An Exopolyphosphatase of *Escherichia coli*  
THE ENZYME AND ITS *ppx* GENE IN A POLYPHOSPHATE OPERON*  

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A gene, *ppx*, that encodes a novel exopolyphosphatase of 513 amino acids (58,133 Da) was found downstream of the gene for polyphosphate kinase, *ppk*. Transcription of the *ppx* gene depends on the *ppk* promoters, indicating a polyphosphate (polyp) operon. Exopolyphosphatase, purified to homogeneity from overproducing cells, is judged to be a dimer of 58-kDa subunits. Orthophosphate is released processively from the ends of polyP ~500 residues long, but chains of ~15 residues compete poorly with polyP as substrate; ATP is not a substrate. Mg**2+** (1 mM) and a high concentration of K**+** (175 mM) support optimal activity.

Inorganic polyphosphate (polyP)**1** includes linear polymers of orthophosphate with chain lengths up to 1000 or more (1). PolyP is ubiquitous, having been found in bacteria, yeast, amebas, and mammals; yet relatively little is known about the enzymes that metabolize polyP or the physiological functions of polyP (2). Among the functions proposed for polyP are (i) phosphate and energy reservoirs with obvious osmotic advantage over P** and ATP, (ii) a substitute for ATP for certain sugar kinases (3–5), (iii) an association with poly-β-hydroxybutyrate and Ca**2+** in a membrane domain of transformable cells (6), (iv) a pH-stat mechanism to counterbalance alkaline stress (7), and (v) a regulator of promoter selectivity by RNA polymerase in stationary phase cells.**8**

An *Escherichia coli* enzyme responsible for polyP synthesis is the homotetrameric polyphosphate kinase (8), which polymerizes the terminal phosphate of ATP into polyP in a freely reversible reaction (**nATP** ⇔ **nADP** + polyP**n** (9), 10).

**ppx** is defined as the amount incorporating 1 pmol [T-32P]ATP at 10 Ci/mol increased 50-fold to 12.5 ml, and [T-32P]ATP was used at 10 Ci/mol.

### EXPERIMENTAL PROCEDURES

**Reagents and Proteins—**Sources were as follows: ATP, S-Sepharose fast flow, Mono Q HRS/5, and Superose-12 HR10/30, Pharmacia LKB Biotechnology Inc.; [γ-**32P**]ATP, Amersham Corp.; DNase I, RNase A, glucose 1-phosphate, and glucose 6-phosphate, Boehringer Mannheim; DE22-cellulose, Whatman; bacterial alkaline phosphatase, U. S. Biochemical Corp.; ADP, tripolyphosphate (polyP**3**), tetrapolyphosphate (polyP**4**), and a mixture (polyP**n**), Sigma; polyethyleneimine-cellulose F and cellulose plates, Merck; and dimethyl adipimidate dihydrochloride and dimethyl aminobisimidate dihydrochloride, Aldrich.

**Bacterial Strains—**Bacterial strains used are derivatives of *E. coli* K12: W3110 (Δ[rnIR], supE44, ΔlacU169/g18 lacZ ΔM15), sodR1, recA1, endA1 gyrA96, thi-1, relA1), and CA36 (Δlac-proAB), supE4, thi-1, relA1). W3110 served as a host strain for pBC10 to overproduce PPX for purification. DH5α or CA36 (11) was used to determine the levels of polyphosphate kinase and PPX overproduction induced by the plasmids. DH5α was also the host strain for preparation of plasmid DNA.

**Construction of Plasmids—**Starting from λ phage 10H6 of the Kohara library of the *E. coli* genome (13), a 5.5-kb EcoRI fragment was subcloned into the Small site of pUC18 in either orientation to produce pBC9 and pBC10 (Fig. 1) (11). Similarly, a 3.8-kb EcoRI fragment was subcloned into the EcoRI site of pUC18 in either orientation to produce pBC5 and pBC6; both lack the *ppk* promoters (Fig. 1). In pBC6 and pBC10, the reading frame of the *ppx* gene is placed in the same orientation as that of the *ppk* gene. Complete digestion of pBC9 by *KpnI* endonuclease and self-ligation resulted in pBC29; digestion of pBC10 by *KpnI* endonuclease, followed by self-ligation, produced pBC30.

