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Evidence for the Existence of a Novel Enzyme System

myo-INOSITOL-1-PHOSPHATE DEHYDROGENASE IN *PHASEOLUS AUREUS**

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A novel enzyme system, *myo*-inositol-1-phosphate dehydrogenase, has been isolated from germinating mung bean seeds. The dehydrogenation and cleavage of *myo*-inositol 1-phosphate by this enzyme leads to the synthesis of a pentose phosphate which appears to be ribulose 5-phosphate. The pH optimum of the enzyme is 8.6; NAD^+ is required as coenzyme and no other nucleotides can replace NAD^+. Mono- or divalent cations are not essential for the enzyme activity. Stoichiometry of the reaction suggests that 2 mol of NAD^+ are reduced per mol of ribulose-5-P generated.

Inositol polyphosphates are widely distributed in the biological kingdom. About 93% of the total polyphosphates occurring in avian erythrocyte has been found to be *myo*-inositol pentaphosphate (1) and this pentaphosphate is thought to be an important regulator of hemoglobin function in avian systems (2). Whereas in matured ungerminated seeds, about 80% of the total phosphate is contributed by *myo*-inositol hexaphosphate, the actual biological function of IP_6[1] in seeds still remained unexplored. Consequently, our interest has been focused mainly on elucidation of the physiological role of IP_6 in seeds. It has been established that during maturation of seeds, glucose 6-phosphate, the key compound of the glycolytic pathway, is utilized for the synthesis of IP_6, which in turn acts as a phosphate donor for the synthesis of ATP during early stages of seed germination (3). This ATP generation from IP_6 is very specific and significant. On the basis of our previous findings, a cyclic pathway involving glucose-6-P and *myo*-inositol phosphates has been postulated (4). The present paper describes the experimental evidence for the existence of a novel enzyme system, *myo*-inositol-1-phosphate dehydrogenase, which oxidizes *myo*-inositol 1-phosphate using NAD^+ as coenzyme. Characterization of the oxidized product, thus far obtained, indicates its identity as ribulose 5-phosphate. With this finding, the complete metabolic pathway involving different *myo*-inositol phosphates during maturation and germination of mung bean seeds is delineated with a slight modification of our previous postulation (4).

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[1] The abbreviations used are: IP_6, *myo*-inositol hexaphosphate; *myo*-inositol-1-P, L-*myo*-inositol 1-phosphate; Ru-5-P, ribulose 5-phosphate.

EXPERIMENTAL PROCEDURES

Glucose 6-phosphate, 6-phosphogluconate, *myo*-inositol 2-phosphate, *myo*-inositol, ribulose-bisphosphate carboxylase, ribulose-5-phosphate kinase, and hexokinase were purchased from Sigma Chemical Co. L-*myo*-Inositol 1-phosphate was enzymatically synthesized by the method of Eisenberg (5) and was further purified by paper chromatography according to Pizer *et al.* (6). D-*myo*-Inositol 1-phosphate was obtained as a gift from Dr. A. L. Majumder. [6-^{14}C]Glucose, [1-^{14}C]glucose, and NaH[^{14}C]O_3 were obtained from Bhabha Atomic Research Centre, Bombay.

myo-Inositol-1-phosphate dehydrogenase has been partially purified by ammonium sulfate fractionation and thermoprecipitation. Surface-sterilized mung bean seeds were soaked in water for 4 h. The soaked seeds were decoated and homogenized in 2 volumes of 0.02 M Tris-HCl (pH 7.4) containing 0.001 M EDTA (Tris/EDTA buffer). The homogenate was centrifuged at 10,000 × *g* for 30 min. The pellet was rejected and the supernatant was subjected to ammonium sulfate fractionation. The 33 to 55% ammonium sulfate fraction was dissolved in Tris/EDTA buffer. It was warmed at 55°C for 2 min, chilled, and centrifuged at 20,000 × *g* for 20 min to remove precipitated protein. The supernatant was dialyzed for 6 h against Tris/EDTA buffer and was used as an enzyme source for the following experiments.

The dehydrogenase activity was measured spectrophotometrically. Unless otherwise stated, the reaction mixture contained 20 mM Tris-HCl (pH 8.6), 0.2 mM NAD^+, 0.25 mM *myo*-inositol-1-P, and an appropriate amount of protein (20 to 30 μg) in a total volume of 1 ml. Reaction was started by adding the enzyme. The increase of absorbance at 340 nm, due to the formation of NADH during *myo*-inositol-1-P oxidation, was followed at 30-s intervals over a period of 3 min at 28°C.

