Purification and Characterization of a Polyphosphate Kinase from *Arthrobacter atrocyaneus*

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SUMMARY

Polyphosphate kinase, an enzyme which incorporated the γ -phosphate of ATP into long-chain polyphosphate molecules, was purified more than 700-fold from *Arthrobacter atrocyaneus* by ammonium sulphate fractionation, DEAE-cellulose column chromatography and Sephadex G-200 gel filtration. The enzyme had a broad pH optimum at 6.0 to 7.0 and required Mn^{2+} or Mg^{2+} , histone, and inorganic phosphate for activity. The K_m for Mn-ATP was 0.53 mM, and for inorganic phosphate was 1.67 mM.

Free ATP concentrations greater than 8 μM inhibited the enzyme. Free Mn^{2+} or Mg^{2+} concentrations greater than 2 mM or 6 mM, respectively, were also inhibitory. Activity was strongly inhibited by 4 mM-ADP, 1 mM-PP_i or 20 mM-NaF. The effect of ADP might have resulted from reversing the equilibrium of the kinase reaction. The activation by phosphate ions might indicate a role for the enzyme in regulating intracellular phosphate levels or maintaining a phosphorus reserve. The level of enzymic activity in the bacteria responded to changes in inorganic phosphate concentration in the medium. Basic proteins, such as protamine, could substitute for histone as activator. Proteins such as casein or bovine serum albumin would also substitute for histone but only in the absence of inorganic phosphate. The presence of a protein might be necessary to form a complex with the product, thus preventing reversal of the reaction *in vitro*.

The reaction product was characterized, and found to be labile in hydroxylamine, base, and acid at 100 °C. It behaved as a long-chain-polyphosphate molecule on chromatography in an Ebel's solvent. The enzymic activity was therefore not that of a protein kinase.

INTRODUCTION

Long-chain polyphosphate was detected in many species of micro-organisms and in some higher plants and animals (Harold, 1966). An enzyme responsible for the formation of this compound, polyphosphate kinase, was detected in yeast (Yoshida & Yamataka, 1953), *Escherichia coli* (Kornberg, Kornberg & Simms, 1956; Li & Brown, 1973) and various other micro-organisms. We have achieved a greater than 700-fold purification of a polyphosphate kinase from the bacterium *Arthrobacter atrocyaneus*. The enzyme from *A. atrocyaneus*, like that from *E. coli* (Li & Brown, 1973), required both histone and inorganic phosphate for activity.

Because of its dependence on histone, the activity was initially thought to be a protein kinase (Schechter, Li & Krulwich, 1972). However, characterizations of the product as well as the enzyme have shown that the latter was a histone-dependent polyphosphate

kinase, which incorporated the γ -phosphate of ATP into long-chain polyphosphate molecules.

METHODS

Organism and growth conditions. *Arthrobacter atrocyaneus* (ATCC13752) was grown on minimal medium MS (Krulwich *et al.* 1967) supplemented with 0.05 M-sodium glutamate as carbon source. For isolation of the polyphosphate kinase, 16 l of culture were grown to the late exponential phase, in carboys, from a 100 ml inoculum, with vigorous aeration, at 30 °C. Phosphate-free medium for physiological studies contained 25 mM-tris pH 7.2, 0.2 % ammonium sulphate, 0.02 % MgSO_4 , plus trace salts (Krulwich *et al.* 1967).

Preparation of crude extracts. Bacteria were harvested by centrifuging, washed with 10 mM-tris pH 7.2, and then resuspended in this buffer. The organisms were disrupted by sonication using a Heat Systems-Ultrasonics W185D sonifier for a total of 2 min. The temperature was kept below 10 °C. The extract thus produced was then treated with DNAase (3 mg/ml) and RNAase (3 mg/ml) for 1 h at 5 °C. The debris was removed by centrifuging for 10 min at 16000 g. When the extract was to be used for physiological studies, DNAase and RNAase treatments were omitted, and extracts were dialysed against 10 mM-tris pH 7.2 for 15 h with one change after centrifuging.

Enzyme assays. The activity of the polyphosphate kinase was determined at 30 °C in a mixture containing (except where noted): 6 mM-potassium phosphate buffer pH 7.0; 2 mM- MnCl_2 ; histone type II from calf thymus (1 mg/ml); 0.4 mM [γ - ^{32}P]ATP (7 to 28 d.p.m./pmol); and 8.8 μg enzyme/ml, in a total volume of 0.1 ml. [^{32}P]polyphosphate was determined by the paper chromatographic procedure of Li & Felmlly (1973), except that the paper strips were not dried before elution and the solvent used was cold 10 % (w/v) TCA. One unit of activity is equivalent to that amount of enzyme which catalyses the incorporation of one nmol [γ - ^{32}P]ATP into product/min. Activity of RNA polymerase was determined by the method of Bonner *et al.* (1968). Protein was determined by the method of Lowry *et al.* (1951).