**DNA Sequencing—**DNA sequencing of the *ppx* gene was performed by Dr. Shauna Brummet (U. S. Biochemical Corp. Custom Sequencing Service) and Alan Smith (PAN facility, Stanford University) as described (14). The strategy for sequencing the *ppx* gene is shown in Fig. 2. For *ppx*, ~97% of the final sequence of 1884 base pairs was determined from both strands (see Fig. 3). The nucleotide and deduced protein sequences were analyzed with the FASTDB program (IntelliGenetics) and with BLASTP and BLASTN (15).

**Assay for Polyphosphate Kinase—**The assay measured the production of acid-insoluble [32P]polyP from [γ-32P]ATP (8). One unit of enzyme is defined as the amount incorporating 1 pmol of phosphate into acid-insoluble polyP/min at 37 °C.

**Preparation of [32P]PolyP—**Synthesis was performed as in the assay for polyphosphate kinase, except that the reaction volume was increased 50-fold to 12.5 ml, and [γ-32P]ATP was used at 0 Ci/mol.

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1 The abbreviations used are: polyP, long-chain polyphosphate; ADA, N-(2-acetamido)-2-iminodiacetic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; polyP**n**, tripolyphosphate; polyP**n**,**m**, tetrapolyphosphate; polyP**n**,**m**, polyphosphate mixture with "average" chain length of 15; PPX, exopolyphosphatase; PAGE, polyacrylamide gel electrophoresis; Tricine, N-tris(hydroxymethyl)methylglycine; kb, kilobase(s).

2 A. Ishihama, personal communication.
FIG. 1. Polyphosphate operon and recombinant plasmids. A, organization of ppk and ppx on E. coli DNA carried in λ phage 10H6 (13). The wavy arrow indicates the start and the direction of transcription of the polyP operon. The stippled box is λ phage vector DNA, and the other boxes are E. coli DNA, EcoRI; B, pUC18. B, DNA fragments carried in the pUC18 vector. The plasmids were constructed as described under “Experimental Procedures.” Arrows indicate the direction of transcription of both the lac and bla promoters of pUC18.

FIG. 2. Strategy for sequencing ppx. Nucleotide sequences in ppx were determined as indicated by the directions and lengths of the arrows. The open reading frames shown are ppk (hatched box) and ppx (filled box). E, EcoRI; Q, KpnI. bp, base pairs.

TABLE I
Polyphosphate kinase and PPX activity of cells harboring various plasmids

<table>
<thead>
<tr>
<th>Host</th>
<th>Plasmid</th>
<th>10^6 units/g cells</th>
<th>fold overproduction</th>
<th>10^6 units/g cells</th>
<th>fold overproduction</th>
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<td>1.0</td>
<td>14</td>
<td>1.0</td>
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<td>0.1</td>
<td>360</td>
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<td>830</td>
<td>59</td>
</tr>
<tr>
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<td>56</td>
<td>14</td>
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<tr>
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<td>pBC30</td>
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<td>1.1</td>
<td>12</td>
<td>0.9</td>
</tr>
<tr>
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<td>pBC6</td>
<td>1100</td>
<td>79</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*PPK, polyphosphate kinase.

(8) With 10^6 units of purified polyphosphate kinase, ~50% of the [32P]polyP was incorporated into acid-insoluble polyP after 3 h at 37°C. The reaction was stopped by adding EDTA (50 mM) and extracted with phenol/CHCl₃ (1:1) once, followed by CHCl₃/isonamyl alcohol (24:1) three times. The long-chain polyP was further purified with slight modifications as described (16). The aqueous phase was mixed with 2 volumes of 2-butanol and placed at ~20°C for 30 min. [32P]PolyP was precipitated by centrifugation at 15,000 × g for 15 min (4°C). The resulting pellet was washed twice with cold 70% acetone and dried. The dried pellet was dissolved twice in 0.5 ml of 50 mM EDTA and precipitated with 2 volumes of 2-butanol. The recovery of acid-insoluble [32P]polyP was 80%, and the purity of polyP was >95%. The average chain length of this [32P]polyP based on polyacrylamide gel analysis (17) was ~500 residues.

