# ORTHOPHOSPHATE AND HISTONE DEPENDENT POLYPHOSPHATE KINASE FROM E. COLI

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SUMMARY:

A polyphosphate kinase has been purified over 100-fold from an extract of E. coli K-12. It requires both orthophosphate and a basic protein (histone or protamine) for maximum activity. Because its activity is stimulated by histone, polyphosphate kinase may easily lead to an error in the determination of protein kinase in the cell extract. Our data suggest that the stimulatory effect of orthophosphate on polyphosphate kinase may be important in the regulation of phosphate metabolism in the microorganism.

## INTRODUCTION

Long chain polyphosphate (poly P) is widespread in many biological systems, ranging from bacteria to mammalian cell nuclei (1). Polyphosphate kinase (poly P kinase), the enzyme which is responsible for the biosynthesis of poly P, has been detected in many microorganisms (1,2). However, none of these enzymes has been purified to a homogeneous state or well characterized. In this communication, we describe the effects of orthophosphate and histone on a partially purified poly P kinase from E. coli.

## METHODS AND MATERIALS

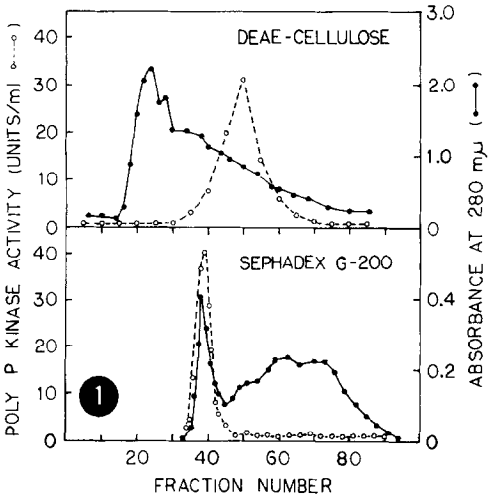
Enzyme Assay: The activity of the poly P kinase was measured at 30° in an incubation volume of 0.1 ml containing 20 mM potassium phosphate (KPi) buffer, pH 7.0, 10 mM $MgCl_2$, 0.2 mg histone (calf thymus Type II, Sigma), 0.4 mM $\gamma$-$^{32}$P-ATP (specific activity from 5 to 28 dpm/pmole, New England Nuclear) and an appropriate amount of the enzyme preparation. $^{32}$P-poly P was isolated and determined by the paper chromatographic method

for protein kinase (3) except that the development of the paper chromatograms was performed with cold 5% trichloroacetic acid. Also, the sample spots were not dried before being placed in the developing solvent. A comparison of this method with millipore filtration was carried out and identical results were obtained. One unit of poly P kinase activity is defined as the amount of enzyme which catalyzes the transfer of 1 nmole of phosphate from ATP (gamma position) into poly P per min at 30° in a 0.1 ml incubation mixture.

Enzyme Purification: Poly P kinase was purified from E. coli Crooke's strain (obtained from Dr. H. V. Rickenberg), grown to a late log phase in a medium containing 8 g nutrient broth, 5 g peptone, 5 g NaCl and 1 g glucose in a liter of distilled water. Fifty grams of frozen cells were disrupted by grinding in alumina powder with a mortar and pestle. The broken cells were extracted with 5 mM Tris-Cl buffer containing 2 mM EDTA, pH 7.5. This crude extract was then incubated with DNase for 1 hr at 0°. After the removal of particulate matter by centrifugation at 35,000 x g for 30 min the soluble proteins were fractionated by the addition of solid $(NH_4)_2SO_4$. The 0-45% $(NH_4)_2SO_4$ fraction was dissolved in 5 mM KPi buffer containing 1 mM EDTA and 10 µM dithiothreitol (DTT), pH 7.0, dialyzed overnight against this buffer, and applied to a DEAE-cellulose column (2.2 x 20 cm.), previously equilibrated with the same buffer. Proteins that adsorbed to the DEAE-cellulose were eluted by a 500 ml linear gradient of increasing KPi concentration (0.005-0.5 M) with both buffers at pH 7.0 containing 1 mM EDTA and 10 µM DTT. The poly P kinase came off at 0.25 M KPi. The active fractions were pooled and concentrated by precipitation with solid $(NH_4)_2SO_4$ at 60% saturation. The precipitate was dissolved in 5 mM KPi buffer, pH 7.0, containing 10 µM DTT, dialyzed overnight against this buffer, and applied to a Sephadex G-200 column (2.5 x 40 cm) equilibrated and eluted with the same buffer. The active fractions were pooled, concentrated by $(NH_4)_2SO_4$ precipitation and dialyzed against 5 mM Tris-Cl buffer, pH 7.4, containing 10 µM DTT. The enzyme so obtained was stored at -20° for use in the following experiments.