Chemicals. ^{32}P -inorganic phosphate and [γ - ^{32}P]ATP were purchased from New England Nuclear Corp. Histone type II, protamine, casein, and bovine serum albumin were purchased from Sigma. Sephadex G-200 was purchased from Pharmacia, and DEAE-cellulose from Bio-Rad. All other chemicals were of the highest quality commercially available.

RESULTS

Purification of the enzyme. The crude extract, prepared as described in Methods, was fractionated using ammonium sulphate, which was added to 45 % of saturation with stirring at 0 to 5 °C. The precipitated protein was centrifuged, dissolved in 5 ml of 10 mM-tris buffer pH 7.2 containing 1 mM-EDTA, and was then dialysed overnight at 5 °C against 2 l of the same buffer with one change. The ammonium sulphate fraction was then applied to a DEAE-cellulose column (1 $\times$ 25 cm) equilibrated with 5 mM-potassium phosphate buffer pH 7.0. The protein was eluted by 500 ml of a linear gradient from 5 mM- to 500 mM-potassium phosphate pH 7.0, containing 1 mM-EDTA, 10 mM-dithiothreitol, and 10 % (w/v) glycerol (Fig. 1). The active fractions (10 ml), which were eluted at approximately 0.22 M-potassium phosphate, were pooled and concentrated by the addition of ammonium sulphate to 45 % of saturation. The precipitate collected was dissolved in 1 ml of 5 mM-potassium phosphate containing 1 mM-EDTA, 10 mM-dithiothreitol and 10 % (w/v) glycerol, and dialysed against 1 l of this buffer overnight. The preparation was

Fig. 1. Elution of polyphosphate kinase from a DEAE-cellulose column. The enzyme fraction (5 ml) obtained after ammonium sulphate precipitation was applied to a DEAE-cellulose column (1×24 cm) equilibrated with 50 mM-potassium phosphate pH 7.0, and was eluted with a linear gradient from 5 mM- to 500 mM-potassium phosphate pH 7.0, containing 1 mM-EDTA, 10 μ M-dithiothreitol, and 10 % (w/v) glycerol at 5 °C. Fractions were assayed as described in Methods. $\circ$, Specific activity; $\bullet$, E_{280} ; —, potassium phosphate concentration.

Table 1. *Purification of polyphosphate kinase*

The purification procedure is described in detail in Results. The preparations were assayed as described in Methods. Recoveries of enzyme are not quoted because of transient stimulations that were seen at some steps.

Purification step	Units/ml	Protein (mg/ml)	Specific activity (nmol/mg/min)	Purification factor
Sonication of crude extract	6.5	5.9	1.1	1
45 % ammonium sulphate precipitation	77.6	6.4	12.1	11
DEAE-cellulose chromatography	33.2	0.3	99.3	89
Ammonium sulphate precipitation	790.6	1.7	472.0	425
Sephadex G-200 chromatography	72.1	0.09	818.8	738

then applied to a Sephadex G-200 column (2.5×40 cm) equilibrated with the same buffer used for dialysis. A sharp peak of activity was eluted approximately at the external void volume (data not shown). The purification procedure is summarized in Table 1. The fractions eluted from the Sephadex G-200 column were used for all subsequent experiments. The enzyme preparation was stable at 5 °C for at least six months.

Characterization of the enzyme. The rate of formation of product remained linear for 15 min (Fig. 2). The enzyme exhibited a broad pH optimum at 6.0 to 7.0 (Fig. 3). The enzyme preparation was free from RNA polymerase activity, although McConnell & Bonner (1972) have reported that the two enzymes from *E. coli* may be purified together.

The polyphosphate kinase apparently required a metal ion-ATP complex as substrate rather than free ATP. Either Mg-ATP^{2-} or Mn-ATP^{2-} was able to serve as substrate.

Fig. 2.

Fig. 3.

Fig. 2. Time course of the polyphosphate kinase reaction. The enzyme was assayed as described in Methods except that a final volume of 0.2 ml was employed. Samples of reaction mixture were removed at appropriate times and radioactively labelled product was determined as described.