Assay for PPX—The assay for PPX measured the loss of acid-insoluble [32P]polyP. The reaction mixture (100 µl) contained 50 mM Tricine/KOH (pH 8.0), 1 mM MgCl₂, 175 mM KCl, 50 mM [32P]polyP (25 µM in phosphate residues), and PPX as indicated. MgCl₂ was added last to avoid precipitation of the polyP. After incubation for 15 min at 37°C, the reaction was stopped by adding 100 µl of 7% HClO₄ and 20 µl of 2 M sodium bovine serum albumin. The [32P]polyP remaining was measured in a liquid scintillation counter after collection on a Whatman GF/C glass-fiber filter and washing with a solution of 1 M HCl and 0.1 M pyrophosphosphate, followed by ethanol. One unit of enzyme is defined as the amount that decreases the acid-insoluble polyP by 1 pmol/min at 37°C.

Assay for Bacterial Alkaline Phosphatase—Bacterial alkaline phosphatase was assayed by measuring hydrolysis of [γ-32P]ATP. The reaction mixture (10, 20, or 100 µl) was 1 M Tris-HCl (pH 8.0), 25 µM [γ-32P]ATP, and bacterial alkaline phosphatase as indicated. After incubation at 37°C for 15 min, the reaction was stopped by adding an equal volume of nonlabeled 10 mM ATP and chilling on ice. A sample (2 µl) was spotted on a polyethyleneimine-cellulose F plate, and chromatography was performed with 0.4 M LiCl, 1 M formic acid. The ATP spot was located by 256 nm UV light and cut out with scissors; the [γ-32P]ATP that remained was measured in a liquid scintillation counter. Bacterial alkaline phosphatase specific activity was measured using p-nitrophenyl phosphate (18). One bacterial alkaline phosphatase unit is defined as the amount hydrolyzing 1 pmol of p-nitrophenyl phosphate/min at 25°C.

Analysis of PPX Hydrolysis Products—The PPX reaction (200 µl) was performed at 37°C; samples (20 µl) were taken periodically. The reaction was terminated by adding 5 µl of 450 mM Tris borate (pH 8.3), 13.5 mM EDTA. The acid-insoluble polyP was detected by HClO₄ precipitation as in the PPX assay. The amount of total polyP was determined as polyP remaining on the origin on a polyethyleneimine plate after development with solvent system A (0.25 M LiCl, 1 M formic acid). Orthophosphate was separated by chromatography on the polyethyleneimine plate with solvent system A. To determine the amount of long-chain polyP, samples were loaded on a polyacrylamide gel (6% acrylamide, 0.3% bisacrylamide, 8 µm urea, 89 mM Tris borate (pH 8.3), 2.7 mM EDTA), and electrophoresis was performed at 750 V with electrophoresis buffer (90 mM Tris borate (pH 8.3), 2.7 mM EDTA) (17). Short chains were determined on a cellulose plate developed for 24 h with isobutyric acid/NH₄OH/water (25:3:12) containing 0.8 mM EDTA (7). As references, sodium phosphate, polyP₄, and polyP₆ were used and, after chromatography, visualized with an ammonium molybdate spray (19).

Other Methods—Cross-linking of the protein with dimethyl adipimide dihydrochloride or dimethyl suberimidate dihydrochloride (20), SDS-PAGE (21), protein concentrations (22) using bovine serum albumin as a standard, and DNA manipulations (23) were performed as described. The amino-terminal sequence of PPX was determined with an Applied Biosystems 470 Gas-phase Protein Sequencer with on-line high pressure liquid chromatography by Alan Smith.

