Guanosine Tetra- and Pentaphosphate Promote Accumulation of Inorganic Polyphosphate in Escherichia coli*

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High levels of guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (ppppGpp), generated in response to amino acid starvation in Escherichia coli, lead to massive accumulations of inorganic polyphosphate (polyP). Inasmuch as the activities of the principal enzymes that synthesize and degrade polyP fluctuate only slightly, the polyP accumulation can be attributed to a singular and profound inhibition by pppppGpp and/or ppGpp of the hydrolytic breakdown of polyP by exopolyphosphatase, thereby blocking the dynamic turnover of polyP. The Kᵢ values of 10 μM for pppppGpp and 200 μM for ppGpp are far below the concentrations of those nucleotides in nutritionally stressed cells. In the complex metabolic network of pppppGpp and ppGpp, the greater inhibitory effect of pppppGpp (compared with ppGpp) leading to the accumulation of polyP, may have some significance in the relative roles played by these regulatory compounds.

Inorganic polyphosphate (polyP),¹ a linear polymer of hundreds of phosphate residues linked by high-energy phosphoanhydride bonds, is ubiquitous having been found in all microbes, fungi, plants, and animals examined (1, 2). In Escherichia coli, polyP, which accumulates up to 20 mM (based on Pi residues) in stationary-phase cells,² is produced from ATP by a membrane-associated enzyme, polyphosphate kinase (PPK) (3).

Mutants lacking PPK are deficient in polyP, fail to adapt to stress, and do not survive in stationary phase (4, 5). A regulatory function is one of the many possible effects of polyP that might account for this essential role. In this regard, the relationship of polyP levels to those of guanosine pentaphosphate (pppGpp), generated in response to amino acid starvation in nutritionally stressed cells, is of particular interest. (pp)ppGpp deserves special attention. Levels of (p)ppGpp increase during the stringent response induced by serine hydroxamate (6, 7). The present study explores the mechanism of polyP accumulation in nutritionally stressed E. coli and the relationships to (p)ppGpp.

EXPERIMENTAL PROCEDURES

Reagents and Proteins—Sources were as follows: [γ-32P]ATP was from Amersham Corp.; nonradioabeled ATP and ADP and bovine serum albumin were from Sigma; polyethyleneimine-cellulose (PEI) TLC plate was from Merck; and creatine phosphate and creatine kinase were from Boehringer Mannheim.

Bacterial Strains—The ∆gppA::kan deletion-insertion allele in strain CF3376 was constructed as follows. A plasmid bearing the wild type gppA region was subcloned from phage λ1039 (9). Using synthetic primers, DNA-encoding, amino acid residues 1–452 of the 496 total in GppA were deleted. A kanamycin resistance (Km-r) cassette derived from plasmid pUC4K (Pharmacia Biotech Inc.) was substituted for the deletion. The insertion-deletion allele was recombined into phage λ1039 by phage growth on a plasmid-bearing strain and the lysate used to lysogenize a wild type K-12 strain (MG1655) selecting for Km-r. Phage curing and recombinative transfer of the ∆gppA allele from phage λ to the chromosome was by heat-pulse curing (10), yielding strain CF3376. Verification of the deletion in genomic DNA was done by: 1) polynucleotide chain reaction amplification from primers flanking the deletion site and visualization of the shortening of the product due to the presence of the kan cassette, 2) measurement of increased abundance of ppGpp relative to ppGpp during the stringent response induced by serine hydroxamate by a nonuniform labeling procedure (11), i.e., ppGpp/ppppGpp + ppGpp = 0.072 (± 0.017) for wild type versus 0.60 (± 0.084) for mutant, and 3) observing expected genetic linkage of the Km-r phenotype after P1 phage cotransduction of Km-r with ilv500::Tn10. Strain CF3382 is an example of a ∆gppA::ilv500::Tn10 recombinant.