Fig. 3. Dependence of the polyphosphate kinase reaction on pH. The assay was performed in a total volume of 0.1 ml, containing histone type II (1 mg/ml), 2 mM-MnCl₂, 6 mM-potassium phosphate pH 7.0, 20 μl water, 0.4 mM-[³²P]ATP and 50 mM-tris-maleate-acetate buffer pH 5 to 9.6. This mixture was incubated for 10 min at 30 °C and radioactively labelled product was determined as described in Methods.

Values could be calculated for the concentrations in our assay of the dissociated components of the complex, Mn²⁺ and ATP, by using the stability constant 56230 M⁻¹ for the Mn-ATP²⁻ complex as determined by Walaas (1958). At high concentrations of Mn-ATP²⁻ complex, i.e. greater than 0.6 mM, the enzyme was inhibited (data not shown). It could be calculated that levels of free ATP (i.e. not in complex with Mn²⁺) of greater than 8 μM caused inhibition of the enzyme.

When the levels of free Mn²⁺ were varied while the Mn-ATP²⁻ complex was maintained at a constant level and the free ATP concentrations were maintained below inhibitory levels, it could be shown that free Mn²⁺ also inhibited enzymic activity (Fig. 4) at concentrations greater than 2 mM. Optimal activity was observed in the presence of 1.0 to 2.0 mM-Mn²⁺. At non-inhibitory concentrations of free ATP and Mn²⁺, a *K_m* of 0.53 mM was calculated for Mn-ATP²⁻. Similar experiments with Mg-ATP²⁻ as substrate gave comparable results.

Phosphate ions were required for enzymic activity. Activity increased with increasing phosphate concentration up to approximately 5 mM (Fig. 5). A *K_m* of 1.67 mM was calculated. Radioactively labelled phosphate was not incorporated into the product.

The enzyme was strongly inhibited by ADP, one of the reaction products. When 4 mM-ADP was added at zero time or after 10 min, the reaction was stopped (Fig. 6). Also shown in Fig. 6 are the inhibitory effects of 1 mM-PP_i or 20 mM-NaF, which are similar to effects reported in *E. coli* (Kornberg *et al.* 1956; Li & Brown, 1973).

The enzyme also required histone for activity (Fig. 7). Other proteins, such as protamine, could substitute for histone to a lesser degree. Bovine serum albumin and casein could also substitute for histone. However, in contrast to the activity observed with basic

Fig. 4.

Fig. 4. Dependence of the polyphosphate kinase reaction on the MnCl_2 concentration. Assays were performed as described in Methods except that the concentration of MnCl_2 was varied. Values for Mn^{2+} , ATP, and Mn-ATP^{2-} were determined from the dissociation constant for the Mn-ATP^{2-} complex. $\circ$, Specific activity; $\bullet$, ATP concentration; Δ , Mn-ATP^{2-} concentration.

Fig. 5.

Fig. 5. Dependence of the polyphosphate kinase reaction on the presence of inorganic phosphate. Assays were performed as described in Methods except that the inorganic phosphate concentration was varied from 0 to 10 mM. The reciprocal plot gave a K_m of 1.67 mM.

proteins, enzymic activity in the presence of these two proteins could be detected only in the absence of inorganic phosphate (Table 2). The requirement for a protein, such as histone, might result in mistaken identification of the enzyme as a protein kinase. This possibility was eliminated for the enzyme from *A. atrocyaneus* by the characterization of the product.

Characterization of the reaction product. Determination of the chemical nature of the product was based on the known properties of the various phosphorylated protein residues and polyphosphate when treated by certain methods. It was initially thought that the reaction product might be either a phosphorylated protein, containing residues of acyl, serine, or imidazole phosphate, or a long-chain polyphosphate. Serine phosphate ester bonds are stable to hydroxylamine treatment, but labile in acid at 100 °C and in base (Kabat, 1970; Agabian, Rosen & Shapiro, 1972). Imidazole phosphate bonds are stable in base but labile in cold acid (Kundig & Roseman, 1971). Acyl phosphate bonds are labile to treatment with NH_2OH , base or acid at 100 °C (Martonosi, 1969; Hokin, Sastry, Galsworthy & Yoda, 1965). Long-chain polyphosphate, having anhydride bonds like the acyl phosphate, would be broken down to inorganic phosphate in acid at 100 °C, in base at 40 °C, and in hydroxylamine.

To obtain the product for characterization, the reaction was conducted for 1 h and stopped by the addition of a solution of TCA to give a final concentration of 10% (w/v). The precipitate was centrifuged, resuspended in 1 ml of 5 mM-potassium phosphate pH 7.0,

Fig. 6.

