Endopolyphosphatases for Long Chain Inorganic Polyphosphate in Yeast and Mammals*

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Whereas exopolyphosphatases have been purified from yeast and a variety of bacteria, this is the first report characterizing endopolyphosphatases that act on long chain inorganic polyphosphate (polyP). The activity from Saccharomyces cerevisiae, localized in vacuoles, has been purified to homogeneity from a strain that possesses vacuolar proteases. The endopolyphosphatase is a dimer of 35-kDa subunits. Distributive action on polyP750 produces shorter chains to an extent of about polyP800 as well as the more abundant release of polyP3; the $K_m$ for polyP250 is 185 nm. Endopolyphosphatases have been identified in a wide variety of sources, except for most eubacteria tested. The activity has been partially purified from rat and bovine brain where its abundance is about 10 times higher than in other tissues but less than $\frac{1}{10}$ that of yeast; the limit product of digestion of the partially purified brain enzyme is polyP3.

Inorganic polyphosphates (polyP) are linear polymers of orthophosphate residues linked by high energy phosphoanhydride bonds. Likely prevalent in prebiotic evolution (1) polyP has been found in all organisms ranging from bacteria to mammals (2). The ubiquitous occurrence of polyP suggests multiple roles depending on the species, cell, subcellular localization, and physiological state.

Our approach toward understanding the functions of polyP has been to identify and isolate the enzymes that synthesize and degrade polyP. Such enzymes have been identified in a variety of microorganisms. PolyP kinase, the enzyme that catalyzes the reversible transfer of the terminal phosphate from ATP to synthesize polyP, has been purified to homogeneity in several bacteria (3–5). Exopolyphosphatases that catalyze the hydrolysis of terminal phosphates from polyP have been purified from Escherichia coli (6), Corynebacterium xerosis (7), and Saccharomyces cerevisiae (8, 9). Phosphotransferases that transfer a phosphate from polyP to AMP (10), NAD (11), glucose (12), and 1,3-diphosphoglycerate (13) have also been described. Endopolyphosphatases (PPN) also called polyP depolymerases or polyphosphorylases catalyze the non-processive cleavage of polyP to release intermediate-size chains during the course of the reaction. PPN are the least studied of the polyP-metabolizing enzymes and have been reported in species of Penicillium and Aspergillus and in S. cerevisiae (14–16).

During the course of investigating polyP metabolism in a variety of cells, we discovered PPN activities in organisms from archeae to mammals but little or none among eubacteria. In this report, we describe the purification and characteristics of PPN from S. cerevisiae, where it is most abundant, using a mutant strain in which a major exopolyPase activity has been deleted (17).

EXPERIMENTAL PROCEDURES

Cells and Tissues—S. cerevisiae CRX (17) was grown in YPD medium (1% yeast extract, 2% tryptone, 2% glucose) at 30 °C to an $A_{600}$ of 14.5. The harvested cells were resuspended in an equal volume of 50 mM Tris-HCl, pH 7.5, 10 mM sucrose, frozen in liquid nitrogen, and stored at $-80$ °C.

Tissues were obtained from 4–6-week-old Fisher 344 rats. Brain tissues at various developmental stages were obtained from Sprague-Dawley rats.

Preparation of Extracts—When microorganisms were the source of enzyme, the cells were suspended in 5 volumes of lysis buffer (0.25 M sucrose in 10 mM Tris-HCl, pH 7.0, and 1 mM EDTA) and sonicated (Branson Instruments) in an ice bath for three 15-s bursts with 30-s cooling between bursts. The homogenates were centrifuged at 25,000 $\times$ g for 30 min and the supernatant used for analysis.

Preparation of $^{32}$P-PolyP—$^{32}$P-PolyP was obtained by synthesis from $^{32}$P-ATP using purified E. coli polyP kinase (18). The polyP was purified (19) and had a chain length of 750 ± 50 residues.

To obtain shorter chains, polyP250 was partially hydrolyzed near pH 2 by adding an equal volume of 20 mM HCl and heating for 2–6 min at 100 °C. Partially hydrolyzed polyP750 was fractionated by 6% urea-polyacrylamide gel electrophoresis and then eluted from gel slices into 50 mM ammonium acetate, followed by ethanol precipitation (4).

