Communication

Chloroplast as a Locale of L-myo-Inositol-1-Phosphate Synthase[1]

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ABSTRACT

Chloroplasts from 5 to 7 day old *Vigna radiata* seedling, grown under alternate light/dark conditions or from green *Euglena gracilis* Z. cells have been found to harbor L-*myo*-inositol-1-phosphate synthase (EC 5.5.1.4) activity. In contrast, dark-grown *V. radiata* seedlings, or streptomycin-bleached *Euglena* cells exhibit either reduced or no enzyme activity. An apparent enhancement of the chloroplastic inositol synthase by growth in presence of light is observed.

An irreversible NAD-dependent oxidation-reduction reaction brings about the cyclization of glucose 6-P (G-6-P) to *myo*-inositol 1-P. The enzyme catalyzing this reaction, L-*myo*-inositol 1-P synthase (EC 5.5.1.4, hitherto referred to as synthase) has been reported from a number of eukaryotic sources (18). In each case, the activity was isolated as constituent of the soluble cytosolic fraction of the cell preparation.

In the green plant chloroplast, about 10% of the membrane phospholipids consist of phosphoinositides (17) which require an endogenous pool of *myo*-inositol for their synthesis. Impermeability of the chloroplast membrane to the cyclitol (23), casts doubt as to the cytoplasmic origin of the chloroplastic inositol and suggests that the plastid is a site for synthesis of *myo*-inositol. Support for this view is found in the report that labeled glucose is enzymically incorporated into *myo*-inositol by acetone-treated chloroplast preparation from peas (17). In addition to an earlier report from this laboratory (8), Loewus *et al.* (19) find inositol synthase activity present in both soluble and chloroplastic fractions of *Euglena gracilis* Z.

The present work was undertaken to determine if the synthase is present in chloroplasts of an alga, *E. gracilis* and a higher plant, *Vigna radiata*.

MATERIALS AND METHODS

Tris, BSA, D-glucose 6-P (disodium salt), D-fructose 6-P (disodium salt), β-NAD, DEAE-cellulose (D-8382), and streptomycin sulfate were from Sigma Chemical Co., Ultrotgel AcA34 was from LKB, France; 2-mercaptoethanol, sodium metaperiodate, sucrose, and riboflavin were from British Drug House. Beef extract paste was from Biochemical and Synthetic Products,

Hyderabad, India; tryptone was from Lab. Chem. Industry, Calcutta, and Vit. B_{12} was from Loba Chemie, Bombay; sodium sulfite was from May and Baker Ltd., and ammonium molybdate was from J. T. Baker Chemical Co. Acrylamide, N,N'-methylene bis-acrylamide and N,N,N',N'-tetramethylenediamine(TEMED) were from Bio-Rad Laboratories. All other reagents were of analytical grade.

Seeds of *Vigna radiata* (variety B-1), from the Oil and Pulse Research Station, Berhampore, West Bengal, India, were allowed to germinate for the required time period at $30 + 2°C$ over moist filter paper in large Petri dishes. Unless otherwise stated, the seeds were exposed to alternate light/dark period each of 12 h duration during germination. Pure cultures of *Euglena gracillis* Z. obtained from the National Chemical Laboratory, Pune, India, were maintained under laboratory conditions as described earlier (8). Nongreen *E. gracilis* cells were obtained by treatment of the green cells with streptomycin-sulfate (16).

Chloroplasts of *E. gracilis* or *V. radiata* leaves were isolated following the method of Hachtel (15) with minor modifications. Seven-d-old cultures of *E. gracilis* or leaves of 6-d-old *Vigna* seedlings were homogenized with 5 volumes of 20 mM Tris acetate (pH 7.5) containing 0.35 M sucrose, 10 mM $MgCl_2$, 100 mM KCl, 1 mM 2-mercaptoethanol, and 2 mg/ml of BSA. The supernatant obtained after centrifugation at 400g for 5 min, was spun at 2000g for 15 min. The resultant chloroplast pellet was washed at least three times with the homogenizing medium. The purity of the chloroplast preparation was checked by phase contrast as well as by transmission electron microscopy. For transmission electron microscopy, isolated chloroplasts from both *V. radiata* and *E. gracilis* were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), post-fixed in 1% O_2O_4 in 0.1 M cacodylate buffer, dehydrated in alcohol and propylene oxide and finally embedded in Epon 812. Ultrathin sectioning were done in the LKB Nova ultramicrotome, thin sections stained in uranyl acetate and Pb-citrate and observed under a JEOL electron microscope at the Regional Scientific Instrumentation Centre, Calcutta. Chloroplasts were further characterized by the ability of the preparation to carry out the characteristic Hill reaction using dichlorophenol-indophenol (DCIP) as the electron acceptor. The isolated chloroplasts were washed with chilled 50 mM Tris-acetate (pH 7.5) containing 0.2 mM mercaptoethanol, and homogenized in 5 volumes of the same buffer. The homogenate was centrifuged at 8,500g in a Remi K24A refrigerated centrifuge for 20 min. The 8,500g supernatant was used as the chloroplastic synthase source in experiments described in Table I. For purification of the activity the low speed supernatant was made 80% saturated with $(NH_4)_2SO_4$. The dialyzed $(NH_4)_2SO_4$ fraction was adsorbed in a DEAE-cellulose column (6 × 1.3 cm), equilibrated previously with 50 mM Tris-

