Polyphosphate Kinase: Distribution, Some Properties and Its Application as an ATP Regeneration System

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The distribution and some properties of polyphosphate kinase, that catalyzes the formation of polyphosphate from ATP, were investigated. High enzyme activity was found in *Alcaligenes faecalis*, *Brevibacterium ammoniagenes*, *Escherichia coli*, *Micrococcus lysodeikticus* and *Pseudomonas aeruginosa*. The enzyme required Mg^{2+} for maximum activity and was activated by basic proteins, polyamines and phosphate polymers of low molecular weight. The enzyme from *E. coli* B could catalyze the reverse reaction, and generated ATP from ADP and metaphosphate. The feasibility of the generation of ATP by the *E. coli* B enzyme was confirmed by means of the coupled reactions of polyphosphate kinase and hexokinase, which produces glucose-6-phosphate.

We have been studying phosphate polymer-utilizing enzymes in microbial cells and have found that such enzymes are frequently found in cells of *Achromobacter*, *Brevibacterium* and *Micrococcus* species.^{1,2)} Polyphosphate glucokinase in *Achromobacter butyri* was used for the production of glucose-6-phosphate (G-6-P) from glucose and metaphosphate.³⁾ Metaphosphate-dependent nicotinamide adenine dinucleotide (NAD) kinase in *Brevibacterium ammoniagenes*⁴⁾ was also useful for the production of nicotinamide adenine dinucleotide phosphate (NADP) from NAD and metaphosphate.⁵⁾ The activities of polyphosphate fructokinase, polyphosphate manokinase and enzymes catalyzing the phosphorylation of purine and pyrimidine nucleosides to the corresponding nucleotides using metaphosphate as a phosphoryl donor were also detected in cell extracts prepared from *Enterobacter aerogenes*, *Brevibacterium ammoniagenes*, *Micrococcus lysodeikticus* and other strains (Murata *et al.*, unpublished data).

Other than these phosphate polymer-utilizing enzymes, microorganisms contain

polyphosphate kinase, that catalyzes the formation of highly polymerized phosphates from the terminal phosphate of adenosine-5'-triphosphate (ATP) (Eq. I). This enzyme activity has been found in various microbial strains such as *Escherichia coli*,⁶⁾ *Corynebacterium xerosis*⁷⁾ and *Enterobacter aerogenes*.⁸⁾ Although the reaction (Eq. I) is probably a

biosynthetic route, the enzyme in *E. coli* has been shown to catalyze the phosphorylation of adenosine-5'-diphosphate (ADP) using phosphate polymers⁶⁾ (Eq. II). The results suggested that

the polyphosphate kinase may be applicable to a bioreactor system as an ATP regeneration system, since substrates (phosphate polymers) for the enzyme are readily available at low cost.

To determine the feasibility of the polyphosphate kinase reaction as an ATP regeneration system, we investigated the distribution and

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some properties of the enzyme in micro-organisms.

MATERIALS AND METHODS

Preparation of cell extracts. All microbial cells were grown in a medium containing 0.5% glucose, 1.0% yeast extract, 1.0% polypeptone, 0.2% meat extract, 0.5% KH_2PO_4 and 0.5% NaCl (pH 7.2). The cultures were reciprocally shaken at 30°C for 16 hr in 100 ml medium in a 2 l Sakaguchi flask. The harvested cells were washed once with a 0.85% saline solution and then resuspended in 5.0 mM potassium phosphate buffer (pH 7.2) containing 0.5 mM sodium deoxycholate. The cell suspension was ultrasonically treated at 90 kHz for 5 min in a Kubota Model 200M Insonator and then the supernatant obtained on centrifugation at $25,000 \times g$ for 40 min was dialyzed against 5.0 mM potassium phosphate buffer (pH 7.2) at 4°C overnight. Protein was determined by the method of Lowry *et al.*⁹⁾

