Biosynthesis of Phosphatidylglycerol in Isolated Mitochondria of Etiolated Mung Bean (Vigna radiata L.) Seedlings

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Phosphatidylglycerophosphate synthase (sn-glycerol-3-phosphate:CDP-diacylglycerol phosphatidyltransferase) and phosphatidylglycerophosphate phosphatase were characterized in mung bean (Vigna radiata L.) mitochondria. The synthase has a rather broad pH optimum between 7 and 9, whereas the phosphatase has one of about 7. Both enzymic activities are stimulated by Triton X-100 and require divalent cations but differ in their cation specificities. The synthase shows apparent $K_m$ values of 9 and 3 $\mu$M for sn-glycerol-3-phosphate and CDP-diacylglycerol, respectively. Phosphatidylglycerophosphate, in contrast to lysophosphatidic and phosphatidic acid, is effectively dephosphorylated by the phosphatase, which exhibits an apparent $K_m$ value of 12 $\mu$M for its substrate. Each enzyme shows higher activities with the dipalmitoyl species of its substrate than with the dioleoyl species. These substrate specificities of both enzymes are predominantly based on differences in apparent $V_{max}$ values.

In plant cells not only plastids and ER but also mitochondria are capable of de novo biosynthesis of glycerolipids. Mitochondria, like plastids, are semiautonomous with regard to the formation of their membrane lipids and have to import phospholipids from the ER. The biosynthesis capacity of mitochondria is largely confined to acylGroPGro and dihydroxyphosphatidylglycerol (cardiolipin), the characteristic and functionally important glycerolipids of these organelles (Moore, 1982; Kinney, 1993; Robinson, 1993).

Depending on plant species and organ, acylGroP is formed either in both mitochondrial membranes or in the outer one (Sparace and Moore, 1979; Frenzten et al., 1990). On the other hand, all enzymic activities that catalyze the reaction sequence from acylGroP to acylGroPGro (Fig. 1), namely CTP:phosphatidate cytidylyltransferase, PGPS, and PGPase, are located in the inner membrane (Douce et al., 1972; Sparace and Moore, 1979). Within this membrane acylGroPGro presumably serves as a substrate for the biosynthesis of cardiolipin (Fig. 1). Recently, the conversion of acylGroPGro into cardiolipin was clearly demonstrated by Schlame et al. (1993) in isolated plant mitochondria for the first time.

Little is known about the properties of the PGPS, PGPase, and cardiolipin synthase of plant mitochondria (Douce and Dupont, 1969; Moore, 1974; Schlame et al., 1993). So far, kinetics and requirements of the PGPS from mitochondria of castor bean endosperm have been determined (Moore, 1974), whereas PGPase activities have not been measured independently of PGPS activities in plant organelles. Furthermore, direct evidence is still missing about whether the plant cardiolipin synthase, as for the enzyme of other eukaryotes (Daum, 1985; Tamai and Greenberg, 1990; Schlame et al., 1993), utilizes CDP-acylGroP in the synthesis of cardiolipin (Fig. 1). In addition, the mechanisms that cause the different fatty acid patterns of acylGroPGro and cardiolipin from plant mitochondria, as well as the origin of polyunsaturated C18 fatty acids, which are esterified in a distinctly higher proportion in cardiolipin than in acylGroPGro (Bligny and Douce, 1980; Fuchs et al., 1981; Edman and Ericson, 1987; Dorne and Heinz, 1989), have not been elucidated.

To approach these problems, we have continued our experiments on de novo biosynthesis of glycerolipids in plant mitochondria. In this paper the properties of both PGPS and PGPase in isolated mitochondria from etiolated mung bean (Vigna radiata L.) seedlings are presented and their substrate specificities are discussed with respect to the fatty acid composition of the glycerolipids synthesized in mitochondria.

MATERIALS AND METHODS

Chemicals

[U-14C]GroP (5.88 kBq nmol⁻¹) was purchased from Amer sham Buchler and CDP-decanoylGroP, CDP-palmitoylGro, CDP-oleoylGro, and CDP-acylGroP from egg lecithin were obtained from Serva Research Laboratories (London, Ontario, Canada) or Sigma.

Palmitoyl- and oleoylGroP[U-14C]GroP were synthesized enzymically from the respective species of CDP-acylGroP and [U-14C]GroP in a way similar to that described by Tamai and Greenberg (1990). The assay contained 0.1 M Tricine-NaOH, pH 8.2, 0.1 M MgCl₂, 0.5% (w/v) Triton X-100, 300 $\mu$M CDP-palmitoylGro or 300 $\mu$M CDP-oleoylGro, 130 $\mu$M [U-14C]-GroP (1.4 kBq nmol⁻¹), and 20 $\mu$g of crude membrane protein of Escherichia coli strain W3110/pPGPL3008, which overproduces PGPS, in a total volume of 2 mL. After a 30-min experiment on de novo biosynthesis of glycerolipids in plant mitochondria. In this paper the properties of both PGPS and PGPase in isolated mitochondria from etiolated mung bean (Vigna radiata L.) seedlings are presented and their substrate specificities are discussed with respect to the fatty acid composition of the glycerolipids synthesized in mitochondria.