RESULTS
Identification of Exopolyphosphatase and Its Gene, ppx—An extract from cells bearing pBC6, a plasmid that carries a 3.8-
Exopolyposphatase

Fig. 3. Sequence nucleotide of ppk. The nucleotide sequence of the noncoding strand of ppk is given from 5' to 3', starting at nucleotide 40 and ending at nucleotide 1581. The predicted amino acid sequence is also shown. The amino-terminal amino acid sequence determined of ppk (underlined) agrees with the deduced amino acid sequence. The 3'-end of the ppk gene is indicated by the box.

kb EcoRI fragment but lacks the intact gene for the polyposphatase kinase (Fig. 1), not only showed a 10-fold decrease in polyprophosphate kinase activity compared with cells harboring the vector pUC18 (Table 1). These data suggested that ppx may have its own very weak promoter, most of the expression of ppx constituting a polyp operon with the lac promoters. Furthermore, insertion of the lac promoter into both the lac promoters (11). When CA36 cells harboring each plasmid were grown (11), overproduction of PPX was 79-fold for pBC9 carrying vector pUC18 (line I), not only showed a 10-fold decrease in polyphosphate kinase activity compared with cells harboring the vector plasmid. These results suggested that a region downstream of ppk carries a gene encoding a phosphatase for polyP.

The ppk gene had been located in a 5.5-kb DNA fragment from λ phage 10H6 (11) and cloned in pUC18 as shown in Fig. 1. High levels of polyposphatase activity were associated with both pBC6 and pBC10, which carry the region downstream of ppk (Fig. 1 and Table 1). Digestion of this region with KpnI endonuclease abolished the phosphatase activity (as in pBC30). DNA sequence analyses revealed that this region contains an open reading frame of 1539 base pairs (nucleotides 40-1578) that spans the two KpnI sites and encodes a protein of 513 amino acids with a calculated mass of 58,133 Da (Fig. 3). This open reading frame (ppk), which encodes the PPX (see below), is located 7 base pairs downstream of ppk and transcribed in the same direction as ppk.