Partial purification of polyphosphate kinase from E. coli B. To a cell suspension (25 ml, 0.5 g cells/ml) of *E. coli* B, 2.0 mg lysozyme was added. After incubation at 37°C for 30 min, 0.1 mg DNase and 0.05 mg RNase were added successively to the mixture and then the incubation was continued for a further 30 min. To the mixture was then added 1.0 mg streptomycin sulfate, and the precipitated material was collected by centrifugation at $25,000 \times g$ for 30 min. The precipitate was dissolved in 5.0 mM potassium phosphate buffer (pH 7.2) (25 ml, 2.1 mg/ml protein) and then fractionated with ammonium sulfate. Solid ammonium sulfate (7 g) was added to the streptomycin sulfate fraction, followed by incubation at 0°C for 30 min. The precipitate was obtained by centrifugation at $25,000 \times g$ for 30 min, dissolved in 2.5 ml of 5.0 mM potassium phosphate buffer (pH 7.2) and then dialyzed against the same buffer at 4°C overnight. The dialysate thus obtained (3.2 ml, 2.4 mg protein) was used as the source of polyphosphate kinase throughout this study. Through the purification steps described above, an approximately 15-fold increase in the specific activity of polyphosphate kinase was attained.

Column chromatography of metaphosphate. The components in metaphosphate were fractionated by Dowex 1 $\times$ 2 (Cl^-) column chromatography as described previously.¹⁾ The metaphosphate solution (10%, pH 6.5) was applied to the column (5 cm $\times$ 30 cm) and then the column was extensively washed with water. The absorbed phosphate polymers were then eluted with a linear gradient of LiCl, the concentration increasing from 0 (400 ml) to 1.0 M (400 ml) (pH 7.2). Fractions were collected at 7 ml/tube/5 min at room temperature. The active fractions (Nos. 48 ~ 52), as a phosphoryl donor for polyphosphate kinase in *E. coli* B, were pooled (35 ml) and designated the "S1

fraction." The S1 fraction contained 25 mM acid labile phosphate, which was estimated by determining the inorganic phosphate concentration after hydrolysis of the phosphate polymers in 1.0 N HCl at 100°C for 7 min. Inorganic phosphate was determined by the method of Fiske and Subbarow.¹⁰⁾

Assay for polyphosphate kinase. The activity as to the forward reaction (Eq. I) of this enzyme was assayed in a reaction mixture (0.1 ml) containing 0.2 μmol MgCl_2 , 100 μg protamine, 0.04 μmol $\gamma\text{-}^{32}\text{P}\text{-ATP}$ (0.4 μCi), 5.0 μmol potassium phosphate buffer (pH 7.2) and cell extract (10 μg as protein). The reaction was carried out at 30°C for 10 min and then aliquots (20 μl) of the reaction mixture were spotted onto 2 cm squares of Toyo Filter Paper (No. 51). Chromatography was conducted at room temperature by the descending method with 10% trichloroacetic acid (TCA) as a solvent. After development, the paper was dried and then the origins were cut out in 2 cm squares for radioactivity determination. The activity as to the reverse reaction (Eq. II) of this enzyme was determined in a reaction mixture (0.1 ml) containing 2.0 μmol (as acid labile phosphate) S1 fraction, 0.5 μmol MgCl_2 , 0.2 μmol ADP, 5.0 μmol potassium phosphate buffer (pH 7.2) and the partially purified polyphosphate kinase (10 μg as protein) from *E. coli* B. After incubation at 30°C for several hours, the reaction was terminated by immersing the test tube in boiling water for 1 min, and then ATP in the supernatant was determined by means of the Luciferase-Luciferin assay.¹¹⁾