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incubation at 37°C, lipids were extracted and acyl2GroPGroP was purified by TLC (Kelly and Greenberg, 1990). The yield was about 220 kBq of acyl2GroP[U-14C]GroP.

Purification of Mitochondria

Mitochondria were isolated from 3-d-old mung bean (Vigna radiata L.) seedlings germinated at 30°C in the dark according to the procedure of Neuburger et al. (1982), but instead of phosphate buffer Mops-NaOH was used in all media. This procedure resulted in an efficient separation of mung bean mitochondria from plastidial and ER membranes. Purified mitochondria were finally resuspended in 20 mM Pipes-KOH, pH 7.2, and 5 mM DTT, mixed with glycerol to a final concentration of 50% (v/v), and either used directly for enzyme assays or stored at -20°C. The protein concentration in mitochondrial fractions was determined according to the method of Bradford (1976).

Enzyme Assays

PGPS was determined by monitoring the incorporation of [U-14C]GroP into chloroform-soluble products in the following standard assay unless otherwise stated. The 100-μL assay consisted of 0.2 mM Pipes-KOH, pH 7.3, 3 mM MnCl₂, 0.1% (w/v) Triton X-100, 8 mM DTT, 30 μM CDP-palmitoylGroGro, 65 μM [U-14C]GroP (1.4 kBq nmol⁻¹), and up to 80 μg of mitochondrial protein. The reaction was terminated and lipophilic products were extracted after 30 min at 30°C according to the method of Hajra (1974). For the determination of total radioactivity and for the analysis of the reaction products, 1 mL of the chloroform layer was used.

To determine PGPase activity, standard incubations were performed for 10 min at 30°C in the presence of 0.1 M Mops-KOH, pH 7, 0.2% (w/v) Triton X-100, 1 mM MgCl₂, 20 μM acyl2GroP[U-14C]GroP (1.4 kBq nmol⁻¹), and up to 20 μg of mitochondrial protein in a total volume of 50 μL. After incubation lipids were extracted with 240 μL of chloroform:methanol (1:1, v/v) containing 50 μg of acyl2GroPGro and 100 μL of 0.2 M H₃PO₄ and 1 M KCl. After phase separation 100 μL of the chloroform layer were used for separating substrate and reaction product by TLC.

Lipid Analysis

Reaction products of the enzyme assays were routinely separated by TLC on Silica-gel 60 plates in chloroform:methanol:glacial acetic acid (65:25:8, v/v). To confirm the identity of the products, they were rechromatographed in different solvent systems, such as chloroform:methanol:glacial acetic acid:water (50:25:4:8, v/v) or chloroform:methanol:concentrated ammonia (65:25:4, v/v). In addition their water-soluble products obtained by deacylation with 0.1 M sodium methoxide were separated on cellulose plates in isopropanol:concentrated ammonia:water (7:3:1, v/v) or in ethanol:1 M ammonium acetate (pH 7.4) (65:35, v/v) (Chang and Kennedy, 1967a; Poorthuis and Hostetler, 1975). Labeling within the polar head groups of the reaction products was confirmed by analyzing their hydrolysis products after phospholipase C digestion (Zwaal and Roelofsen, 1974). Radioactivity on plates was detected by scanning the plates with an automatic TLC linear analyzer and quantified by scintillation counting.

RESULTS AND DISCUSSION

Assay Conditions of PGPS

Assay conditions of PGPS from mung bean mitochondria were optimized using [U-14C]GroP as tracer molecules. Incorporation of GroP into lipophilic reaction products was strictly dependent on exogenously added CDP-acyl1Gro (Table I), suggesting that the endogenous CDP-acyl1Gro pool within the mitochondrial membranes was negligibly low. The analysis of the reaction products revealed that acyl1GroPGroP and acyl2GroPGro were labeled (Table I) and that both products carried the labeling in their polar head groups. These results

<table>
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<th>Table I. Labeling rates of total lipophilic products and the individual ones by mung bean mitochondria from [U-14C]GroP and CDP-palmitoyl1Gro</th>
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<tbody>
<tr>
<td>Standard Assay</td>
</tr>
<tr>
<td>Complete</td>
</tr>
<tr>
<td>Minus CDP-acyl1Gro</td>
</tr>
<tr>
<td>Minus DTT</td>
</tr>
<tr>
<td>Plus HgCl₂</td>
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<tr>
<td>50 μM</td>
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<td>500 μM</td>
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are consistent with the biosynthesis of acyl\textsubscript{1}GroPGro from GroP and CDP-acyl\textsubscript{1}Gro via PGPS and PGPase activity (Fig. 1). Under standard assay conditions acyl\textsubscript{1}GroPGro was formed as the main product (Table I). These results suggested that mung bean mitochondria displayed distinctly higher PGPase than PGPS activity as described for the organelles of castor bean endosperm (Moore, 1974). But the proportion of the two products and, thus, the relative activities of the two enzymes varied in dependence on the assay conditions (Table I; Fig. 2).