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Table I. *Plastidial myo-Inositol 1-P Synthase Activity from V. radiata Seedling and E. gracilis*

Plastids from different sources were isolated as described in "Materials and Methods." Isolated plastids were homogenized in 5 volumes of 50 mM Tris-acetate buffer (pH 7.5) containing 0.2 mM mercaptoethanol and the homogenate centrifuged at 8,500 g for 20 min. The supernatant was assayed for the enzyme activity.

Source of Plastids	Growth Conditions	Stage	Range of Specific Activity	Average of Specific Activity
			nmol myo-inositol-1-P-formed/h·mg protein	
V. radiata				
Normal	Alternate light/dark (12 h each)	6-day seedling	150–450 (7)[a]	315
Etiolated	Continuous darkness	6-day seedling	48–150 (4)	84
E. gracilis				
Normal	Alternate light/dark (12 h each)	7-day culture	40–95 (5)	75
Streptomycin sulphate bleached	Alternate light/dark (12 h each)	7-day culture	0–0 (3)	0

[a] Number in parentheses indicate number of experiments performed.

acetate (pH 7.5) containing 0.2 mM mercaptoethanol and washed with two bed volumes of the same buffer. Synthase was eluted from the column with a linear gradient of 0 to 0.5 M NH₄Cl in Tris-acetate (pH 7.5). Fractions of 1.0 ml/4 min were collected and the active fractions were pooled and concentrated in preparation for filtration through Ultrogel ACA34 gel, equilibrated in 50 mM Tris-acetate (pH 7.5), containing 0.2 mM mercaptoethanol. The enzyme was eluted with the same buffer.

The synthase was assayed colorimetrically by the periodate oxidation method of Barnett *et al.* (3) and further corroborated by the inositol-1-phosphatase assay as described earlier (8). The procedure was as follows: assay mixture contained 50 mM Tris acetate (pH 7.5), 14 mM NH₄Cl, 0.8 mM NAD⁺, 5 mM mercaptoethanol, 5 mM glucose 6-P and enzyme in a total volume of 0.5 ml. Suitable blanks in which addition of enzyme was omitted, and zero time controls were also run. After incubation at 37°C for 1 h, the reaction was terminated by 0.2 ml of 20% (w/v) TCA. To the deproteinized supernatant (0.7 ml, total volume) was added an equal volume of 0.2 M NaIO₄, followed by incubation at 37°C for 1 h for oxidation of the synthase reaction product, *myo*-inositol 1-P, with concomitant release of inorganic phosphate. The excess periodate was destroyed by 1.4 ml. of 1 M Na₂SO₃. Parallel to the periodate set, appropriate nonperiodate controls, in which NaIO₄ and Na₂SO₃ treatment were omitted were also run. Inorganic phosphate was estimated from both sets, periodate and nonperiodate, by the method of Chen *et al.* (7). The set without NaIO₄ gave the amount of inorganic phosphate released by nonspecific phosphatases and the difference between the sets with and without NaIO₄ gave the actual synthase activity measured in terms of nmol of *myo*-inositol 1-P produced per mg protein per h. Alternatively, inositol 1-P was quantitated by the inositol 1-P phosphatase catalysis method (12). There was an agreement between the two methods of assay as reported earlier (5). Assay of the enzymes, *e.g.* ribulose 1,5-bisP carboxylase (EC 4.1.1.39), NADP-glyceraldehyde 3-P dehydrogenase (EC 1.2.1.12), and glucose 6-P dehydrogenase (EC 1.1.1.49) were performed as described (4, 14, 22). The enzymes, inositol-1-phosphatase and L-*myo*-inositol 1-P dehydrogenase were prepared and assayed by the method of Eisenberg (12) and De and Biswas (10), respectively. The method of Lowry *et al.* (20) was followed for estimation of protein. Chl content was determined by the method of Arnon (1).