Coupled reactions of polyphosphate kinase and hexokinase. To produce G-6-P from glucose, the ATP generation reaction (reverse reaction) catalyzed by polyphosphate kinase was coupled with the hexokinase reaction. The reaction mixture (0.1 ml), consisting of 2.0 μmol ADP, 5.0 μmol glucose, 2.0 μmol (as acid labile phosphate) S1 fraction, 0.5 μmol MgCl_2 , 5.0 μmol potassium phosphate buffer (pH 7.2), 5.0 units of hexokinase and 10 μg (as protein) of the partially purified polyphosphate kinase from *E. coli* B, was incubated at 30°C for several hours. The reaction was terminated by boiling at 100°C for 1 min and then G-6-P in the supernatant was determined enzymatically by the method of Hohorst.¹²⁾

Chemicals. Polyphosphate and metaphosphate were purchased from Katayama Chemicals Industry, Co., Ltd., Osaka, Japan. Ribonuclease, deoxyribonuclease, trimetaphosphate and tetrametaphosphate were from Sigma Chemical Co., St. Louis, MO. $\gamma\text{-}^{32}\text{P}\text{-Adenosine } 5'\text{-triphosphate}$ was purchased from New England Nuclear, Mass.

RESULTS

Assay conditions for polyphosphate kinase

The assay conditions for polyphosphate ki-

FIG. 1. Chromatographic Separation of ^{32}P -ATP from ^{32}P -polyphosphate.

A: A chromatogram of a 20 μl aliquot of the polyphosphate kinase reaction mixture incubated at 30°C for 10 min. B: A chromatogram of the corresponding control using the boiled enzyme solution. C: A chromatogram of the reaction mixture incubated in the absence of Mg^{2+} . After development, the chromatographic paper was dried and then cut into 1 cm sections except for the origins which were cut out in 2 cm squares for radioactivity determination. Other conditions for the reaction and chromatography are given under MATERIALS AND METHODS.

nase activity (Eq. 1: forward reaction) were investigated using cell extract of *Arthrobacter atrocyaneus* ATCC13752 showing polyphosphate kinase activity. Figure 1 shows the chromatographic patterns of the reaction products. In the complete system containing ATP, Mg^{2+} and protamine (Fig. 1A), a large amount of radioactivity was found at the origin. On the other hand, the radioactivity at the origin was low when the boiled extract was used (Fig. 1B). Omission of Mg^{2+} from the reaction mixture resulted in a decrease in the radioactivity at the origin (Fig. 1C). In the complete system (Fig. 1A), the radioactivity at the origin increased with reaction time (up to 10 min) and protein concentration (up to 0.3 mg/ml).

The reaction mixture for the complete system was chromatographed with Ebel's solvent.¹³⁾ Radioactivity was again found at the origin, as was observed in the case of development with 10% TCA. On the chromatogram, intermediate products of phosphate polymers of low molecular weight were not detected during the reaction (Fig. 2A). The reaction mixture for the complete system was treated with 1.0N HCl for 7 min at 100°C and then chromatographed with Ebel's solvent (Fig.

FIG. 2. Chromatographic Analysis of the Reaction Products.

The polyphosphate kinase reaction (forward) was carried out as described under MATERIALS AND METHODS. A: An aliquot (10 μl) of the reaction mixture was spotted on to Toyo Filter Paper (No. 51) and then developed with Ebel's solvent at room temperature. Ebel's solvent contained 350 ml *iso*-propanol, 20 g TCA, 2.5 ml 25% NH_4OH and 150 ml of water. The chromatogram (ascending) was developed for approximately 20 hr. To locate the positions of authentic phosphate compounds, the chromatographic papers were sprayed with acid molybdate solution, heated at 80°C for 10 min and then exposed to ultraviolet radiation. For the determination of radioactivity, the chromatographic papers were dried and cut into 0.5 cm sections, and then the radioactivity in each section was determined. B: An aliquot (40 μl) of the reaction mixture was diluted with an equal volume of 2.0N HCl, and then the mixture was incubated at 100°C for 7 min. An aliquot (40 μl) of the sample after treatment was spotted, chromatographed and then analysed under the same conditions as above. The arrows in A and B indicate the positions of (a) orthophosphate, (b) pyrophosphate and (c) triphosphate.