To study the decarboxylation reaction, the assay mixture was incubated in screw cap tube at 28°C for 30 min; the evolved CO_2 was trapped in NaOH-soaked paper. The paper was counted for radioactivity using a toluene fluor. The radioactive substrates *myo*-[6-^{14}C]inositol-1-P and *myo*-[1-^{14}C]inositol-1-P used in this experiment were prepared enzymatically. [1-^{14}C]Glucose was used for the synthesis of [1-^{14}C]glucose-6-P (7). The reaction was stopped by adding 20% trichloroacetic acid, the supernatant, after removing the precipitated protein, was neutralized, charged onto Dowex 1-Cl^- (200 to 400 mesh) column (0.5 × 8 cm) and was extensively washed with water to remove unreacted [1-^{14}C]glucose. [1-^{14}C]Glucose-6-P was eluted with 0.05 N HCl. It was concentrated, neutralized, and served for the synthesis of *myo*-[6-^{14}C]inositol-1-P using synthase from rat testis (5). Similarly, for the synthesis of *myo*-[1-^{14}C]inositol-1-P, [6-^{14}C]glucose was used as the starting material.

The coupled enzymatic reaction for the characterization of the product was carried out by the method of Latzko *et al.* (8). The putative product from the *myo*-inositol-1-P dehydrogenase system was incubated for 30 min at 20°C with ribulose-5-phosphate kinase and ribulose bisphosphate carboxylase in the presence of NaH[^{14}C]O_3 in a total volume of 0.5-ml reaction mixture. 0.1 ml of 20% perchloric acid was added to stop the reaction and also to destroy the excess NaH[^{14}C]O_3. A 0.02-ml aliquot was spotted on Whatman No. 3MM paper and counted for radioactivity in toluene fluor.

RESULTS

Properties of the Enzyme—Table I shows the activity of *myo*-inositol-1-P dehydrogenase toward a number of phosphosugars and sugars. It is apparent that the enzyme is highly specific for L-*myo*-inositol-1-P and can distinguish its optical isomer. The oxidation of *myo*-inositol-1-P, as measured by NADH formation, was linear with time for at least 3 min. Stoichiometry of the NAD^+ reduction, measured spectrophotometrically, and pentose phosphate formation, assayed by orcinol reagent and phosphate estimation after separation from other components of the reaction mixture by paper chromatography using solvent system A as mentioned in Fig. 1, revealed a ratio of 2:1. The optimum pH for enzyme activity

TABLE I

Substrate specificity of myo-inositol-1-P dehydrogenase

The 1-ml reaction mixture contained Tris-HCl (pH 8.6), 20 mM; NAD^+, 0.2 mM; an appropriately diluted aliquot of enzyme (20 to 30 μg of protein), and the concentration of the sugars and phosphosugars used were 1 mM.

Substrate	NAD^+ reduced
	nmol min^{-1} mg^{-1}
L-*myo*-Inositol 1-phosphate	340
D-*myo*-Inositol 1-phosphate	0
myo-Inositol 2-phosphate	0
Glucose 6-phosphate	0
Gluconate 6-phosphate	0
myo-Inositol	0
Glucose	0

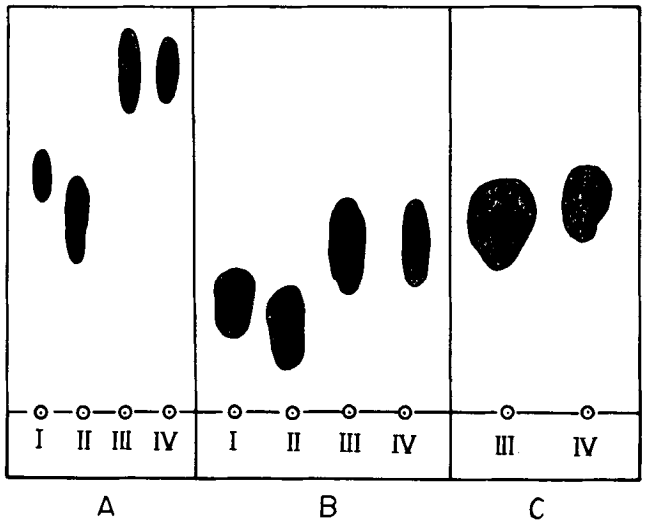

FIG. 1. **Paper chromatography of the product from *myo*-inositol-1-P dehydrogenase system in different solvents.** Paper chromatograms *A* and *B* were developed in descending manner for 24 h at 28°C. Chromatogram *C* was developed in ascending manner for 12 h at 28°C. Solvent compositions were: 95% ethanol:1 M ammonium acetate (7:3) for *A*, isopropyl alcohol:ammonia:water (7:1:2) for *B*, and isobutyric acid:ammonia:water (66:1:33) for *C*. Staining solution was alkaline $AgNO_3$. *I*, *myo*-inositol-1-P; *II*, 6-phosphogluconate; *III*, ribulose-5-P; and *IV*, the putative product from *myo*-inositol-1-P dehydrogenase.