The ∆ppks::kan deletion-insertion allele in strain CF5802 consists of a deletion of the C-terminal portion of ppk fused with a N-terminal deletion of ppx, again with a substitution of a kanamycin resistance cassette. In the ppks operon this deletion is deduced to remove 98.5% of PPK and 78% of PPX. The source of the residual ppk sequences was plasmid pBC29 (after SacI cleavage), while the source of the residual ppx sequences was the plasmid pBC6 (after PvuII cleavage). After insertion of the pUC4K kan cassette, the plasmid was linearized and used to transform a recB recC sde strain of E. coli selecting for Km-r, yielding strain CS5772. The ∆ppks deletion-insertion was transferred into strain MG1655 by transduction with phage P1, selecting for Km-r recombinants, which yielded strain CF5892. Verification of the replacement of wild type ppks locus by the deletion-insertion allele in CF5892 was by: 1) polynucleotide chain reaction with flanking primers, as above, 2) observation of dramatically decreased levels of PPK and PPX enzymatic activities in extracts, and 3) observing the expected genetic linkage of Km-r after phage P1 transduction to recipients bearing quaBA::Tn10 (88%) or ·ff208::Tn10 (10%).

Although the ∆gppA and ∆ppks allelices are both Km-r, they could be combined in a single strain by transduction of CF5892 with phage P1 grown on a ∆gppA ilv500::Tn10 donor (CF3382), selecting for Rec-r and screenin recombinants for the presence of high ppGpp/ppppGpp ratios.

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References

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2. The abbreviation used are: polyP, inorganic polyphosphate; polyPase, inorganic polyphosphatase; ppGpp, guanosine 5′-diphosphate 3′-diphosphate; pppGpp, guanosine 5′-triphosphate 3′-diphosphate; (p)ppGpp, ppGpp and pppGpp; PEI, polyethyleneimine, PPK, polyphosphate kinase; PPX, exopolyphosphatase, PPX*, overexpressed PPX; GppA, guanosine pentaphosphate phosphohydrolase; Km-r, kanamycin resistance; MOPS, 4-morpholinepropanesulfonic acid.


PolyP Accumulation under Stringent Condition

**FIG. 1.** PolyP accumulation and PPK and PPX activities under stringent conditions. E. coli MG1655 was grown on MOPS medium containing 0.4 mM Pi, (10 μCi/ml [32P]orthophosphate) and 40 μg/ml of amino acids. At an A595 near 0.2, serine hydroxamate (SHX) was added (0.5 mg/ml) for induction of amino acid starvation and accumulation of pppGpp and ppGpp. At the times indicated after addition of serine hydroxamate, 1-ml cultures were collected and used for the measurement of polyP amount (A) and PPK and PPX activities (B). Symbols are with (■) and without (○) serine hydroxamate. Units of PPK and PPX in B are 10² units of enzyme activity.

(11), yielding strain CF5986. The ilv506::Tn10 present in CF5986 was removed by transduction with phage P1 grown on a wild type MG1655 parental strain, selecting for prototrophs and screening recombinants for retention of the gpp-phenotype, yielding strain CF6032. 

**FIG. 2.** Inhibition of PPX by pppGpp and ppGpp. PPX hydrolysis of [32P]polyP was assayed in the presence of pppGpp (A) and ppGpp (B). The reaction mixture (10-μl) in A contained purified PPX (6 ng) and 50 μM [32P]polyP and in B contained purified PPX (3 ng) and 20 μM [32P]polyP. Further details are described under “Experimental Procedures.” of ppGpp and ppGpp plus pppGpp was calculated by the PhosphorImager scanner.