PPN Activity Assay—The reaction mixture (10 µl) contained 100 mM Tris-HCl, pH 7.5, 5 mM MgCl$_2$, and 350 µM $[^{32}$P]polyP (as P, residues). The reaction was carried out at 37 °C for various times, followed by the addition of 2.5 µl of 5 × electrophoresis sample buffer (50% sucrose, 0.125% bromphenol blue, 2.25 mM Tris borate, pH 8.3, and 67.5 mM EDTA) to stop the reaction. Controls were incubated on ice or at reaction temperatures without addition of extracts. The reaction products were electrophoresed on 6% urea-polyacrylamide gel (21), exposed to a PhosphorImager screen (Molecular Dynamics), and analyzed. The assay is linear over a 60-min period under the described assay conditions at protein concentrations up to 0.2 µg (when crude extracts are used as the enzyme source).

Units of PPN Activity—PPN activity was calculated by measuring the percentage decrease in counts at the origin with respect to total counts in the lane. The percentage was then converted to pmol of polyP utilized (as P, equivalents). A unit was defined as pmol of polyP (as P, residues) utilized per min; units/mg of protein gives the specific activity.

Gel Filtration of PPN—Gel filtration for determination of native molecular weight of PPN was at 4 °C on a TSK-Gel G2000SWxl column (TosoHaas) equilibrated in buffer B (100 mM Tris-HCl, pH 7.0, 100 mM KCl, 1 mM diethiothreitol, and 0.1 mM EDTA). Elution was performed with buffer B at a flow rate of 0.7 ml/min. Marker proteins were detected by monitoring UV absorption ($A_{280}$ nm). Fractions containing PPN activity were detected by assay. Molecular mass markers were

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The abbreviations used are: polyP, polyphosphate; PP, inorganic pyrophosphate; PPN, endopolyphosphatase; exopolyPase, exopolyphosphatase; PAGE, polyacrylamide gel electrophoresis.
abblase (158 kDa), hexokinase (104 kDa), bovine serum albumin (66 kDa), and ovalbumin (43 kDa). Void volume was determined by measuring elution volume of apoferritin (443 kDa).

Other Methods—Protein was estimated by the method of Bradford (22) using bovine serum albumin as a standard. SDS-PAGE of proteins was according to Laemmli (23) with size standards from Bio-Rad. Protein bands were visualized by silver staining (24).

RESULTS

PPN Activity Assay—Early assays for detecting PPN activity were based on measurement of changes in viscosity of polyP which decreased as the reaction proceeded (25). However, the main limitation of this method is its inability to detect cleavages in substrates with a chain length shorter than ~100 P_1 residues. A linked spectrophotometric assay using polyP gluokinase allows for calculating the number of polyP chains formed by PPN action and distinguishes between exo- and endopolyPase activity (26). An assay method based on the ability of polyacrylamide gels to separate polyP of different chain lengths was developed by Wieckowski and Wood (27) to measure PPN activity in crude yeast extracts. This allows separation of substrate polyP from medium and short chain products that are formed as a result of cleavage of internal phosphohydroxyl bonds. Activity is determined by measuring the decrease in concentration of the initial substrate. We used a modification of this method to assay PPN in various crude extracts as well as to follow the purification of yeast PPN. Sensitivity was increased over previously described assays by using [32P]polyP having a length of 750 residues with small size variations (±50) compared with the previously used commercial substrates.

However, the main drawback of this assay is its inability to provide a quantitative measure of the number of cleavage events during the PPN reaction. However, when the assay is done under conditions where less than 50% of the substrate is removed, linearity with respect to both time and enzyme concentration was observed (data not shown). Also, by scanning across the length of each lane one can determine the relative amounts of products formed during the course of the reaction; this could be used as a basis for calculating the number of cleavages in the original substrate per unit time. However, for routine assays, the amount of substrate removed was determined to calculate the activity of the enzyme.

Abundance in Various Organisms—The ability of extracts prepared from various organisms to cleave long chain polyP was tested. Various amounts of extracts were used to hydrolyze 350 μM [32P]polyP, and activity was determined based on linear events during the PPN reaction. However, when the assay is done under conditions where less than 50% of the substrate is removed, linearity with respect to both time and enzyme concentration was observed (data not shown). Also, by scanning across the length of each lane one can determine the relative amounts of products formed during the course of the reaction; this could be used as a basis for calculating the number of cleavages in the original substrate per unit time. However, for routine assays, the amount of substrate removed was determined to calculate the activity of the enzyme.

### Table I

| Organism                        | Specific activitya |
|---------------------------------|-------------------|
| Synnechococcus sp.              | 45                |
| Sulfolobus acidocaldarius       | 22                |
| Pyrococcus islandicum           | 13                |
| Giardia duodenalis              | 6                 |
| Chlamydomonas sp.               | 5                 |
| Phaeodactylum tricornutum       | 46                |
| Saccharomyces cerevisiae        | 211               |
| Neurospora crassa               | 26                |
| Dictyostelium discoideum       | 17                |
| Caenorhabditis elegans          | 5                 |
| Drosophila melanogaster         | 13                |

a Standard error of 5% or less; units were corrected for the appearance of P_i in the assay presumably due to exopolyPase action.