Fig. 7.

Fig. 6. Inhibition of the polyphosphate kinase reaction by ADP, NaF, and pyrophosphate. Assays were performed as described in Methods in a volume of 0.2 ml, with the following added to each of three tubes at time zero: ●, 4 mM-ADP; ×, 20 mM-NaF; ○, 1 mM-pyrophosphate. Δ, ADP (4 mM) was added to one tube after 10 min (arrow). The control (▲) contained no additions.

Fig. 7. Dependence of the polyphosphate kinase reaction on the histone concentration. Assays were performed as described in Methods except that the histone concentration was varied from 0 to 2.0 mg/ml.

and washed twice with 10 % (w/v) TCA. It was then resuspended in 1 ml of the same buffer, neutralized, and dialysed overnight at 5 °C against a large volume of 5 mM-potassium phosphate pH 7.0. The product was then subjected to the following conditions: (i) 1.2 M-HCl for 15 min at 100 °C; (ii) 0.5 M-KOH for 2 h at 40 °C; (iii) 1 M-hydroxylamine for 30 min at room temperature; (iv) papain (2.5 mg/ml) for 30 min at 37 °C. After treatment, each sample was spotted on strips of Whatman No. 1 filter paper. The strips were eluted for 16 h with an Ebel's solvent containing 735 ml isopropanol, 50 g TCA, 2.5 ml conc. NH_4OH (sp.gr. 0.88) and 265 ml water; 100 ml of the solvent was diluted with 25 ml water before using (Ohashi & Van Wazer, 1963). On elution, long-chain polyphosphate remained at the origin while inorganic phosphate migrated rapidly. Shorter polyphosphate molecules migrated from the origin with intermediate R_F values depending on chain length. After elution the strips were air-dried and counted in a Baird Atomic strip counter, which was adjusted to give maximum sensitivity while keeping background radioactivity low.

Radioactive ATP and inorganic phosphate standards were applied to the paper as were the samples, and eluted similarly. They migrated rapidly on the paper and separated well from each other (data not shown). Untreated radioactive product remained at the origin after chromatography. The chromatogram contained no residual radioactive ATP

Table 2. *Effect of inorganic phosphate on polyphosphate kinase reaction with different proteins*

The 700-fold purified enzyme was assayed as described in Methods, except that different proteins were substituted for histone where indicated and assays were conducted with or without inorganic phosphate as shown.

Protein (1 mg/ml)	Inorganic phosphate (6 mM) added	Specific activity (nmol/mg/min)
Histone	+	254.1
	—	19.9
Protamine	+	135.0
	—	0.6
Casein	+	0.5
	—	4.1
Bovine serum albumin	+	6.6
	—	59.0

from the reaction, which indicated that washing twice with TCA was sufficient to remove unreacted substrate. After chromatography of the sample treated with acid, no counts remained at the origin; the product was completely hydrolysed to inorganic phosphate. After KOH treatment and elution with solvent, 19 % of the counts remained at the origin. This suggested that the product was not protein containing imidazole phosphate bonds, which are stable to base under the conditions employed. Hydroxylamine caused the release of all but 8 % of the counts from the origin. A serine phosphate ester product would be unaffected by this treatment. Seventy-two per cent of the product was unaffected by papain treatment. The loss of about 30 % of the product after papain treatment might be due to some degradation of the histone needed to stabilize the product. However, a phosphorylated protein would show greater degradation after proteolytic treatment. This distinguished the reaction product from an acyl phosphorylated protein which resembled polyphosphate on the basis of the other criteria tested. The labile nature of the product was in accord with results found by other investigators (Kornberg *et al.* 1956; Li & Brown, 1973; Mühlradt, 1971) and was compatible with the identification of the reaction product as polyphosphate rather than a phosphorylated protein.

Physiological studies. Phosphorus storage may be the physiological role of the polyphosphate kinase. Activity of the enzyme was determined under different conditions in dialysed crude extracts of the bacteria. Extracts which were frozen and thawed once before assaying sometimes showed an increase of from three- to fivefold over the activity of the same extract before freezing. One possible explanation of this observation was that the enzyme might be loosely associated with the bacterial membrane. Freezing and thawing might have caused its release when sonication had not already done so.