## RESULTS AND DISCUSSIONS

Column chromatographic separation of a poly P kinase ac-



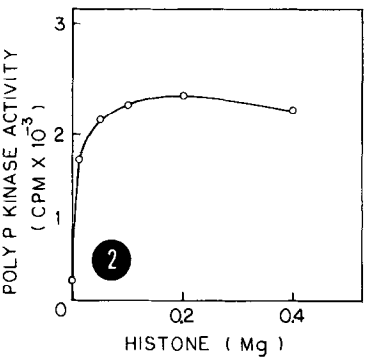




Fig. 1. Column chromatography of E. coli poly P kinase on DEAE-cellulose (fractions of 6 ml were collected) and on Sephadex G-200 (fractions of 2 ml were collected). Poly P kinase activity was determined with 10 μl aliquots from the indicated fractions, as described under "Methods and Materials".

Fig. 2. Effect of histone on poly P kinase activity. Poly P kinase was assayed as described under "Methods and Materials" except the concentration of histone was varied.

tivity from E. coli extract is shown in Fig. 1. The enzyme activity was eluted as a single, sharp peak at the void volume of the Sephadex G-200 column. The specific activity of the G-200 eluate was around 150 units per mg protein. This represented more than a 100-fold purification from the crude extract.

The effect of histone concentration on the enzymatic reaction is shown in Fig. 2. A variety of proteins were tested for their ability to stimulate the poly P kinase activity (Table I). Protamine and histone were found to be the most effective stimulators. These two basic proteins are generally the most effective substrates for 3',5'-adenosine monophosphate-dependent protein kinases from animal tissues (4) and E. coli (5) as well as for a protein acyl kinase from C. crescentus (6). Because of this, one can easily be misled to consider the poly P kinase from

Table 1. Effect of various proteins on poly P kinase activity incubation conditions were as described in the text except for the replacement of 0.2 mg of histone by 0.2 mg of the indicated proteins. After incubation for 5 min, 25 µl of aliquots were withdrawn from the 0.1 ml incubation mixture for analysis.

| Protein added | Poly P - Formation cpm/5 min/25 µl |
|---|---|
| None | 245 |
| Casein | 620 |
| Bovine Serum Albumin | 645 |
| Histone | 2,092 |
| Protamine | 2,932 |

E. coli as a protein kinase or protein acyl kinase. In the presence of histone, the enzyme activity was low when assayed in Tris-Cl buffer. However, the activity was proportionally increased by raising either sodium or potassium phosphate concentration. The maximum activity was obtained in 20 mM phosphate. Further increase in phosphate concentration caused a decrease in poly P formation.

The effects of phosphate and histone on poly P kinase activity are summarized in Table 2. Both phosphate and histone were required for maximum enzyme activity. Phosphate or histone alone was inhibitory. When poly P kinase was mixed with a basic protein (histone or protamine), the solution became turbid and the enzyme activity could be sedimented by centrifugation.

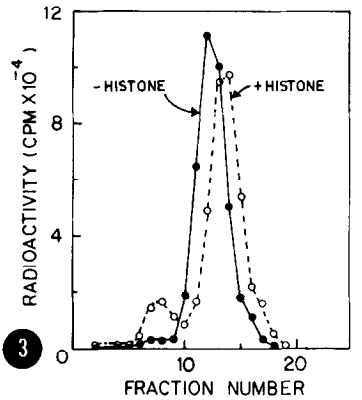
In a reaction mixture containing phosphate buffer, histone, ATP and $MgCl_2$, the catalytic properties of the enzyme were consistant with those reported previously (7,8). The enzymatic reaction exhibited a bell-shaped pH profile with optimum pH around 7.0, $MnCl_2$ could replace $MgCl_2$, the Km of ATP was 1.4 mM, and

Table 2. Effect of orthophosphate and histone on poly P kinase activity. Incubation conditions were as described in the text. After incubation for 5 min, 25 µl of aliquots were withdrawn from 0.1 ml incubation mixture for analysis.