PolyP Operon Made Up of ppk and ppx—PPX was not overproduced from pBC6 in lac' cells (CA36), in which the lac repressor is present at levels higher than in wild-type cells (data not shown). In view of the overproduction of PPX from pBC6 in lac' cells (DH5α), there would appear to be a loose repression of the lac promoter. To study the expression of ppx without the influence of strong promoters in the vector DNA, ppx was oriented in the direction opposite to transcription of both the lac and bla genes (Fig. 1). The resultant plasmids were pBC5 with a 3.8-kb EcoRI fragment lacking a 0.9-kb BglII-EcoRI fragment presumably carrying the ppk promoters and pBC9 with a 5.5-kb BglII fragment containing the ppx promoters (11). When CA36 cells harboring each plasmid were grown, overproduction of PPX was 79-fold for pBC9 and only 3.7-fold for pBC5 (Table 1). Thus, although ppx may have its own very weak promoter, most of the expression of ppx depends on the 0.9-kb fragment with the presumed ppx promoters. Furthermore, insertion of the kan gene into ppx on the chromosome disrupted not only polyP synthesis but also the PPX activity. These data suggested that ppx constitutes a polyP operon with the ppk gene. Whereas a Shine-Dalgarno sequence was identified upstream of ppx, none is apparent for ppx (Fig. 3). Purification of PPX—Inasmuch as ppx is cotranscribed in polyphosphate kinase, this open reading frame did not appear to be a loose repression of the lac promoter. To study the expression of ppx without the influence of strong promoters in the vector DNA, ppx was oriented in the direction opposite to transcription of both the lac and bla genes (Fig. 1). The resultant plasmids were pBC5 with a 3.8-kb EcoRI fragment lacking a 0.9-kb BglII-EcoRI fragment presumably carrying the ppk promoters and pBC9 with a 5.5-kb BglII fragment containing the ppx promoters (11). When CA36 cells harboring each plasmid were grown, overproduction of PPX was 79-fold for pBC9 and only 3.7-fold for pBC5 (Table 1). Thus, although ppx may have its own very weak promoter, most of the expression of ppx depends on the 0.9-kb fragment with the presumed ppx promoters. Furthermore, insertion of the kan gene into ppx on the chromosome disrupted not only polyP synthesis but also the PPX activity. These data suggested that ppx constitutes a polyP operon with the ppk gene. Whereas a Shine-Dalgarno sequence was identified upstream of ppx, none is apparent for ppx (Fig. 3). Purification of PPX—Inasmuch as ppx is cotranscribed in polyphosphate kinase, this open reading frame did not appear to be a loose repression of the lac promoter. To study the expression of ppx without the influence of strong promoters in the vector DNA, ppx was oriented in the direction opposite to transcription of both the lac and bla genes (Fig. 1). The resultant plasmids were pBC5 with a 3.8-kb EcoRI fragment lacking a 0.9-kb BglII-EcoRI fragment presumably carrying the ppk promoters and pBC9 with a 5.5-kb BglII fragment containing the ppx promoters (11). When CA36 cells harboring each plasmid were grown, overproduction of PPX was 79-fold for pBC9 and only 3.7-fold for pBC5 (Table 1). Thus, although ppx may have its own very weak promoter, most of the expression of ppx depends on the 0.9-kb fragment with the presumed ppx promoters. Furthermore, insertion of the kan gene into ppx on the chromosome disrupted not only polyP synthesis but also the PPX activity. These data suggested that ppx constitutes a polyP operon with the ppk gene. Whereas a Shine-Dalgarno sequence was identified upstream of ppx, none is apparent for ppx (Fig. 3). Purification of PPX—Inasmuch as ppx is cotranscribed
Strain W3110/pBC10 was grown in LB medium (23) containing 50 µg/ml ampicillin at 30 °C. An absorbance (A_{600nm}) of 0.05, the temperature was shifted to 37 °C, and growth was continued for 4 h to an A_{600nm} of ~1.4. Unless indicated, all manipulations were 0-4 °C. The harvested cells were resuspended in an equal volume of 50 mM Tris-HCl (pH 7.5), 10% sucrose; frozen in liquid nitrogen; and stored at -80 °C. Dithiothreitol (to 2 mM) and lysozyme (to 300 µg/ml) were added to the thawed cell suspension (300 g), and the mixture was immediately transferred to Beckman Ti-45 centrifuge tubes and incubated at 0 °C for 30 min. The cells were lysed by exposure to 37 °C for 4 min, followed by immediate chilling in ice water. The particulate fraction was collected by centrifugation in a Ti-45 rotor at 20,000 rpm for 1 h (46,500 × g_{max}). The resulting pellet (144 g) was dispersed by sonication in the presence of 50 mM Tris-HCl (pH 7.5), 10% sucrose, 5 mM MgCl₂, 10 µg/ml DNase I, and 10 µg/ml RNase A. The temperature during the sonication procedure was maintained at or below 4 °C. To assure the dissociation of PPX from the membrane fraction, solid KCl was dissolved to 1.0 M, followed by addition of 0.1 volume of 1 M potassium phosphate (pH 7.0), 10% glycerol, 1 mM dithiothreitol, and 1 mM EDTA. The dialyzed extract (1100 ml) was stored at -80 °C.

Potential of PPX from overproducing cells

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Exopolyphosphatase

**FIG. 6.** PPX response to MgCl₂, salts, temperature, and pH. Purified PPX (fraction V, 25 fmol as dimer) was added to 100 µl of the PPX assay (see "Experimental Procedures"). The conditions for the assay were varied independently as follows. A, MgCl₂; B, the indicated salts; C, temperature for incubation; D, pH. The buffers (50 mM) used for each pH (D, 0) were MES (pH 5.5 and 6.0), ADA (pH 6.5), MOPS (pH 7.0), HEPES (pH 7.5 and 8.0), Tricine (pH 8.5), and CHES (pH 9.0). With Tricine, various pH values were also assayed (D, 0).

