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FURTHER CHARACTERIZATION OF PHOSPHOINOSITOL KINASE ISOLATED FROM GERMINATING MUNG BEAN SEEDS*

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Abstract—Phosphoinositol kinase isolated and purified from germinating mung bean seeds has been further characterized. The rate of phosphorylation varies with different inositol phosphates and this is consistent with the K_m and V_{max} for each of the substrates. The phosphate transfer from ATP has been found to be mediated by a phosphoprotein intermediate. In a particular step of the reaction the immediate product of the reaction has been found to be most inhibitory, other products being less or non-inhibitory. The inhibition has been found to be competitive in nature. The K_is have been found to range between 0·6 and 1×10^{-4} M. ADP also inhibited non-competitively with respect to IP_5. K_i for this has been found to be $2{\cdot}3 \times 10^{-4}$ M. The purified enzyme migrated as a single protein band on polyacrylamide gel electrophoresis. In the presence of sodium dodecyl sulphate it is dissociated into 3 subunits in the ratio 1:1:1. The MW of the three subunits are approx. 86 000, 56 000 and 35 000. The MW of the enzyme has been found to be approx. 177 000.

INTRODUCTION

IN A PREVIOUS communication[1] we have reported the isolation, purification and partial characterization of PI-kinase from germinating mung bean seeds. The present paper deals with the further characterization of the enzyme.

RESULTS

Relative Affinity of PI-Kinase for Different Inositol Phosphates and Inositol

The activity of the PI-kinase with different inositol phosphates and with inositol was investigated, using ^{14}C-labelled inositol and ^{32}P-labelled inositol phosphates as substrates.

TABLE 1. PHOSPHORYLATION OF DIFFERENT INOSITOL PHOSPHATES BY PHOSPHOINOSITOL KINASE

Substrate used	nmol conversion to the next higher phosphorylated component/mg protein	Substrate used	nmol conversion to the next higher phosphorylated component/mg protein
1 *myo*-inositol	0	4 IP_3	29·0
2 IP	18·4	5 IP_4	20·0
3 IP_2	20·4	6 IP_5	21·0

0·1 μmol substrate was used in each case. ^{14}C-*myo*-Inositol of specific activity 5×10^7 cpm/μmol was used. For details of the assay for phosphorylation of *myo*-inositol see Experimental. Specific activities of the different ^{32}P-inositol phosphates were $1{\cdot}2 \times 10^2$, 2×10^2, $1{\cdot}36 \times 10^2$ and $1{\cdot}75 \times 10^2$ cpm/nmol for IP_2, IP_3, IP_4 and IP_5 respectively.

* Part VI in the series "Metabolism of Inositol Phosphates". For Part V see *Indian J. Exptl Biol.* (In press).

[1] A. N. LAHIRI MAJUMDER, N. C. MANDAL and B. B. BISWAS, *Phytochem.* **11**, 503 (1972).

The phosphorylated compounds produced are shown in Table 1. It is seen that the enzyme cannot phosphorylate inositol, but it can phosphorylate the lower homologues of the inositol phosphates to their respective higher homologues. The rate of phosphorylation with different inositol phosphates, however, differs. It is seen that in a limited sense the rate increases up to IP_3, the rate with IP_2, IP_4 and IP_5 being almost the same but lower than that with IP_3.

Effect of Different Reaction Products in Different Steps of PI-Kinase Reaction

The preliminary observation that the presence of IP_5 in the reaction mixture inhibits IP_4 conversion to IP_5 by PI-kinase, and that ADP, another reaction product, is inhibitory, suggested that the different reaction products can affect the conversion of a particular inositol phosphate to its immediate higher homologue. Conversion of a particular inositol phosphate was therefore assayed in the presence of ADP and of insoitol phosphates higher in the series.

TABLE 2. EFFECT OF DIFFERENT REACTION PRODUCTS IN DIFFERENT STEPS OF PHOSPHOINOSITOL KINASE REACTION

Condition of the experiment	Phosphoinositol kinase activity (nmol conversion/mg protein) IP_2–IP_3	IP_3–IP_4	IP_4–IP_5	IP_5–IP_6
1 Complete	27·0	30·0	21·0	18·8
2 Complete plus 0·2 μmol IP_3	4·8	—	—	—
3 Complete plus 0·2 μmol IP_4	6·8	8·3	—	—
4 Complete plus 0·2 μmol IP_5	26·2	20·0	4·2	—
5 Complete plus 0·2 μmol IP_6	26·5	24·0	9·8	8·7
6 Complete plus 0·1 μmol ADP	23·4	24·0	15·3	—
7 Complete plus 0·2 μmol ADP	15·1	13·0	4·8	4·8

Enzyme assay (see Experimental) with 50 μg enzyme per incubation. Specific activities of IP_2, IP_3, IP_4 and IP_5 were $1{\cdot}5 \times 10^2$, $1{\cdot}75 \times 10^2$, $2{\cdot}8 \times 10^2$ and 3×10^2 cpm/nmol, respectively.