### Table II

| Tissue | Specific activitya |
|--------|-------------------|
| Brain  | 13.7              |
| Heart  | 1.4               |
| Kidney | 1.3               |
| Lung   | 0.5               |
| Liver  | 0.4               |

a Standard error of 5% or less.

b Bovine brain had 9.8 × 10^3 units/mg.

### Table III

| Stage and day | Specific activitya |
|---------------|-------------------|
| Prenatal      | 27.5              |
| Postnatal     |                   |
| 3             | 14.2              |
| 15            | 7.7               |
| 35            | 4.8               |

a Standard error of 5% or less.

### Table IV

**Purification of PPN**

A paste of CRX cells was suspended in an equal volume of 50 mM Tris-HCl, pH 7.5, 10% sucrose, frozen in liquid nitrogen, and stored at ~80 °C. All subsequent steps were at 0–4 °C. Frozen paste (150 g) was thawed, mixed with an equal volume of 0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and sonicated in a Branson Sonifier in 100-ml aliquots (60% duty cycle, 70% power) for five 1-min pulses with 2-min cooling intervals. The sonicate was centrifuged at 30,100 × g for 30 min, made to 1 mM with MgCl_2, incubated at 30,100 × g for 1 h. The pellet was resuspended in 50 ml of buffer A (50 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol) and dialyzed against 4 liters of buffer A (three times for 2 h each). The dialysate (fraction II) was spun at 30,100 × g for 30 min, made to 1 mM with MgCl_2, incubated for 12 h on ice with DNase I and RNase A, each at 5 μg/ml, and applied to a 100-ml heparin-Sepharose CL-4B column equilibrated with buffer A. The column was washed sequentially with 2 bed volumes of buffer A and buffer B containing 0.6, 0.8, and 1 mM KCl. To the PPN activity, most abundant in the 0.8 mM KCl wash, was added cytochrome c to 0.1 mg/ml and concentrated in an Amicon ultrafiltration cell to 10 ml (fraction III). Fraction III was dialyzed against buffer A as described above and loaded on a phosphocellulose column equilibrated with buffer A. The column was washed with 2 bed volumes of buffer A followed by 2 bed volumes each of buffer A containing 0.8 mM sodium phosphate, 0.1 mM sodium pyrophosphate, 1 mM KCl, and 1 mM MgCl_2. To the PPN activity in the MgCl_2 eluate was added cytochrome c to 0.1 mg/ml and concentrated by ultrafiltration to 1 ml. The enzyme was dialyzed against 1 liter of buffer A (three times for 2 h each, fraction IV) and used for all further analyses.

| Fraction | Protein | Activity | Specific activity | Recovery | Purification |
|----------|---------|----------|------------------|----------|--------------|
|          | mg      | units × 10^−7 | units × 10^−3/mg | %        | fold         |
| I        | 1.092   | 19.0     | 170              | 100      | 21           |
| II       | 1.080   | 16.5     | 150              | 87       | 0.9          |
| III      | 6       | 9.0      | 15,000           | 48       | 87           |
| IV       | 0.072   | 4.3      | 600,000          | 23       | 3,490        |

a Standard error of 5% or less.
earity with respect to protein concentration and time. Inasmuch as widely varying sources were examined, the standard assay of PPN in each may not represent the true levels. Table I summarizes the results of the survey under the described assay conditions. Among euarchaota, only *Synechococcus* (a cyanobacterium), had PPN activity; none was detected in *E. coli*, *Bacillus subtilis*, and *Thermus aquaticus*. Among the archaea, species belonging to crenarcheota had moderate levels of activity while the euryarcheota tested (*Pyrococcus furiosus* and *Thermococcus litoralis*) had no detectable activity. All eu- karyotes tested, ranging from *Giardia*, a protozoan considered to be the earliest diverging member within the eukaryotic lineage (28), to bovine brain had significant amounts of PPN activity.

**Abundance in Rat Tissues**—PPN activity was found in all tissues tested (Table II). Brain had 10 times the specific activity of heart and kidney and 30 times that of lung and liver. Brain tissue from 15-day rat embryos had higher activity than that of developed brain (Table III). The crude brain enzyme appears to act by a mechanism similar to the action of the yeast PPN as judged by product distribution over a period of time after PPN action on polyP<sub>750</sub> (see Fig. 4, C and D).