RESULTS AND DISCUSSION

While continuing our study on the cytosolic inositol synthase from the green alga *Euglena gracilis* (8), it was noted that a considerable portion of the enzyme activity could be recovered in a low-speed particulate fraction. Similar studies with light-grown *Vigna radiata* seedling revealed that while in the 2-d-old seedling most of the synthase activity could be localized in the high-speed supernatant (*i.e.* cytosolic) fraction, a large proportion of synthase activity could be recovered in the green low-speed particulate fraction from the 5- to 7-d-old seedlings. These results prompted us to investigate as to whether the chloroplasts from both the sources harbor the inositol synthase, in addition to the soluble cytosolic activity.

A critical aspect of the localization of the inositol synthase, as described in the present work, has been the identification of the subcellular fractions obtained from both the systems. In addition to the phase contrast and electron microscopic methods employed for this purpose and described in "Materials and Methods," such identification was made by assay of the marker enzymes for both chloroplastic and the cytosolic fractions. Ribulose 1, 5-bisP carboxylase and NADP-glyceraldehyde 3-P dehydrogenase activities were found predominant in the chloroplastic fractions, the former being undetectable and the latter present only to an extent of less than 10% in the cytosolic fractions. The cytosolic fraction, on the other hand, showed appreciable glucose 6-P dehydrogenase activity in contrast to the chloroplastic fraction exhibiting near-absence of the same.

Soluble supernatant (8500g) fractions obtained (as described in "Materials and Methods") from chloroplasts isolated from leaves of 6-d-old *V. radiata* seedling and 7-d-old cultures of *E. gracilis* exhibit appreciable inositol synthase activity (Table I). The organelle-bound activity was also determined in plastids from dark-grown *V. radiata*, although the activity in this case was 30 to 50% of that obtained from alternate light/dark grown seedlings. In contrast, plastids isolated from *Euglena* cells, bleached completely with streptomycin sulfate, exhibit no synthase activity (Table I). The results suggest the presence of synthase in the chloroplasts as also their enhanced activity in higher plant and algal chloroplasts under normal and/or light-mediated growth.

The authenticity of the synthase in both *E. gracilis* and *V. radiata* was established as follows: synthase from both fractions were specifically and absolutely dependent on glucose 6-P and
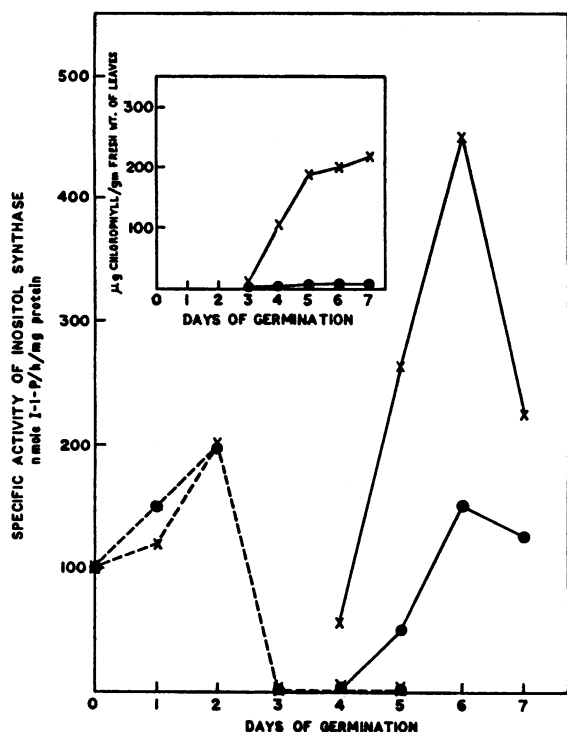
FIG. 1. Cytosolic and plastidial myo-inositol 1-P synthase activity in V. radiata seedling under variable conditions. (x– – –x) and (●– – –●) represent cytosolic inositol synthase activity in germinating Vigna seeds grown under alternate light/dark (12 h each) conditions and complete darkness, respectively. (x——x) and (●——●) indicate plastidial synthase activity under alternate light/dark and complete dark conditions. Inset presents the total content of Chl from seedling of Vigna grown under alternate light/dark conditions (x——x) and complete darkness (●——●). For obtaining a cytosolic fraction, seeds of V. radiata were germinated under conditions as indicated, homogenized in 5 volumes of 20 mM Tris-acetate buffer (pH 7.5) containing 1 mM 2-mercaptoethanol and centrifuged at 50,000 rpm for 60 min in a Beckman L5-50 ultracentrifuge. Supernatant was dialyzed against 100 volumes of homogenizing buffer for 6 h with one change and used as the cytosolic synthase source. Assay of the synthase from the chloroplasts was performed with the 8,500g supernatant obtained from isolated chloroplasts as described in "Materials and Methods."

