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INOSITOL HEXAPHOSPHATE GUANOSINE DIPHOSPHATE PHOSPHOTRANSFERASE FROM *PHASEOLUS AUREUS**

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Key Word Index—*Phaseolus aureus*; Leguminosae; mung bean; inositol hexaphosphate guanosine diphosphate phosphotransferase.

Abstract—Inositol hexaphosphate guanosine diphosphate phosphotransferase which transfers phosphate from inositol hexaphosphate to guanosine diphosphate, synthesizing guanosine triphosphate, has been isolated from germinating mung bean. A purification of 86-fold with 33% recovery has been obtained and the protein was made homogeneous after polyacrylamide gel electrophoresis. The MW of this enzyme was *ca* 92000. The optimal pH was 7·0 and Mn^{2+} was stimulatory. Inositol hexaphosphate was the most active donor of the phosphoryl group (P) to GDP. Inositol penta- or tetra-phosphate (mixed) was partially active, but inositol pentaphosphate produced in this reaction did not act further as phosphate donor. The transfer of P from inositol hexaphosphate was mediated through a phosphoprotein. Polyphosphate (poly Pi), pyrophosphate (PPi) and orthophosphate (Pi) were inactive in this reaction. ADP, CDP and UDP could not substitute for GDP, neither could dGDP nor GMP accept P from inositolphosphate. GTP inhibited the reaction, but ATP did not interfere with the reaction. The products have been shown to be [GMP-^{32}P] and inositol pentaphosphate by several criteria. The reaction is practically irreversible. K_m values for GDP and inositol hexaphosphate were $1{\cdot}1 \times 10^{-4}$ M and $1{\cdot}6 \times 10^{-6}$ M respectively.

INTRODUCTION

During an investigation of the role of phytin (Ca^{2+} and Mg^{2+} salt of inositol hexaphosphoric acid, IP_6, a compound which probably acts as a reservoir of phosphate [1]), a novel phosphoinositol phosphotransferase system has been detected in germinating mung bean seeds. The partially purified enzyme was specific for GDP, synthesizing GTP [2, 3]. ATP: phosphoinositol phosphotransferase has been demonstrated in wheat endosperm [4], but this system has not yet been purified and details of the reaction are lacking. The purpose of the present work was to purify and characterize the enzyme IP_6–GDP–phosphotransferase isolated from the cotyledons of mung bean seeds.

RESULTS AND DISCUSSION

Purification

The enzyme was purified by the procedure described in Experimental, and summarized in Table 1. A purification of 86-fold with 33% recovery was obtained. Further purification was only possible after gel electrophoresis. At least three protein bands were present in the Biogel-P-100 preparation; band 1 was weak, band 2 very strong and band 3 intermediate. Most of the phosphotransferase activity was found in the third (fastest) protein band.

Effect of pH and cations

The enzyme had a pH optimum at 7·0 measured over the range of 6–8. The course of the reaction was studied by noting the transfer of ^{32}P from IP_6 [^{32}P] to GDP by the purified enzyme (Biogel preparation). It has been observed that at 37° the transfer of ^{32}P increases linearly up to 20 min after which it reaches a plateau over 40 min. Activity is entirely dependent on divalent cations and monovalent cations have essentially no effect on the reaction. Mn^{2+} has been found to be more effective than Mg^{2+}. Low activity has been recorded in presence of Ca^{2+}.

Specificity

Neither deoxy-guanosine diphosphate (dGDP) nor GMP accepted P from phytate. With a high

* Part IX in the series 'Metabolism of Inositol Phosphates'. For Part VIII see (1974) *Phytochemistry* **13**, 1047.

Table 1. Summary of purification procedure

Treatment	Total volume (ml)	Total protein (mg)	Sp. act. (units/mg protein)	Total activity (unit)
1. Crude extract	250	2420	2·5	6050
2. 20–45% Ammonium sulphate fraction	24	630	9	5670
3. DEAE column chromatography	2	56	50	2800
4. Biogel P-100 filtration	6	9·3	216	2000

Protein was estimated by the procedure described earlier and phosphotransferase was assayed by the procedure given in experimental. One unit of the enzyme is defined as that amount of protein which can transfer 0·1 nmol [^{32}P] from phytate to GDP under the conditions described.

concentration of GTP the reaction is inhibited. ATP does not influence the reaction.

