (g). NF58: control (h); valine (500 µg/ml), 25 min
(i), and 40 min (j); then 3.5 mM picolinic acid for an additional 5 min
(k), and 15 min (l).

FIG. 4. Decay of ppGpp after Mn⁺⁺ addition. N720 cells, at 0.2
A₅₀₀ were treated with valine (500 µg/ml) for 15 min. Picolinic acid
(3.5 mM) was added for an additional 15 min with or without picolinic acid (3.5
mM). Chloramphenicol (100 µg/ml) was then added (zero time). AT3:
control, O—O picolinic acid-treated, O—O AT16: control,
■—■ picolinic acid-treated.

FIG. 3. Degradation of ppGpp in spoT and spoT⁺ cells treated
with picolinic acid. AT3 (spoT⁺) and AT16 (spoT⁻) were treated for
15 min with valine (500 µg/ml) to elevate ppGpp. They were then
incubated for an additional 15 min with or without picolinic acid (3.5
mM). Mn⁺⁺ was not detected in this experiment (Fig. 5), growth
inhibition in SPOT cells is surprising and is not easily
explained by alterations in ppGpp metabolism, the only
known defect in SPOT cells. This suggests that the spoT
mutation may exert pleotropic effects on cells.

We previously proposed that the primary cause for growth
inhibition by chelator treatment is reduced RNA synthesis. In vitro, 1,10-phenanthroline inhibits RNA polymerase by
chelation of essential metal ions on RNA polymerase (24). In vivo inhibition could be due to a similar mechanism. Alter-
natively, an increase in ppGpp could be responsible for RNA
synthesis inhibition (25, 26). However, growth inhibition in
spoT cells, where ppGpp does not increase following picolinic acid addition, suggests that an elevation of ppGpp is not
responsible.

The longer lag period of growth inhibition after chelator
addition (Fig. 5) and the decreased rate of ppGpp degradation
(11, Fig. 3) in spoT cells could be explained by one defect, a
lower level of Mn⁺⁺ in spoT cells. If a decreased intracellular Mn⁺⁺ concentration is the sole defect in spoT cells, other
properties of the spoT phenotype, such as lack of ppGpp accumulation during a stringent response (10, 11), should be
corrected by growing cells in a high concentration of Mn⁺⁺ (27,
28). We find that this explanation is probably not correct,
however, since spoT cells do not accumulate ppGpp during
isoleucine starvation when pregrown for 24 h in medium
containing 1 mM MnCl₂.

DISCUSSION

The metabolism of ppGpp during a stringent response in
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wild type cells is thought to proceed through the following pathway (15-19, 29).

\[
\text{pppGpp} \rightarrow \text{ppGpp} \rightarrow \text{ppGpp} \rightarrow \text{GDP}
\]

**Scheme 1**

According to this scheme, ppGpp is formed by a pyrophosphoryl transfer from ATP to GTP (7) (Reaction 1). ppGpp is dephosphorylated to ppGpp (Reaction 2), and ppGpp is degraded (Reaction 3) to GDP (15, 16). Under normal growth conditions, *E. coli* maintains low, but detectable levels of ppGpp. Synthesis of this basal ppGpp is independent of relA mutations (25) and is not clearly understood. Presumably, relX is required for basal synthesis. The ppGpp increase during an energy shutdown is due to a decrease in degradation and not to an increase in synthesis (25, 26). Mutant relX cells have low basal ppGpp levels and are unable to increase ppGpp during a glucose-to-sucinate shutdown (12). relX may directly catalyze ppGpp (or ppGpp) formation, or it may regulate basal activity of the "stringent factor" in some unknown manner independent of relA mutations.

spoT cells have elevated basal ppGpp levels (18, 19, 30). This increase is due to a decrease in the rate of degradation (11); however, the increase in ppGpp is less than that expected for the decrease in degradation. It follows that the rate of basal synthesis is also decreased in spoT cells (17-19). Extracts from spoT cells contain much less ppGpp degrading activity than do extracts from wild type cells (15, 16). This decreased activity is partially restored by Mn⁴⁺ (15). These in vitro results suggest a direct role for the spoT gene product and also for Mn⁴⁺ in ppGpp degradation. The observed effects on synthesis have not been explained.

When wild type cells under normal growth conditions are treated with chelating agents, ppGpp increases. This effect is presumably due to chelation of trace metals that are essential for degradation of ppGpp: 1) the increase can be explained by the measured inhibition of ppGpp decay; 2) structurally similar, poorly chelating analogs of these agents are ineffective; 3) a divalent ion, Mn⁴⁺, overcomes the action of the chelators. It should be emphasized that these results demonstrate that Mn⁴⁺ can activate degradation, but it does not necessarily mean that Mn⁴⁺ is the normally required ion.⁴

³ Mn⁴⁺ is poorly chelated by 1,10-phenanthroline (22). To the best of our knowledge, the ability of picolinic acid to chelate Mn⁴⁺ has not been analyzed. Our results suggest that Mn⁴⁺ is poorly chelated by picolinic acid. Therefore, small amounts of Mn⁴⁺ are sufficient to overcome the ion deficiency induced by chelator addition.

ppGpp binds Mn⁴⁺ (31). Thus the possibility that the complex ppGpp · Mn⁴⁺ is the substrate for degradation should be considered. Alternatively, the affinity of ppGpp for Mn⁴⁺ could aid binding of ppGpp to an enzyme · Mn⁴⁺ complex.

Genetic and metabolic studies show that the increase in ppGpp is separable from relA-dependent ppGpp synthesis. Identical responses are observed in relA⁻ and relA cells. Isoleucine does not decrease ppGpp in picolinic acid-treated relA⁻ cells starved for isoleucine before picolinic acid addition. Also, the increase following chelator addition occurs while RNA synthesis is inhibited; relA-dependent ppGpp synthesis requires mRNA in vitro (7) and is inhibited in vivo by rifampicin (32).

Our results demonstrate that chelator addition to intact cells inhibits ppGpp decay. This is expected from previously reported requirements for Mn⁴⁺ in the degradation of ppGpp (13-16). In addition, our results show a complexity in ppGpp metabolism that has not previously been observed. ppGpp increases in relA relX spoT cells after chelator addition. This raises the question of how ppGpp is synthesized in these cells. Even more intriguing is the fact that there is no increase in relA⁻ relX⁻ spoT⁻ cells when similarly treated. These results suggest that the spoT gene product either influences synthesis (17-19) through some undetermined regulatory mechanism, or that the spoT gene itself catalyzes ppGpp (or ppGpp) formation.

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