RESULTS

Influence of pppGpp and ppGpp on Activities of PPK and PPX—Accumulation of polyP by E. coli lacking or overproducing PPX has established that this activity is responsible for the synthesis of polyP (5). Similarly, the removal of polyP can be attributed to the presence or absence of PPX, the principal polyphosphatase of E. coli (13). Combination of these two opposing activities can account for a turnover of polyP in growing cells of 12 min or less. It was paradoxical then that 100-fold increases in the level of polyP in response to the nutritional stress of amino acid starvation were not accompanied by significant changes in the activities of PPK or PPX as measured in crude cell extracts (Fig. 1). Adding increased amounts of pppGpp and ppGpp had no influence on the activity of PPX: at 100 μM pppGpp and at 400 μM ppGpp, neither the synthesis of polyP from ATP nor the conversion of polyP to ATP were detectably (≥5%) affected. However, the effects of these compounds on PPX activity were strikingly inhibitory (Fig. 2). At 100 μM, pppGpp inhibited PPX by 90%; ppGpp also inhibited PPX, but less strongly.

Kinetic Features of Inhibition of PPX by pppGpp and ppGpp—Inhibition of PPX, examined at several levels of pppGpp, was consistent with its behavior as a competitive inhibitor of the polyP substrate with a Ki value of 10 μM (Fig. 3). A similar Lineweaver-Burk plot for inhibition of PPX by ppGpp yielded a Ki value of 200 μM. Binding of ppGpp to PPX was judged to be reversible in that a prior incubation of the nucleotide at 400 μM with the enzyme (200 ng) for 10 or 20 min at 37 °C yielded the expected level of inhibition upon a subsequent 10-fold dilution for assay of enzyme activity.

Stimulation of polyP accumulation by ppGpp and ppGpp could be demonstrated in the course of polyP synthesis by PPK and hydrolysis by PPX (Fig. 4). The presence of either of these nucleotides from the outset or an addition later in the reaction led to a large increase in the amount of polyP produced. Further addition of 500 μM ppGpp did not lead to a significant increase in the amount of polyP produced in the presence of 100 μM pppGpp (data not shown).
the far lesser effect of ppGpp makes it important to examine the enzymatic routes whereby pppGpp is hydrolyzed to ppGpp. The principal known route is the action of GppA (16), which also proved to have exopolyPase activity (17). In view of the considerable sequence homology between GppA and PPX (18), the latter was examined and also proved to have ppGpp hydrolase activity (Fig. 5). The $K_m$ value of 7 $\mu$M for ppGpp as substrate (Table I) as expected was virtually identical to its $K_i$ value of 10 $\mu$M as an inhibitor of polyPase activity and even lower than the $K_m$ of 110–130 $\mu$M ppGpp determined for GppA (16, 17). Yet, as indicated by the $k_{cat}$ value, PPX still qualifies more as a polyPase than as a ppGppase (Table I).

Relative ppGppase Contributions by GppA and by PPX—To evaluate the relative activities of GppA and PPX in the hydrolysis of ppGpp, extracts of several strains with various levels of these activities were compared. The strain lacking both ppkx and GppA was strikingly deficient in the hydrolytic activity compared with the strain that lacked only ppkx (Fig. 6). However, gppA and ppx mutants complemented with multicopy plasmids bearing ppx did recover ppGppase activity. Thus, GppA appears to be the major source of ppGppase activity, but PPX may function in an auxiliary role.

DISCUSSION

Whereas the polyP levels in E. coli may increase 1000-fold in response to nutritional stress, the levels of the enzyme activities responsible for polyP synthesis (PPK) and polyP degradation (PPX) hardly change at all.2 This was shown to be true when the stringent response was provoked using an amino acid analog serine hydroxamate (Fig. 1). In this study, we show that this phenomenon can be explained by the profound and singular inhibition of PPX by the stress-responsive nucleotides, ppGpp and pppGpp, without any effect on PPK (Figs. 2 and 3). Thus, the continued synthesis of polyP without degradation results in its extensive accumulation.