2B). With this treatment, the radioactivity at the origin disappeared and was found at the same position as that of orthophosphate.

Effects of basic proteins and polyamines

The effects of various proteins and polyamines on the polyphosphate kinase reaction were investigated (Table I). Of the proteins tested, basic proteins such as protamine and histamine stimulated the enzyme reaction. Polyamines such as putrescine, spermine and spermidine were also effective as to stimulation of the polyphosphate kinase reaction.

TABLE I. EFFECTS OF BASIC PROTEINS AND POLYAMINES ON THE POLYPHOSPHATE KINASE ACTIVITY OF *A. atrocyaneus*

The forward reaction was carried out in the presence of basic proteins or polyamines. The assay conditions are given under MATERIALS AND METHODS. The relative activity is given in parenthesis.

Addition	Concentration	Activity ($\times 10^{-3}$ cpm)
None		1.82 (1.00)
Protamine	1.0 mg/ml	2.96 (1.63)
Histamine	1.0 mg/ml	2.47 (1.36)
Casein	1.0 mg/ml	1.77 (0.97)
Bovine serum albumin	1.0 mg/ml	1.68 (0.92)
Putrescine	1.0 mM	2.76 (1.52)
Spermine	1.0 mM	2.91 (1.60)
Spermidine	1.0 mM	3.10 (1.70)

Effects of phosphate polymers

The effects of phosphate polymers of low molecular weight were tested as to their ability to act as "templates" for polyphosphate synthesis. Of the phosphate polymers tested, tetrapolyphosphate, trimetaphosphate and tetrametaphosphate significantly increased the polyphosphate synthesis (Table II).

Distribution of polyphosphate kinase in bacteria

To select the most suitable strains for the application of the polyphosphate kinase reaction as an ATP regeneration system, the distribution of the enzyme activity among bacteria was investigated (Table III). The enzyme activity (Eq. I: forward reaction) was found in all the bacterial strains tested, being higher in *A. faecalis*, *B. ammoniagenes*, *E. coli* B, *M. lysodeikticus* and *P. aeruginosa*. The reverse reaction (Eq. II) of the enzyme was investigated among the strains listed in Table III, but only *E. coli* B cell extract showed detectable activity as to the formation of ATP from ADP and the S1 fraction. *E. coli* B was selected as the source of polyphosphate kinase for ATP regeneration.

Phosphoryl donor in metaphosphate

Metaphosphate is a mixture of various ring phosphate polymers with different chain lengths. To obtain the intrinsic phosphoryl

TABLE II. EFFECTS OF PHOSPHATE POLYMERS ON THE POLYPHOSPHATE KINASE ACTIVITY OF *A. atrocyaneus*

The forward reaction was carried out in the presence of various phosphate polymers. The relative activity is given in parenthesis.

Phosphate polymer	mm	Activity ($\times 10^{-3}$ cpm)
None		2.12 (1.00)
Pyrophosphate	1.0	2.20 (1.04)
	3.0	2.31 (1.09)
	5.0	0.91 (0.54)
Triphosphate	0.1	2.22 (1.05)
	0.3	3.11 (1.47)
	0.5	2.05 (0.97)
Tetraphosphate	0.1	5.81 (2.74)
	0.3	6.25 (2.95)
	0.5	3.62 (1.71)
Trimetaphosphate	0.01	1.90 (0.98)
	0.03	3.50 (1.65)
	0.10	2.83 (1.35)
Tetrametaphosphate	0.1	4.21 (1.99)
	0.3	5.12 (2.42)
	0.5	4.65 (2.19)

TABLE III. DISTRIBUTION OF POLYPHOSPHATE KINASE ACTIVITY AMONG BACTERIA

The forward reaction was carried out using crude cell extracts, as described under MATERIALS AND METHODS. Activity is expressed as nmole of 32 P incorporated per mg of protein per min. Other reaction conditions are given under MATERIALS AND METHODS.