NAD as substrate and co-factor, respectively. Identification of the reaction product as L-myo-inositol 1-phosphate was obtained by enzymic assay of the putative synthase product, pooled from a number of incubation mixture, by its specific cleavage by inositol 1-phosphatase (12), and by specific NAD-dependent utilization by L-myo-inositol 1-P dehydrogenase (10).

An enriched fraction (10-fold purified) of the chloroplastic synthase was obtained from the leaves of 6-d-old V. radiata seedlings grown under alternate light/dark condition by the procedure outlined in "Materials and Methods." On polyacrylamide gel electrophoresis under nondissociating conditions (9), the preparation revealed presence of at least 10 to 12 protein bands of which the fourth band from top was associated with the synthase activity.

The partially purified chloroplastic synthase from V. radiata records nonspecific phosphatase activity to a maximum of 7 to 10% compared to the inositol synthase activity. the K_m for glucose 6-P and NAD have been determined to be around 1.8 and 0.13 mM, compared to the values of 1.18×10^{-3} M and 8×10^{-6} M for the Saccharomyces cerevisiae enzyme (11) and 3.8 and 17.9 mM for the enzyme from rat testes (21). Plastidial inositol synthase from V. radiata operates optimally at a pH of

7.5 to 7.75. A 2-fold stimulation of the enzyme was recorded in presence of 9 mM NH_4Cl.

A specific light effect of the chloroplastic synthase in contrast to the cytosolic enzyme in germinating V. radiata seedling is demonstrated in Figure 1. In these experiments, both cytosolic and plastidic synthase(s) were monitored in germinating V. radiata seeds grown under alternate light/dark conditions or complete darkness up to the 7-d seedling stage. As evident, the cytosolic synthase activity was detectable even in the ungerminated seeds, increases at the 2-d stage, and drops to an undetectable titre from 3-d onwards, a stage when the green leaves begin to emerge. At stage 3 to 4 d, soluble extracts from whole tissue showed no detectable enzyme activity, under the assay conditions. Although unexpected, no explanation can be offered for this behavior at this stage. The titer or the pattern of the cytosolic synthase activity remains unaltered in seeds germinated under alternate light/dark or complete darkness. Such growth conditions, however, bring about a remarkable change in the chloroplastic synthase activity. The plastidial synthase from dark-grown seedling was detected between 5- to 7-d stage, showing highest enzyme activity in the 6-d plastids. The activity in this case was enhanced at least 2- to 3-fold in seedlings grown under alternate light/dark conditions. The greenness of the light-grown and etiolated seedlings were monitored by determination of the Chl content of the leaves at the 6-d stage (inset, Fig. 1).

A large number of chloroplastic enzymes are known to be photoregulated and various mechanisms have been postulated to account for their activation (6). The apparent light-effect of the chloroplastic inositol synthase, as demonstrated here, requires further study for complete elucidation of the mechanism.

The present work leads to an interesting question. Is it possible that besides being dephosphorylated to free myo-inositol, the L-myo-inositol 1-P, generated by the synthase, may lead to the formation of ribulose 5-P by a mechanism analogous to the L-myo-inositol 1-P dehydrogenase reaction? Such activity, although reported from germinating seeds (10, 13), is yet to be demonstrated in chloroplasts. Provided that the green plant chloroplasts harbor such an activity, it is tempting to speculate that the synthase-dehydrogenase couple may emanate as an alternate route for ribulose 5-P generation for the subsequent phosphorylation (2) to ribulose 1,5-bisP, the photosynthetic CO_2 acceptor.

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