At pH 7, the enzyme transfers the P from phytate to GDP, forming the corresponding lower inositol phosphate and GTP. The forward reaction thus results in the formation of GTP from GDP. Reversibility was tested at three different pHs 6, 7 and 8·4 and also using ATP as donor in place of GTP. There is a transfer of P from GTP to IP_5 to a very limited extent and the rate of transfer increases slightly at alkaline pH. ATP is ineffective as a donor.

To determine whether IP_2, IP_3, IP_4, and IP_5 in addition to IP_6 acts as substrates, activity was measured in each case, taking one of the lower inositol phosphates in place of IP_6 as donor of the P group; other components in the assay system were kept constant. Difficulty arises in routine assay procedure because the lower inositol phosphates were eluted with GTP from the Dowex-formate column used. However, GTP can be separated from inositol phosphates by paper electrophoresis with 0·05 M ammonium formate pH 3·5 (see Experimental). Affinity for PPi, poly-Pi and Pi were also tested. Results are given in Table 2. Besides IP_6, IP_5, and IP_4 (mixed) can also be used as substrates. The enzyme has little affinity for IP_3 and no affinity for IP_2, Pi, PPi or poly-Pi. The rate of transfer of P tends to decrease as the number of P groups decreases. However, this is not true when IP_5, obtained as a product of this reaction, was used for P transfer (Table 2). The reactivities of certain axial groups may contribute to this type of transfer of P from IP_6 [5, 6]. However, the homologues of IP_5 produced in this reaction remain to be ascertained.

Effect of GDP and IP_6 concentration on P transfer

With IP_6 at 50 nmol/ml, the K_m value for GDP as determined from a double reciprocal plot was $1·1 \times 10^{-4}$ M at 37°. V_{max} was 30 nmol Pi transferred/mg protein. With the GDP concentration constant at 0·2 μmol/ml, the K_m value for IP_6 was $1·6 \times 10^{-6}$ M at 37° and V_{max} 40 nmol P transferred/mg protein.

Mechanism of P transfer

Under experimental condition 3·5 nmol of P was transferred. This suggests that the transfer of P from IP_6 to GDP is mediated through a phosphoprotein intermediate.

EXPERIMENTAL

Purification. 100 g of mung bean seeds (*Phaseolus aureus* c.v. NP-23) were germinated for 36 hr at 35° (see Ref. 7). The cotyledons (190 g) were separated from the embryos and homogenized in a pestle and mortar with an eq. vol. of 50 mM Tris–HCl buffer pH 7 and 15 g acid washed sand. All operations were done at 0–4°. After grinding for 15–20 min the homogenate was strained through two layers of cheese cloth and centrifuged at 10000 *g* for 40 min. The supernatant was designated crude extract. This was made 20% sat. with $(NH_4)_2SO_4$, kept for 30 min with frequent stirring and centrifuged at 10000 *g* for

Table 2. Substrate specificity of the phosphotransferase

^{32}P-phosphate donor	Concentration (μmol/ml)	nmol ^{32}P Transferred/ mg protein
IP_6*	0·08	19·6
IP_5	0·1	8·8
IP_5†	0·02	0·0
IP_4	0·1	7·2
IP_3	0·1	1·8
IP_2	0·1	0·0
IP_1	0·5	0·0
Poly-(Pi)	0·23	0·0
PPi	0·25	0·0
Pi	0·52	0·0

The sp. act. of different inositol phosphates were between the range $0·3–0·6 \times 10^3$ cpm/nmol P. Specific activity of PPi, polyphosphate and Pi are $2·78 \times 10^7$ cpm/μmol, $5·0 \times 10^6$ cpm/μmol and $2·97 \times 10^6$ cpm/μmol respectively. The incubation mixture, except in case of PPi, polyphosphate and Pi, contained the following in μmol in a total volume of 0·5 ml; Tris, pH 7, 50; Mg^{2+}, 2; GDP, 0·1; enzyme 50 μg; inositol phosphates, as indicated.

* IP_6, IP_5, IP_4, IP_3, IP_2, represent inositol hexa, penta, tetra, tri, diphosphate respectively.