To examine the effects of inorganic phosphate in the medium on polyphosphate kinase activity, *A. atrocyaneus* was grown on the phosphate-free medium described in Methods with 50 mM-malate as carbon source. Potassium phosphate was added to three separate cultures at 5, 25 and 100 mM concentrations. Dialysed crude extracts were frozen and thawed before assaying. The specific activities for organisms grown in the presence of 5, 25 and 100 mM inorganic phosphate were 2.6, 1.4, and 0.3 nmol of ^{32}P incorporated into product/min/mg protein, respectively. The results indicated a direct relationship between the availability of phosphate in the medium and the activity of the polyphosphate kinase.

To demonstrate derepression of this enzyme, organisms were grown on medium MS plus 50 mM-malate overnight. These bacteria were harvested, washed, resuspended in 2 l of phosphate-free medium and returned to the shaker at 30 °C. At 0, 10, 20 and 30 min, samples (250 ml) were removed and crude extracts were prepared, dialysed, frozen and thawed for assay. The specific activities of the extracts were 0.08, 3.52, 3.88 and 4.78 nmol/min/mg protein, respectively. Thus, an extremely rapid increase in activity took place when organisms were aerated in the absence of inorganic phosphate. It is not clear whether the increase in activity was due to synthesis of new protein or to activation of inhibited enzyme already present.

DISCUSSION

Studies of enzyme induction in *A. atrocyaneus* led to the purification and characterization of a histone-dependent polyphosphate kinase. The requirement for histone to detect enzyme activity might be misleading. Unless the reaction product is purified, it might appear that the enzyme catalysed the phosphorylation of histone. When investigating protein kinase activity, one must therefore first eliminate the possibility of the presence of a histone-dependent polyphosphate kinase. The additional finding that inorganic phosphate inhibited or activated the enzyme when different proteins were used might also be used as a criterion for polyphosphate kinase activity where it is suspected. This characteristic has also been found for the *E. coli* enzyme (H.-C. Li & G. G. Brown, personal communication). A possible role of histone or other proteins in the reaction might be to complex with the polyphosphate and prevent reversal of the reaction, thereby facilitating detection of the product. Depending on the protein, phosphate ions might interfere with this function or enhance it. Alternatively, the histone might interact with the enzyme, altering its activity in the presence of inorganic phosphate.

It is possible that ADP inhibited because it caused the equilibrium of the reaction to favour the reversal of polyphosphate synthesis. The results in Fig. 6 appear to contradict this, since radioactivity was not lost from the product on addition of ADP after 10 min. However, if the role of histone were to complex with the product and prevent reversal of the reaction, it might mask the effect of ADP inhibition on product which was already formed. The reversal of the polyphosphate kinase reaction by ADP was shown in *E. coli* by Kornberg (1957) and Li & Brown (1973).

The activation by inorganic phosphate might be consistent with a role for the enzyme, which has been suggested by others, for the accumulation of a phosphorus reserve. In many organisms, polyphosphate can be utilized as a phosphorus source for nucleic acid and phospholipid biosynthesis during spore germination (Nishi, 1961) or when organisms are starved for phosphorus (Harold, 1966; Baker & Schmidt, 1964; Miyachi *et al.* 1964; Mudd, Yoshida & Koike, 1958; Winder & Denneny, 1957). Sufficient amounts of polyphosphate might accumulate to allow the bacterial mass to double several times under conditions of phosphate starvation. The pattern of polyphosphate synthesis was studied in *Aerobacter aerogenes* by Harold (1966) with wild-type organisms and mutants of this bacterium deficient in polyphosphate metabolism. He found that under conditions of nucleic acid synthesis, polyphosphate synthesis was inhibited, possibly by competition for ATP, and degradation of polyphosphate was enhanced. When nutrient limitation stopped nucleic acid synthesis, inhibition of polyphosphate synthesis was relieved and polyphosphate degradation was inhibited. Kaltwasser (1962) suggested that accumulation of polyphosphate regulated the intracellular levels of inorganic phosphate. Conditions under which polyphosphate was synthesized were those where macromolecular synthesis was inhibited by

nutrient starvation or specific inhibitors while uptake of inorganic phosphate from the medium continued. If the physiological role of polyphosphate were to serve as a phosphorus reserve, then it would be advantageous to the organism to have a polyphosphate-synthesizing enzyme which was sensitive to the levels of inorganic phosphate available.

Experiments designed to investigate the physiological role of the polyphosphate kinase strongly implicated involvement in phosphate metabolism of the bacteria. The levels of inorganic phosphate in the medium clearly correlated with levels of polyphosphate kinase, and derepression of the enzyme occurred when inorganic phosphate was removed from the medium. The role of the enzyme, therefore, might be to store phosphate internally at the expense of ATP when phosphate was available only in limiting amounts in the environment.

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