**Purification of the Enzyme**—PPN was purified from *S. cerevisiae* CRX, a strain mutant in the *ppx* (exopolyPase) gene. Soluble enzyme was obtained only when the cells were broken by sonication; breakage with glass beads (29) or by Dounce homogenization of spheroplasts yielded preparations that were largely insoluble.<sup>2</sup> Removal of nucleic acids by DNase I and RNase A was essential for obtaining PPN as a single fraction from heparin-Sepharose (Table IV). Activity could not be eluted from a phosphocellulose column with increasing concentrations of phosphate or KCl up to 1 M; however, elution with 1 M MgCl<sub>2</sub> was effective in yielding a homogeneous protein as visualized on SDS-PAGE after silver staining (Fig. 1). In essence, two affinity columns led to a 3,500-fold purification. Cytochrome c was added to stabilize the enzyme when protein concentrations were less than 0.01 mg/ml. The molecular mass of denatured PPN was estimated to be 35 kDa by SDS-PAGE (Fig. 1); the native mass was judged to be 80 kDa by gel filtration on a TSK-Gel G2000SWXL high-performance liquid chromatography column (Fig. 2).

**Requirements for Activity**—The pH profile showed an optimum near 7.5 (Fig. 3A). Activity required a metal ion; 10 mM EDTA resulted in total inhibition. Mn<sup>2+</sup> was more active than Mg<sup>2+</sup> with optima near 2.5 mM (Fig. 3B); CaCl<sub>2</sub> and ZnCl<sub>2</sub> at 1 mM inhibited by 35%. NaF at 10 mM and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and NaCl, each up to 50 mM, had no effect on activity (data not shown). The addition of P<sub>1</sub> resulted in a 50% inhibition at 20 mM, and pyrophosphate at 10 mM inhibited completely (Fig. 3C). Inas-

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<sup>2</sup> P. Ramulu and A. Kornberg, unpublished observations.
much as lysine and arginine are present in large amounts in the yeast vacuole along with almost 99% of the cellular polyP, these amino acids were tested up to 100 mM but without any effect on activity (data not shown).

**Effect of Chain Length**—The enzyme activity was determined for various sizes of polyP chains used as substrates. Initial rates were plotted against a series of substrate concentrations for each chain length, and the $K_{m}/V_{max}$ was determined. No significant interpretation could be drawn from the $K_{m}/V_{max}$ values, which ranged from 20 to 50 min$^{-1}$ mM$^{-1}$. With lengths below 60 residues virtually no substrate was utilized (<1% at concentrations up to 12 mM). PPN did not hydrolyze polyP$_{60}$ at 15 mM and polyP of sizes 3, 4, and 5 at concentrations of 50 mM (in terms of polymer).

**Analysis of Reaction Products**—As observed on its action on polyP$_{750}$, PPN action was nonprocessive, giving rise to polyP of intermediate chain lengths throughout the course of the reaction. The products, characterized by electrophoresis on 6% polyacrylamide gel (Fig. 4), showed a progressive decrease in length along with an appearance of polyP$_{3}$ (Fig. 4A). The sequential increase in intermediate size products indicated a clear preference for the longer chains. Based on PhosphorImager data, hydrolysis virtually stopped when the chains reached about 60 residues. The ratio of polyP$_{3}$ products (≥60) to polyP$_{3}$ as polymer was rather constant with a value near 3 at each time point (Table V and Fig. 4, A and B). Thus, only one of four cleavages releases a large molecule of polyP, the other three release polyP$_{3}$ from the chain end. Additional polyP species of sizes between 3 and 60 made up ~10% of the total products. These could result from the random intramolecular cleavage of polyP chains during the course of PPN action.

**Localization of PPN**—PPN activity in extracts of *S. cerevisiae* CBX (17), a strain lacking vacuolar proteases (in addition to exopolyPase), was 20-fold lower than that found in extracts of CRX. PPN activities in vacuolar extracts prepared (30) from each of these strains showed a 14-fold enrichment in those from vacuoles compared with the whole cell lysates from CRX strains, suggestive of a vacuolar location for PPN. No difference in PPN activity was detected between vacuolar extracts and the whole cell lysates of protease-deficient mutant CBX.

**DISCUSSION**

Depolymerases of nucleic acids, proteins, and polysaccharides have proven to have important metabolic roles, acting internally (endo) or at the ends of chains (exo). With regard to inorganic polyP, exopolyPases have been identified and characterized in bacteria (6, 7) and yeast (8, 9), but not in animal cells. As for PPNs, little is known about them from any source. In our attempts to discover enzymes responsible for the synthesis of polyP in animal cells and tissues, we encountered instead formidable PPN activity. This activity was present in all rat tissues, particularly in brain (Table II); during fetal development the levels in brain were higher still (Table III). Brain tissues enriched in glial cells (spinal cord and optic nerve) were further enriched. Even though bovine brain could be used as a source material, the relatively low abundance of the PPN activity would make purification of the enzyme rather difficult.