† IP_5, [^{32}P]-IP_5 as isolated from the reaction product.

20 min. The supernatant was made 45% sat. with $(NH_4)_2SO_4$, kept for 30 min and centrifuged similarly. The pellet obtained was dissolved in 20 ml of 50 mM phosphate buffer pH 7·4 and dialysed against 20 mM phosphate buffer of same pH for 18 hr. This was designated the $(NH_4)_2SO_4$ fraction.

The $(NH_4)_2SO_4$ fraction containing the enzyme was passed through a DEAE cellulose column (42 × 1·8 cm) previously equilibrated with 20 mM phosphate buffer, pH 7·4. $(NH_4)_2SO_4$ fraction (630 mg protein) was chromatographed with 50 mM phosphate buffer pH 7·4 until the effluent was protein-free. Gradient elution was started with 300 ml of 10 mM phosphate buffer pH 6·5 in the mixing vessel and 300 ml of 0·5 M same buffer in the reservoir. 6 ml fractions were collected at 6 ml/10 min. A at 280 nm when plotted against fraction number showed four distinct peaks. Fractions from each peak were separately pooled and made 100% sat. with $(NH_4)_2SO_4$ for 30 min and centrifuged at 10000 *g* for 20 min. The pellets were dissolved in 50 mM Tris–HCl buffer pH 7 and dialysed 18 hr against 10 mM Tris–HCl. Each fraction was separately assayed and most of the activity was found to be associated with the first peak. This was designated DEAE cellulose fraction. 56 mg DEAE fraction in 2 ml was then passed through Biogel P-100 (0·9 × 25 cm) previously equilibrated with 50 mM Tris buffer pH 7. The protein was eluted with the same buffer (1 ml fraction) and A at 280 nm was plotted against the phosphotransferase act. The act. was associated with the small peak which followed the first broader peak. The enzyme protein (peak II) was pooled together, conc against aquacide and stored for further use.

Preparation of ^{32}PPi and [^{32}P]-polyphosphates. ^{32}PPi and [^{32}P]-polyPi were prepared from $H_3{}^{32}PO_4$ by the method of Bergman *et al.* [8]. Inositol phosphates [^{32}P] were synthesized and purified as described in Ref. 9.

Isolation of phosphoprotein. About 80 μg of the purified enzyme was incubated at 37° for 20 min in the presence of 50 μmol, Tris–Cl^- buffer (pH 7·0); 2 μmol, Mn^{2+} and 30 nmol, ^{32}P-labelled IP_6. (sp. act. 1·5 × 10^5 cpm/μmol Pi) in a total vol of 0·5 ml. The reaction was stopped by cooling to 0°. The incubate was chromatographed on Biogel P-100 as previously described. Fractions 20–24 containing enzyme were radioactive. These were pooled and conc under vacuum in the cold to 0·5 ml. Disc electrophoresis was performed according to the method of Davis [10] with slight modifications [11]. Gels were sliced and eluted as described earlier [12]. 50 μg protein was subjected to electrophoresis on 5% polyacrylamide gel and the protein stained with 1% amido black. The association of radioactivity with the enzyme indicated a transfer of (^{32}P) from IP_6 to the enzyme and not to other protein bands present with the enzyme. Phosphoprotein (600 cpm) was incubated with GDP under standard assay condition without addition of [^{32}P]-IP_6.

Assay. In a typical assay system the following are the components expressed in μmol in a total vol. of 1 ml; Tris–HCl buffer, pH 7; 100; $MgCl_2$, 4; GDP, 0·2; Na–IP_6-[^{32}P], 0·05; and enzyme, an appropriate vol. The mixture was incubated for 20 min at 37°. The reaction was stopped by heating at 100° for 15 sec and the ppt. was removed by centrifugation. The supernatant was conc under vacuum after adding non-radioactive GTP (0·1 μmol) as carrier. An aliquot was spotted on a Whatman No. 1 paper (30 × 18 cm). Electrophoresis was carried out in 50 mM ammonium formate buffer pH 3·5 for 2·5 hr (8 V/cm). The electrophoretogram was dried at room temp. and GTP was located by UV quenching. The GTP spot was counted using a liquid scintillation counter [9].

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