**FIG. 7.** Substrate specificities of PPX and bacterial alkaline phosphatase. Reaction mixtures were incubated at 37 °C for 15 min. Hydrolysis was detected as in the bacterial alkaline phosphatase (BAP) assay for [γ-³²P]ATP and the PPX assay for [³²P]polyP (see "Experimental Procedures"). A, PPX specificity. Purified PPX (fraction V) and either 25 µM [γ-³²P]ATP or 50 nM [³²P]polyP as polymer were present in a reaction mixture of 100 µl. B, bacterial alkaline phosphatase specificity. Bacterial alkaline phosphatase and 10 µM [γ-³²P]ATP or 10 µM [³²P]polyP as polymer were present in a reaction mixture of 10 µl.

**PPX Appears to Be a Dimer.**—Comparison of the Superose-12 elution volume of PPX with those of reference proteins indicated a molecular mass of ~100 kDa (Fig. 5), suggestive of a dimer. With the bifunctional reagent dimethyl adipimide dihydrochloride or dimethyl suberimidate dihydrochloride, PPX was cross-linked, appearing as a 120-kDa polypeptide on SDS-PAGE; monomeric ovalbumin remained as a 43-kDa band as expected (data not shown).

**FIG. 8.** Inhibition of PPX and bacterial alkaline phosphatase reactions. PPX (fraction V, 160 units, 60 fmol as dimer) hydrolysis of [³²P]polyP and bacterial alkaline phosphatase (BAP) (100 units, 14 fmol as dimer) hydrolysis of [γ-³²P]ATP were assayed as described under "Experimental Procedures" in the presence of each bacterial alkaline phosphatase substrate (unlabeled); the reaction volume of the bacterial alkaline phosphatase assay was 20 µl.

**Requirements for PPX.—**Under optimal pH (~8) and temperature (37 °C), 1 mM MgCl₂ was required for PPX activity (Fig. 6). KCl at 175 mM stimulated PPX 21-fold (Fig. 6), but was inhibitory at higher levels; NaCl was not as effective. Ammonium sulfate at 50 mM stimulated 7-fold. PPX was completely inhibited by 20 mM phosphate, but was unaffected by 1 mM NaF (data not shown).

**Substrate Specificity of PPX.**—The chain length of the [³²P]polyP substrate synthesized by polyphosphate kinase is ~500 as judged by polyacrylamide gel analysis. Under optimal conditions, 0.2 pmol (as dimer) of PPX hydrolyzed 5 pmol (as polymer) of polyP (Fig. 7A), yet showed no activity on 2.5 nmol of [γ-³²P]ATP (25 µM). Assuming the length of [³²P]polyP to be 500, the Kₘ of polyP for PPX is ~9 nM as polymer, a value well below that used in the assays. Upon raising the polyP level in the bacterial alkaline phosphatase assay to 10 µM as polymer, comparable to the Kₘ for ATP (18), polyP hydrolysis by bacterial alkaline phosphatase was still undetectable (<1%) during an incubation in which 10 µM ATP was hydrolyzed completely (Fig. 7B). The hydrolysis of 12.5 nmol of phosphate residues/pmol of PPX is 50 times that of ATP molecules/pmol of bacterial alkaline phosphatase. The capacity of bacterial alkaline phosphatase substrates to compete with polyP for PPX was also examined (Fig. 8). Glucose 1-phosphate, glucose 6-phosphate, ATP, and ADP were ineffective inhibitors for PPX (Fig. 8), but served as inhibitors for bacterial alkaline phosphatase.

**Inhibition by Substrate Analogs.**—In the short-chain polyP series, polyP₃ showed little inhibition of PPX hydrolysis of...
polyP even at a 2000-fold polymer molar excess over the long-chain polyP substrate (Fig. 9A); polyP inhibited 50% at a 2000-fold ratio, and polyP<sub>10</sub> showed this level of inhibition when present at a 200-fold ratio. Inhibitory effects by these millimolar levels of short-chain polyphosphate compounds were observed even when the Mg<sup>2+</sup> concentration was increased to 10 mM in the PPX assay. These short-chain polyphosphate compounds were far more effective inhibitors of bacterial alkaline phosphatase (Fig. 9B). A 50% inhibition of ATP hydrolysis was achieved at an ~50-fold polymer ratio for polyP<sub>3</sub>, a 20-fold ratio for polyP<sub>4</sub>, and a 4-fold ratio for polyP<sub>6</sub>.