It is seen (Table 2) that with 0·2 μmol IP_3, IP_2 conversion to IP_3 is inhibited maximally; with higher members of the series, there is practically no inhibition. IP_4 (0·2 μmol) reduces the IP_3 conversion to IP_4 considerably, while with higher inositol phosphates, inhibition is very low. It is evident that 0·2 μmol IP_5 inhibits IP_4 conversion to IP_5 to about 80%, whereas with IP_6 at the same concentration in the reaction mixture, the conversion is inhibited to about 53%. With 0·2 μmol IP_6 more than 50% inhibition of the IP_5 conversion was found. In all cases tested inclusion of ADP (0·2 μmol) caused inhibition.

Effect of Substrate Concentration and the Nature of Inhibition by the Immediate Higher Member in the Different Steps of PI-Kinase Reaction

To see the effect of different concentrations of inositol phosphates at different steps of the phosphoinositol kinase reaction, experiments were performed for each step in which different concentrations of a particular inositol phosphate were used as substrate and its conversion to the immediate higher homologue was assayed. From the Lineweaver–Burk plot the K_m and V_{max} for the different inositol phosphates were calculated. These have been summarised in Table 3.

From the earlier results it is evident that in any step of the PI-kinase reaction, the immediate product is highly inhibitory. Experiments were then performed similarly with 0·1 μmol of the next highest inositol phosphate in each, keeping one control set. The nature of the inhibition in each case was found to be competitive. The K_i for the competitive inhibition were determined. The results are presented in Table 3.

TABLE 3. SUMMARY OF K_m, V_{max} AND K_i VALUES FOR DIFFERENT INOSITOL PHOSPHATES

Reaction	Substrate	K_m (M × 10^4)	V_{max} (nmol/mg)	Inhibition by	K_i (M × 10^4)
IP → IP_2	IP	1·5	55	—	—
IP_2 → IP_3	IP_2	1·7	62·5	IP_3	0·77
IP_3 → IP_4	IP_3	2·7	100	IP_4	1·00
IP_4 → IP_5	IP_4	3·6	66	IP_5	0·66
IP_5 → IP_6	IP_5	2·5	71	IP_6	1·00

V_{max}, K_m and K_i were obtained from a double reciprocal plot for each substrate.

Nature of Inhibition by ADP in the Conversion of IP_5 to IP_6

Different concentrations of IP_5 were used and its conversion to IP_6 was determined in the presence and absence of 0·1 μmol ADP. When the Lineweaver–Burk plot was made, two straight lines were obtained indicating a non-competitive inhibition by ADP the K_i for which was calculated to be $2·3 \times 10^{-4}$ M.

Mechanism of Phosphate Transfer

To determine whether the phosphate transfer from ATP to inositol phosphate is mediated through a phosphorylated protein intermediate, in two tubes 0·2 ml each of the phosphoprotein (see Experimental) were taken with 100 μmol Tris-HCl buffer (pH 7·4), 3 μmol Mg^{2+}, 5 μmol mercaptoethanol and 0·1 μmol unlabelled IP_4 in a total vol. of 1 ml. In one tube the reaction was stopped at 0 min and the other incubated at 37° for 20 min. The supernatant obtained after termination of the reaction was neutralized, adsorbed on a Dowex-1 (Cl^-) column and IP_5 and IP_6 were eluted as described earlier.[1] Radioactivity was detected in these fractions due to the transfer of phosphate from the phosphoprotein to IP_4 which was equivalent to a transfer of 0·84 nmol γ-^{32}P from labelled ATP. This indicates that the transfer from ATP in the PI-kinase reaction is mediated through a phosphoprotein intermediate.

Electrophoretic Mobility of PI-Kinase on Polyacrylamide Gel

Biogel purified enzyme (30 μg) was subjected to polyacrylamide gel electrophoresis at pH 8·0 using 5% acrylamide and stained in amido-black. A single protein band was detectable. A duplicate separation was sliced, each slice being 2 mm thick and kept in 0·05 M Tris-HCl buffer (pH 7·4) overnight. The slices were then homogenized, centrifuged, and the supernatant taken for PI-kinase assay. The enzyme activity was found to correspond the protein band.

Subunits of PI-Kinase and MW

To determine whether PI-kinase contains subunits, the enzyme was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. About 20 μg of

the purified protein was used for this purpose (see Experimental). It was seen that the enzyme is dissociated into three subunits a, b and c. When the migration of each band was compared with a standard curve obtained with proteins of known MW, the MW of a, b and c were found to be approx. 86 000, 56 000 and 35 000 respectively in the ratio 1:1:1. The MW of the enzyme is therefore approx. 177 000.

DISCUSSION

It is apparent that phosphoinositol kinase can use all the inositol phosphates as substrates. Reaction kinetics suggest that the conversion of IP_3–IP_4 occurs at a comparatively higher rate than those observed with other homologues, which is consistant with the V_{max} obtained for each of the substrates. ADP exerts a non-competitive inhibition with respect to IP_5, the only substrate tested.