In keeping with these in vitro findings are several in vivo observations. (i) Accumulation of polyP follows the buildup of ppGpp and ppGpp in response to amino acid starvation.2, 3 (ii) In amino acid-starved cells, the levels of ppGpp increases from 20 $\mu$M to 1 mM and that of pppGpp from 0.5 $\mu$M to 200 $\mu$M; these elevated levels far exceed those required to inhibit PPX in vitro (i.e. a $K_i$ value of 200 $\mu$M for ppGpp and 10 $\mu$M for ppGpp (Figs. 2, 3)). The fact that the activity of PPX was unchanged in extracts of stressed cells can be attributed to the large dilution of ppGpp and ppGpp suffered in the preparation of the cell extracts. (iii) Turnover of polyP (resulting from its cyclic synthesis by PPK and hydrolysis by PPX) was found to be 12 min...
reaction mixtures containing 15 μl at 37 °C; amounts of [32P]ppGpp were measured by TLC as described or less,4 a result simulated by the accumulation of polyP in a type strains and mutants. mppGppase activities were measured in that fail to produce ppGpp and pppGpp (Fig. 4). (iv) Finally, mutants mixture containing purified PPK and PPX responding to the fail to accumulate any polyP 2, 3 when treated with serine orless,4 a result simulated by the accumulation of polyP in a mixture containing purified PPK and PPX responding to the presence of ppGpp and pppGpp (Fig. 4). (iv) Finally, mutants that fail to produce ppGpp and pppGpp (e.g. relA and spoT) also fail to accumulate any polyP2,3 when treated with serine hydroxamate.

While ppGpp is more abundant than pppGpp, the relative effects of these two regulatory nucleotides have not been thoroughly examined. In the case of polyP accumulation, pppGpp appears to be more effective than ppGpp (Fig. 2). The levels of these nucleotides depend on the synthesis of ppGpp by RelA and SpoT, the hydrolysis of pppGpp to ppGpp, and the conversion of ppGpp to GDP by SpoT. With regard to the generation of ppGpp from pppGpp, the action of the GppA enzyme is likely the principal route (Fig. 6). However, the facts that GppA is an exopolyphosphatase and also bears strong amino acid homology to PPX promoted our discovery that PPX, like GppA, can generate ppGpp from pppGpp. Other possibilities for minor contributions to PPX also hydrolyze pppGpp to ppGpp (Fig. 5).

Based on studies with extracts of various mutants, the PPX generation of ppGpp from pppGpp may be auxiliary to the action of GppA (Fig. 6). Other possibilities for minor contributions toward the formation of ppGpp from pppGpp include the translation elongation factors, EF-G and EF-Tu (6).

The kinetic parameters of PPX actions (Table I) show the same Km value for ppGpp as a substrate as the Km value as an inhibitor of exopolyphosphatase, indicating that the same active center is employed for both activities.

A scheme of polyP metabolism (Fig. 7), based on information mentioned in this report, is surely incomplete. For example, the role of RpoS, the sigma factor induced by (p)ppGpp and responsible for the expression of some 50 genes important in the response to starvation, needs to be included. Complementation of ppk mutants by a multicopy rpoS plasmid restored hydrogen peroxidase II activity, indicating an interaction relevant to polyP metabolism (4). Another example is the behavior of phoB, the regulatory gene of the phosphate regulon (19). Mutants of phoB, which produce (p)ppGpp in a stringent response, nevertheless fail to accumulate polyP.5 Attempts to reconstitute transcriptional systems with an RNA polymerase holoenzyme containing RpoS acting on an RpoS-activated gene (e.g. katE, the hydroperoxidase II gene) have resulted in a profound inhibition by polyP rather than the anticipated activation.5 One must conclude that factors, such as a polyP-binding protein, may be operating in vivo and are lacking in the reconstituted systems. Clues might also be supplied from studies of other microbial systems, such as Myxococcus xanthus (8) and Pseudomonas aeruginosa, in which starvation responses, heralded by increased levels of (p)ppGpp are followed, as in E. coli, by a large accumulation of polyP.

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REFERENCES

H. Wurst and A. Kornberg, unpublished results.
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