**PPX Releases Orthophosphate from Ends of PolyP**—In the course of hydrolysis of [32P]polyP by PPX, P<sub>1</sub> release matched the loss of high molecular weight polyP (Fig. 10A). Inorganic pyrophosphate, polyP<sub>3</sub>, and polyP, were not detected among the products (data not shown). Inasmuch as no perceptible decrease in polymer size occurred during the course of its extensive conversion to P<sub>1</sub> (Fig. 10B), the hydrolysis from the chain ends appears to be highly processive. A chain in the size range of 10–20 residues, as an end product of hydrolysis of each polyP<sub>200</sub> chain, could have been missed because of heterogeneity and relatively low abundance. The level of phosphate residues in each member of a short-chain polyphosphate series may be <1% of the initial polymer and thus would contribute to an undetected smear on the polyacrylamide gel.

**DISCUSSION**

In pursuit of understanding the metabolism of polyP, we isolated the *E. coli* enzyme polyphosphate kinase (8). Polyphosphate kinase can generate long-chain polyP from ATP or convert it back to ATP in the presence of ADP. In the course of identifying the *ppk* gene that encodes polyphosphate kinase, we discovered a related activity, an exopolyphosphatase, encoded by an adjacent gene (*ppx*) (Fig. 1 and Table I). The facts that expression of *ppk* and *ppx* is controlled in a single operon and that the levels of polyphosphate kinase and PPX activities are quite similar suggest some physiological relationship between the functions of these enzymes. To explore this question, studies are underway to examine the consequences of overexpressing these genes or eliminating their activities. Whereas the phenotypes of the overproducing cells and those lacking the functional genes do not appear to be striking, it is already clear that the mutant cells possess low molecular weight polyphosphate<sup>4</sup> and an additional route for the hydrolysis<sup>5</sup> of polyP.

Several characteristics of PPX distinguish it from the known phosphatases of *E. coli* (24). It has a strong preference for long-chain polyP and fails to act on ATP (Fig. 7). Based on inhibition studies (Fig. 9), it has only a feeble affinity for smaller size polyP of ~15 residues. In addition to its pH optimum of 8 and requirement for Mg<sup>2+</sup>, exopolyphosphatase

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<sup>4</sup> E. Crooke and A. Kornberg, unpublished data.

<sup>5</sup> J. Keasling, E. Crooke, L. Bertsch, and A. Kornberg, unpublished data.
is strongly stimulated by potassium salts and is not inhibited by sodium fluoride. Direct comparisons of PPX with bacterial alkaline phosphatase reveal their striking differences (Figs. 7–9).

Whether PPX of E. coli is closely related to the polyphosphatase identified in other microorganisms will require further characterization of these other enzymes. Exopolyphosphatases have been obtained from Corynebacterium xerosis (25), Saccharomyces cerevisiae (1), Aerobacter aerogenes (26), and Neurospora crassa (1). The enzymes from A. aerogenes and N. crassa also require Mg²⁺ and a high concentration of K⁺, characteristics also similar to those of the E. coli PPX. Whether these factors influence the structure of the polyP substrate or the enzyme is not known.

The action of PPX is directed to the ends of the polyP chain, from which it removes orthophosphate processively (Fig. 10). During the course of the reaction, the size of the remaining large-chain polyP is undiminished, and only orthophosphate is released. Inasmuch as polyP chains of ≈15 residues are weak competitors with the long-chain polyP substrate and would presumably be poorly attacked, it would seem that they should also accumulate as an end product. However, with only one such small chain produced per long-chain substrate and the likely heterogeneity of these small-chain products, the failure to observe any discrete bands in the small size range by electrophoretic gel analysis would be expected. The mechanism that enables PPX to distinguish the ends of a long chain from those of a short one is obscure and intriguing. As for the cellular salvage and disposal of short chains, they may be used as primers for polyphosphate substrate or the enzyme is not known.

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REFERENCES

* J. Griffith, personal communication.