The interesting point in the PI-kinase reaction is that inositol phosphates are also inhibitory for the conversion of IP_2–IP_6; more specifically the conversion of IP_2–IP_3 is highly inhibited by IP_3, IP_4 being less inhibitory and IP_5 and IP_6 not inhibitory at the concentrations tried. This pattern of inhibition, i.e. maximum inhibition by the immediate product is exhibited in each step of the reaction from IP_2 to IP_6 and seems to be competitive in nature. In the conversion of IP_3–IP_4, the effect of IP or IP_2 could not be tested since these are also converted simultaneously to the initial substrate thus increasing the substrate concentration. Since several steps of the reaction are catalyzed by a single enzyme, different products might be accumulated in different amounts. In each step of the reaction the immediate product seems to be inhibitory almost to the same extent. It is expected that either IP_6 is removed continuously *in vivo* from the reaction centre after its synthesis or it is complexed with metal ions, proteins or lipids thus favouring the synthesis of IP_6. Actually such complexes of phytic acid have been reported from a variety of sources.[2–8]

What emerges from the present finding is that the reaction kinetics of phosphoinositol kinase are such that, except IP_6, at any time the accumulation of other inositol phosphates are not favoured and for this reason the lower homologues could not be detected in appreciable quantities.[9] Participation of a hypothetical complex X-IP_6 as an intermediate in phytic acid biosynthesis has been proposed by Asada *et al.*[10] Their supposition was based on the fact that they could not detect the presence of lower intermediates in the ripening seeds where synthesis is a predominant process. They studied the incorporation of ^{32}Pi into inositol phosphates by ripening rice and wheat and could detect the radioactivity in phytic acid only. The observation seems to indicate that, (i) inositol mono-, di-, tri-, tetra- and penta-phosphates are not the intermediates in the formation of phytic acid or that, (ii) these lower phosphorylated inositols could not be detected because of the rapid rate of turn over. To rule out the second possibility Asada *et al.*[10] fed a mixture of mono- to penta-phosphates of inositol to a detached ear of wheat before administration of ^{32}Pi and found

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that this did not affect the normal incorporation of ^{32}Pi into phytate. In an analogous experiment Mandal and Biswas[11] administered ^{32}P-labelled IP_4 and IP_5 into the seeds (pre-soaked for 6 hr) in the presence of the respective labelled compounds in a limited amount of water. It was noticed that when 80% of the water has been imbibed 100% of the added radioactivity could still be recovered in the remaining solution. The result of Asada *et al.* indicating that the presence of the lower inositol phosphates could not affect the normal incorporation of ^{32}Pi into phytic acid can be explained on the basis of this observation.

EXPERIMENTAL

Sources of plant material, radio-isotopes and details of the methods of isolation and purification of the enzyme PI-kinase, preparation of ^{32}P-inositol phosphates, and polyacrylamide gel electrophoresis have been reported in the earlier communication.[1]

Assay of IP phosphorylation. Non-labelled IP and 0·1 μmol of β, γ-^{32}P-ATP (specific activity $1{\cdot}5 \times 10^3$ cpm/nmol γ P) with other components of the reaction mixture were incubated for 40 min at 37°. The reaction was stopped by adding 1 ml 0·4 M TCA, the supernatant neutralized and adsorbed on a Dowex-1 (Cl^-) column (0·5 × 8 cm). AD^{32}P was eluted with 50 ml of HCl. ^{32}P transferred to IP_2 was the measure of phosphoinositol kinase activity.[1]

Isolation of phosphoprotein. About 100 μg of the purified enzyme was incubated at 37° for 20 min in presence of 100 μmol Tris-Cl^--buffer (pH 7·4); 2 μmol Mg^{2+} and 20 nmol of β, γ-^{32}P-ATP (specific activity $5{\cdot}4 \times 10^2$ cpm/nmol γP) in a total vol. of 0·5 ml. After incubation, the tube was placed in ice, the contents layered on a Biogel P-200 column (6 × 1 cm) and fractions of 1 ml were collected at a rate of 1 ml/20 min by washing the column with 0·05 M Tris-HCl buffer (pH 7·4). Aliquots from each fraction were checked for radioactivity. The fractions (7–9) in which the enzyme is generally eluted were found to have the maximum radioactivity. These were pooled (3 ml) and concentrated under vacuum in the cold to 0·4 ml. The association of radioactivity with the enzyme indicates a transfer of ^{32}P from ATP to the enzyme thus forming a phosphorylated protein.

Polyacrylamide gel electrophoresis of proteins in presence of sodium dodecyl sulphate. The method followed was that of Weber and Osborn.[12] The gels were run for 90 min (8 mA/tube), stained in coomassie blue and destained electrophoretically. The standard proteins used as markers were β-amylase (150 000), bovine serum albumin (69 000), egg white albumin (42 000), β-lactoglobulin (36 000) and pancreatic RNase (14 000).

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