Strain	Activity
<i>Acetobacter xylinum</i>	2.01
<i>Achromobacter butyri</i>	1.84
<i>Alcaligenes faecalis</i>	13.2
<i>Arthrobacter atrocyaneus</i>	1.32
<i>Bacillus subtilis</i>	1.30
<i>Brevibacterium ammoniagenes</i>	6.10
<i>Brevibacterium flavum</i>	3.21
<i>Corynebacterium sepeidonicum</i>	1.30
<i>Enterobacter aerogenes</i>	2.77
<i>Escherichia coli</i> B	7.20
<i>Flavobacterium arborescens</i>	0.11
<i>Micrococcus flavus</i>	4.30
<i>Micrococcus lysodeikticus</i>	11.4
<i>Pseudomonas aeruginosa</i>	10.2
<i>Proteus mirabilis</i>	0.23
<i>Serratia marcescens</i>	0.42

donor in metaphosphate for the polyphosphate kinase reaction, metaphosphate was fractionated by column chromatography and

FIG. 3. Elution Pattern of the Phosphoryl Donor in Metaphosphate.

A 10% metaphosphate solution (pH 6.5) was applied to a Dowex 1 $\times$ 2 (Cl^-) column and then eluted with a linear LiCl gradient as described under MATERIALS AND METHODS. The phosphoryl donor activity in each fraction was determined using the partially purified polyphosphate kinase from *E. coli* B. ●, phosphoryl donor activity; ○, inorganic phosphate; ○, acid-labile phosphate; ---, LiCl concentration.

the phosphoryl donor activity in each fraction was determined using the partially purified polyphosphate kinase of *E. coli* B (Fig. 3). Only one active peak was eluted, with 0.5M LiCl. The active fractions were pooled and used as the substrate for polyphosphate kinase, as described under MATERIALS AND METHODS. This substrate fraction, designated as the "S1 fraction," contained 25mM acid-labile phosphate, and a blue color was produced when the S1 fraction was mixed with Fiske-Subbarow reagent, indicating that the phosphate polymer in the S1 fraction is a ring phosphate polymer, since ring phosphate polymers react with Fiske-Subbarow reagent to give a blue color.¹⁾

ATP regeneration through the polyphosphate kinase reaction

To regenerate ATP from ADP and phosphate polymers, the partially purified polyphosphate kinase from *E. coli* B was incubated with ADP in the presence or absence of the S1 fraction. Although the reaction was not stoichiometric, ATP was found to be synthesized in association with the degradation of phosphate polymers in the S1 fraction (Fig. 4A), which was indicated by the decrease in acid-labile phosphate during the

FIG. 4. ATP Regeneration through the Polyphosphate Kinase Reaction and Its Application to G-6-P Production.

A: The ATP regeneration reaction was carried out in the absence (---) and presence (—) of the S1 fraction as described under MATERIALS AND METHODS. At prescribed times, an aliquot of the reaction mixture was taken, and then the concentrations of ATP (●) and acid-labile phosphate (○) were determined. B: The ATP regeneration reaction above was coupled with the hexokinase reaction, and then G-6-P produced in the absence or presence of the S1 fraction was determined. The concentration of the S1 fraction was 0 (○), 5 (●) and 10 (◐) mM. Other conditions for the reactions in A and B were as described under MATERIALS AND METHODS.

reaction. The ATP formation observed in the absence of the S1 fraction was due to the adenylate kinase activity contaminating in the polyphosphate kinase preparation.