TABLE II

Decarboxylation of myo-inositol-1-P

The assay mixture contained Tris-HCl (pH 8.6), 20 mM; NAD^+, 0.2 mM; *myo*-[6-^{14}C]inositol-1-P or *myo*-[1-^{14}C]inositol-1-P, 0.2 mM; and an appropriate aliquot of the enzyme (50 μg of protein). Specific activity of *myo*-[6-^{14}C]inositol-1-P and *myo*-[1-^{14}C]inositol-1-P were 3.3×10^5 cpm/μmol and 3.5×10^5 cpm/μmol, respectively.

Substrate	Experimental condition	$[^{14}C]O_2$ liberated
		cpm
myo-[6-^{14}C]Inositol-1-P	Complete	1460
	−Enzyme	75
	$-NAD^+$	400
myo-[1-^{14}C]Inositol-1-P	Complete	54
	−Enzyme	65
	$-NAD^+$	70

was 8.6 using Tris-HCl buffer throughout the pH range of 7.0 to 9.0. The monovalent and divalent ions: Na^+, K^+, NH_4^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, and the chelating agent EDTA, had no effect on enzyme activity. The enzyme reacted specifically with NAD^+ which could not be replaced by any of the other nucleotides thus far tested, *e.g.* $NADP^+$, FAD, and FMN.

Characterization of the Product—Table II shows the decarboxylation activity of *myo*-inositol-1-P dehydrogenase when specifically labeled substrates, *myo*-[1-^{14}C]inositol-1-P and *myo*-[6-^{14}C]inositol-1-P, were used. This study demonstrates the involvement of carbon atom 6 (C_6) of the inositol ring in dehydrogenation and decarboxylation by this enzyme system, because $[^{14}C]O_2$ was evolved only when *myo*-[6-^{14}C]inositol-1-P was used as the substrate. The small amount of $[^{14}C]O_2$ evolution when NAD^+ was omitted, as shown in Table II, is due to a slight contamination of *myo*-inositol-1-P with NAD^+. The product was separated from *myo*-inositol-1-P by paper chromatography using different solvents and R_F values were compared with different authentic compounds (Fig. 1). In all three solvent systems, the product has the same R_F value as that of authentic Ru-5-P. The product was assayed with the orcinol reagent (9) and the cysteine-carbazole reagent (10), respectively. In the orcinol assay, the spectrum of the colored complex gave two absorption peaks, one at 580 nm (small) and other at 660 nm (large) which were identical with those of Ru-5-P. It was also observed that, upon hydrolysis of the phosphate group, the absorption at 580 nm increased. In the cysteine-carbazole

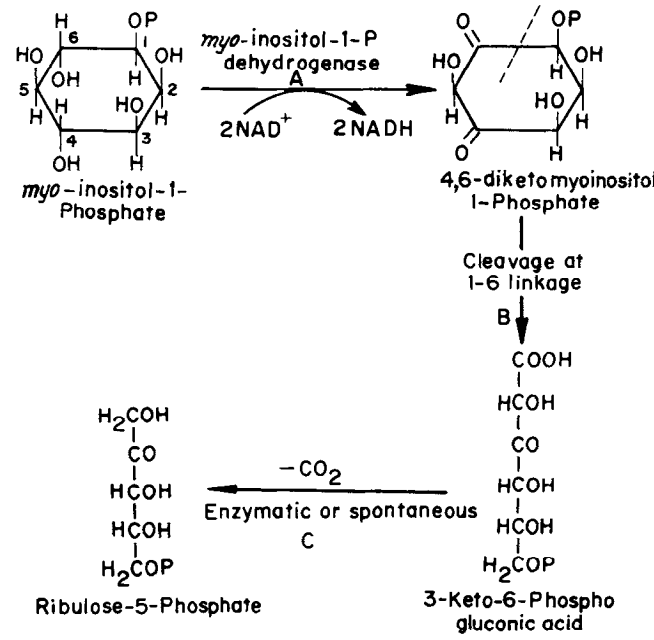

FIG. 2. **Suggested pathways for conversion of *myo*-inositol 1-phosphate to ribulose 5-phosphate.** The pathways are represented by *A*, *B*, and *C*. The *myo*-inositol molecule is cleaved between carbon atoms 1 and 6. The hydroxyl group at C_2 of *myo*-inositol is the axial one; the remaining five hydroxyl groups are equatorial.