A survey of microbial and other sources for PPN activity revealed a particularly high level in yeast (Table I); remarkably little or none was detected in *E. coli*, in which exopolyPase activities are abundant (6). For the PPN activity to be manifest in yeast, it is essential that strains possess vacuolar proteases, indicative of a need for proteolytic processing of the enzymes. For ease of assay and purification, a strain was chosen in which a potent exopolyPase was eliminated by mutation (17). The PPN activity, as judged by subcellular fractionation, was concentrated in the vacuoles, although purification of the enzyme proceeded with extracts prepared from sonication of the whole cell.

Purification of the PPN activity entailed a 3,500-fold enrichment with a 23% yield (Table IV). The procedure was based largely on affinity to polyanionic exchange resins: heparin-Sepharose and phosphocellulose. A noteworthy feature of the chromatography on phosphocellulose was the rather novel use of 1 M MgCl$_2$ as the eluant after a high concentration of a monovalent salt (1 M KCl) had failed. The final preparation appeared as a single polypeptide of 35 kDa on SDS-PAGE (Fig. 1) but gave a molecular mass of 80 kDa on gel filtration, indicating its possible existence as a dimer.

The divalent metal ion required for activity can be supplied by Mg$^{2+}$ at 5 mM or by Mn$^{2+}$ at a lower concentration (Fig. 3B); Ca$^{2+}$ and Zn$^{2+}$ were inhibitory. Both P$_i$ and PP$_i$ reduced activity (Fig. 3C); 50% decrease was observed with P$_i$ at 20 mM and with PP$_i$ at 2 mM. Sodium fluoride, ammonium sulfate, and sodium chloride did not influence activity, nor did arginine or lysine, regarded as vacuolar counterions (31).

The affinity of polyP$_{750}$ measured by $K_m$, was 185 nM. The $K_{cat}/K_m$ values were similar (between 20 and 50 min$^{-1}$ nm$^{-1}$) over a chain length range of 100–750 residues. No activity was detected with polyP$_{60}$, one of the two end products of the reaction. With regard to the course of the reaction, a nonprocessive mode of action produces chains of decreasing size until the final accumulation of predominantly polyP$_{60}$ and polyP$_{3}$ (Fig. 4A). Additional evidence supports the inability of PPN to hydrolyze polyP ≤ 60; no hydrolytic products were observed when polyP over a range of 3–60 were used as substrates. Kowalczyk and Wood (32) have reported that PPN activity in partially purified *S. cerevisiae* extracts decreased with decreasing chain lengths (range of 30–700 P$_i$ residues) and dramatically dropped with polyP of lengths below 100 residues. With every chain cleavage internally, it appears that three polyP$_{3}$ molecules are cleaved from the chain ends until a length of 60 residues is reached (Fig. 4B).

**Table V.** Products of PPN action

|         | 15 | 30 | 60 | 120 | 240 |
|---------|----|----|----|-----|-----|
| pmol (as polymer) |
| PolyP$_{60}$ | 3  | 6  | 8  | 15  | 39  |
| PolyP products (≥60 < 750) | 10 | 15 | 19 | 32  | 49  |
| PolyP$_{3}$ | 32 | 43 | 56 | 87  | 138 |
| Ratio of PolyP$_{3}$/PolyP products | 3  | 3  | 3  | 3   | 3   |
FIG. 4. Products of PPN action. The reaction was performed as described under “Experimental Procedures,” and the hydrolysis products electrophoresed on 6% urea-polyacrylamide gel. For reference, unlabeled polyP of various lengths were electrophoresed and stained with toluidene blue. Panel A, products of yeast PPN were determined by autoradiography of a 6% gel. Panel B, PhosphorImager analysis of the gel pattern with three chain lengths given for each lane as indicated. Panel C, autoradiogram of products of rat brain PPN. Panel D, PhosphorImager analysis of gel pattern as described for the yeast enzyme.
in the vacuole and other cellular locations. Were the fate of polyP hydrolysis by PPN simply to furnish P_i, then the polyP_60 and polyP_3 end products could be degraded further by known exopolyPases (8, 9) and tripolyPase (34). Were there specific functions assigned to these products, few clues exist from studies of other cells and known from the yeast cell. Clearly, more information is needed about the metabolism of a polymer that in yeast may account for 20% of its dry weight (20) and the vast bulk of its phosphate.

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