| Buffer | Poly P Formation (cpm/5 min) | |
|---|---|---|
| | -Histone | +Histone |
| 20 mM Tris·Cl, pH 7.5 | 1,703 | 762 |
| 20 mM Tris·Cl, pH 7.2 + 20 mM potassium phosphate, pH 7.2 | 981 | 6,490 |
| 20 mM potassium phosphate, pH 7.2 | 797 | 6,455 |

$^{14}$C-ATP could not substitute for $\gamma$-$^{32}$P-ATP for the formation of $CCl_3COOH$ precipitable radioactive product. ADP, pyrophosphate, and NaF at 0.2 mM, 1 mM and 20 mM, respectively, inhibited the enzymatic reaction completely. NaCl and KCl were also inhibitory. 3',5'-Adenosine monophosphate did not stimulate the enzyme activity. In the absence of histone the reverse reaction could be demonstrated by adding ADP to a standard incubation mixture containing enzymatically synthesized $^{32}$P-poly P and enzyme. Histone inhibited the reverse reaction completely.

The radioactive product formed by the enzyme in the presence and absence of histone was examined further. The product was isolated by incubating a 10-fold larger amount of the standard assay mixture for 30 min and filtered through a Sephadex G-25 column that was presaturated with a solution containing 8 M urea, 0.2 M KCl, 10 mM EDTA and 10 mM HCl. The elution patterns of



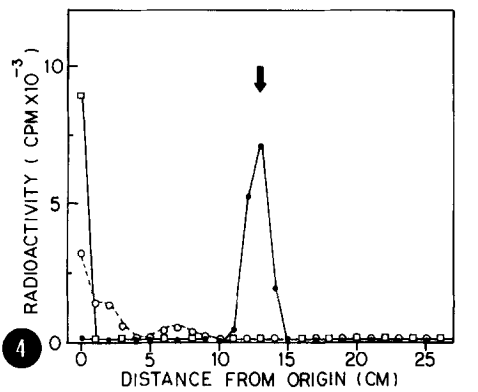




Fig. 3. Isolation of $^{32}$P-poly P by Sephadex G-25 column. The open circle (o-o-o) represents the chromatogram of the complete reaction mixture, while the closed circle (●-●-●) represents the chromatogram of the reaction mixture minus histone.

Fig. 4. Paper chromatogram of the histone-containing $^{32}$P-product after treating with (1) pronase (□——□), (2) 0.5 N NaOH at 40° for 2 hrs (o----o), and (3) 1 N HCl at 90° for 15 min (●——●). The arrow indicates the position of the $^{32}$P-orthophosphate standard on the paper chromatogram.

the reaction mixture with and without histone are shown in Fig. 3. Fractions at the void volume were pooled and extensively dialyzed against distilled water at 4°. Both products, containing histone or not, showed the characteristic properties of poly P (7). They were non-dialyzable, bound tenaciously to ion-exchange resin and did not move on the paper chromatogram. All the radioactivity of both products remained at the origin of the chromatogram after 20 hrs development with Ebel's solvent (9). As shown in Fig. 4, Pronase digestion of the histone-containing product did not change the mobility of the radioactivity. After treating with 0.5 N NaOH at 40° for 2 hrs, the radioactivity was found to spread out in the chromatogram. No radioactivity was in the orthophosphate position. These results indicate that the long chain polyphosphate was hydrolyzed to heterogeneous fragments other than orthophosphate. The product was readily and com-

pletely hydrolyzed to orthophosphate at 90° in 1 N HCl for 15 min. All radioactivity was found to have an $R_f$ value identical to orthophosphate standard. These data exclude the possibility that the stimulatory effect of histone on poly P kinase activity is a result of the covalent phosphorylation of a hydroxyl or carboxyl residue on the histone as reported in the protein kinase (4) or protein acyl kinase (6) reaction.

The present finding that poly P kinase requires orthophosphate for maximum activity suggests that the enzyme may be involved in the regulation of the cellular level of phosphate. Since the increase of phosphate level will result in the stimulation of poly P kinase activity which in turn will accelerate the flow of phosphate into the poly P pool thus keeping the phosphate level relatively constant. It has been suggested that poly P serves as a phosphorus reservoir (1), and the poly P cycle is involved in the regulation of the cellular level of phosphate in microorganisms (10). Our data on the control of polyphosphate kinase activity by phosphate tends to support that hypothesis.

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