TABLE III

Characterization of the product using coupled enzymes as a probe

A 0.5-ml reaction mixture contained Tris/EDTA buffer (pH 7.6), 60 mM; $MgCl_2$, 10 mM; $NaH[^{14}C]O_3$, 10 mM (specific activity, 1.5×10^6 cpm/μmol); ATP, 1 mM; reduced glutathione, 4 mM; ribulose-bisphosphate carboxylase, 4 units; ribulose-5-phosphate kinase, 8 units; and Ru-5-P, 0.025 μmol in the control tube. In the experimental tube, Ru-5-P was substituted by 0.025 μmol of the product (assayed by phosphate estimation) from *myo*-inositol-1-P dehydrogenase system. The blank tube contained all the above mentioned constituents except Ru-5-P and the product from *myo*-inositol-1-P dehydrogenase system.

System	$[^{14}C]O_2$ incorporated	
	cpm/20 μl	*total corrected cpm*
Blank	62	
Control	490	12,800
Experimental	540	14,300

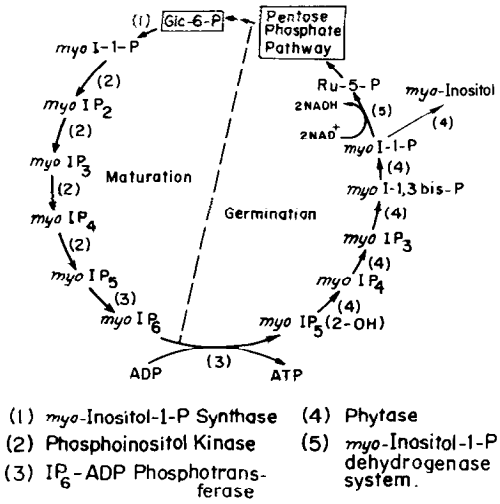

FIG. 3. **Proposed metabolic cycle involving *myo*-inositol phosphates during formation and germination of seeds.** *myo-I-1-P*, *myo*-inositol 1-phosphate; *myo-IP*$_2$, *myo-IP*$_3$, *myo-IP*$_4$, *myo-IP*$_5$, and *myo-IP*$_6$ correspond to di-, tri-, tetra-, penta-, and hexaphosphates of *myo*-inositol, respectively.

reaction, the absorption maximum was observed at 540 nm. The product was further confirmed as Ru-5-P by a coupled enzymatic reaction (Table III). The experiment was based on the principle that [^{14}C]O_2 would be fixed by the coupled enzyme system leading to the synthesis of 3-keto-6-phosphogluconic acid only when Ru-5-P was used as substrate. It is evident from Table III that [^{14}C]O_2 is incorporated into the product of *myo*-inositol-1-P dehydrogenase by the coupled enzyme system.

DISCUSSION

It is evident that the putative product of this enzyme system is Ru-5-P. Further, the stoichiometry of the reaction indicates that two sites of the inositol ring are oxidized simultaneously. Since in the decarboxylation reaction it is observed that C_6 is eliminated as CO_2, the involvement of C_6 as one of the sites of oxidation is thus clearly demonstrated. The other site of oxidation is most probably the C_4 of the inositol ring giving rise to Ru-5-P as the final product. It is yet to be resolved as to whether the decarboxylation is mediated by the same or by a different enzyme or whether it takes place spontaneously by a β-keto decarboxylation of the presumed intermediate, 3-keto-6-phosphogluconic acid (Fig. 2).

Oxidation of the axial as well as the equatorial hydroxyl group of the inositol ring has been demonstrated (9, 11). Our results indicate that the oxidation catalyzed by this enzyme involves not the axial but two equatorial hydroxyl groups. As a possible mechanism for the formation of Ru-5-P, we would like to suggest a cleavage of the bond between carbon atoms 1 and 6 of the inositol ring. The presence of this enzyme system provides a link between the metabolic pathway of the inositol phosphates during germination of seed with the pentose phosphate cycle as depicted in Fig. 3. That the pentose phosphate cycle is most active during the early period of germination (12) could also be explained by the proposed cycle. Further purification of the enzyme and characterization of the product and its intermediates are currently under investigation.

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