The polyphosphate kinase (partially purified) reaction, as an ATP regeneration system, was coupled with the hexokinase reaction for the production of G-6-P (Fig. 4B). These two kinds of reactions were coupled well, and G-6-P was found to be produced using ATP regenerated from ADP and the S1 fraction through the polyphosphate kinase reaction. G-6-P formation in the absence of the S1 fraction was presumably due to the adenylate kinase reaction, as mentioned above. The partially purified polyphosphate kinase preparation showed no polyphosphate glucokinase activity, which phosphorylates glucose to G-6-P using metaphosphate.¹⁾

DISCUSSION

The polyphosphate kinase catalyzing the polymerization of phosphorus was found in all the bacterial strains listed in Table III, and *E.*

coli B was selected as the source of the enzyme, since the enzyme in this organism could catalyze the reverse reaction (Eq. II: ATP-synthesizing activity, from ADP and phosphate polymers). The products of the enzyme reaction were established to be phosphate polymers from the paper-chromatographic mobility and from the generation of orthophosphates on hydrolysis (Fig. 2A, B).

The *E. coli* B enzyme reaction was stimulated in the presence of basic proteins, polyamines and phosphate polymers of low molecular weight (Tables I and II). Basic proteins and polyamines can bind to phosphate polymers and may protect the phosphate polymers from attack by the phosphate polymers from attack by polyphosphate-degrading enzymes. The interaction of phosphate polymers with basic proteins or polyamines is of interest as to the physiological significance of phosphate polymers. A recent study of Offenbacher and Kline provided evidence that certain nuclear histone (basic) proteins (NHPs) are strongly and covalently associated with phosphate polymers.¹⁴⁾ Furthermore, a previous investigation demonstrated that free polyphosphate causes destabilization of chromatin and enhancement of transcription *in vitro*.¹⁵⁾ Therefore, the existence of polyphosphate-containing NHPs may indicate the roles of polyphosphates as positive modifier in gene expression.

The phosphate polymers of low molecular weight also stimulated the polyphosphate kinase reaction. This was presumably due to the "template effect" of these phosphate polymers on polyphosphate synthesis. It has been suggested that phosphate polymers may be synthesized on a "template," which might be an enzyme surface or some other macromolecule such as RNA.¹⁴⁾ The high energy phosphates could become attached to the "template" surface and fairly large molecules synthesized without any intermediates being formed. This is similar to the mechanism proposed for the synthesis of proteins from amino acids and polynucleotides. This would explain the inability to detect any soluble intermediates dur-

ing the synthesis of the high molecular weight phosphate polymers (Fig. 2A).

The structure and molecular size of the intrinsic phosphoryl donor in metaphosphate is of great interest as to the reverse reaction (Eq. II) of polyphosphate kinase. Chromatographic separation of metaphosphate showed the existence of only one substrate (phosphoryl donor) for the reverse reaction of polyphosphate kinase (Fig. 3). This substrate, which was eluted with 0.5 M LiCl, seemed to be different from those for polyphosphate glucokinase and metaphosphate-dependent NAD kinase. In case of polyphosphate glucokinase, at least three kinds of phosphoryl donors were found in metaphosphate, and they were all very unstable.¹⁾ On the other hand, only one kind of phosphoryl donor was found for NAD kinase, which was eluted with 0.1 M LiCl from Dowex 1 $\times$ 2 (Cl⁻) column (Murata *et al.*, unpublished data). It is, therefore, striking that each enzyme could use phosphate polymers by discriminating the degree of condensation of phosphorus, although nothing is known of the shape of the polyphosphate molecule in solution, nor of its tendency to form aggregates.

The reaction of polyphosphate kinase in *E. coli* B was coupled with the hexokinase reaction for the production of G-6-P. The ATP regeneration reaction catalyzed by polyphosphate kinase worked well when both the partially purified enzyme and the substrate-enriched S1 fraction were used (Fig. 4). Therefore, the polyphosphate kinase reaction seems to be promising as an ATP regeneration system for the production of useful compounds. However, for the utilization of the polyphosphate kinase reaction for practical processes, partial purification of the enzyme and isolation of the intrinsic phosphoryl donor in metaphosphate seems to be indispensable, since the intracellular activity of the enzyme and the phosphoryl donor content of metaphosphate are extremely low.

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