Total incorporation rates of GroP as a function of the pH of the reaction mixture showed that the mitochondrial PGPS has a rather broad pH optimum of about 7.5 (Fig. 2A). Acyl\textsubscript{1}GroPGro was, however, formed as the main product at a pH value of about 7 only (Fig. 2A). These results indicate that the PGPase has a sharper pH optimum than the PGPS (see below). Highest PGPS activity was measured in Pipes-KOH buffer, whereas its activity was slightly lower in Mops-KOH and distinctly lower in phosphate buffer. On the other hand, Pipes-KOH concentrations from 50 to 300 mM hardly altered the PGPS activity.

As depicted in Figure 2C, PGPS activity was appreciably stimulated by Triton X-100, and maximal activity was measured with 0.1% (w/v) Triton X-100. Furthermore, the PGPS activity required divalent cations (Fig. 2B). Highest activities were obtained in the presence of Mn\textsuperscript{2+} in concentrations of about 3 mM. Stimulation of PGPS activity by Mg\textsuperscript{2+} was distinctly lower than with Mn\textsuperscript{2+} (Fig. 2B). Even at high Mg\textsuperscript{2+} concentrations of up to 100 mM, the activity was about 3-fold lower than in the presence of 3 mM Mn\textsuperscript{2+}. Concentrations of Ca\textsuperscript{2+} up to 10 mM did not stimulate the enzymic activity. DTT within the reaction mixture affected the incorporation rates of GroP only slightly (Table I), but it was found to stabilize PGPS activity during storage at -20°C.

Maximal incorporation rates of GroP were attained at 60 \mu M GroP and 20 \mu M CDP-palmitoyl\textsubscript{1}Gro. Under these conditions the incorporation rates were constant for at least 30 min and proportional to the mitochondrial protein added up to 80 \mu g. First kinetic analyses were carried out by determining the PGPS activity at various concentrations of CDP-palmitoyl\textsubscript{1}Gro at different fixed levels of GroP. The initial velocity pattern thus obtained indicates a sequential reaction mechanism as described for the PGPS of E. coli (Hirabayashi et al., 1976). Apparent \( K_{m} \) values of 9 and 3 \mu M were calculated for GroP and CDP-palmitoyl\textsubscript{1}Gro\textsuperscript{P}, respectively. These values are very similar to those reported for the mitochondrial PGPS from castor bean endosperm, which also displayed highest activities at pH 7.5 in the presence of Mn\textsuperscript{2+} and Triton X-100 (Moore, 1974). Thus, the mitochondrial PGPS from plant tissue investigated so far have very similar properties. With regard to their strict dependence on divalent cations they resemble the respective enzymes from plastids and microsomes (Marshall and Kates, 1972; Moore, 1974; Andrews and Mudd, 1984), as well as from bacterial cells (Chang and Kennedy, 1967a; Hirabayashi et al., 1976; Carman and Wieczorek, 1980). Their properties, however, differ from the mitochondrial PGPS from yeast and mammalian cells, the activities of which are hardly influenced by divalent cations (Carman and Belunis, 1983; Daum, 1985; Karkhoff-Schweizer et al., 1991).

**Assay Conditions of PGPase**

As mentioned above, the proportion of the two reaction products acyl\textsubscript{1}GroP and acyl\textsubscript{1}GroPGro formed by mung bean mitochondria from [U-\textsuperscript{14}C]GroP and CDP-acyl\textsubscript{1}Gro varied in dependence on the assay conditions (Fig. 2) and, thus, indicates differences in the properties of PGPS and PGPase. To determine the PGPase activity independently of the PGPS activity, mitochondrial fractions of mung bean seedlings were incubated with exogenously added palmitoyl\textsubscript{1}GroP[U-\textsuperscript{14}C]-GroP. The analyses of the reaction products showed that palmitoyl\textsubscript{1}GroP[U-\textsuperscript{14}C]Gro\textsuperscript{P} was converted to labeled palmitoyl\textsubscript{2}GroPGro only. As depicted in Figure 3A, the PGPase displayed a pH optimum of about 7 as deduced from experiments with in situ synthesized acyl\textsubscript{1}GroPGro (Fig. 2A). Similar to PGPS, PGPase was stimulated by divalent cations (Fig. 3B) and by Triton X-100 (Fig. 3C). In contrast to PGPS, PGPase showed highest activities in the presence of 1 mM Mg\textsuperscript{2+}, whereas Mn\textsuperscript{2+} in concentrations higher than 1 mM even inhibited the enzymic activity (Fig. 3B). Substituting Co\textsuperscript{2+} or Ca\textsuperscript{2+} for Mn\textsuperscript{2+} gave results similar to those obtained with Mn\textsuperscript{2+} (Fig. 3B).

A dependence on Mg\textsuperscript{2+} has also been reported for the PGPase activity in pea chloroplasts (Andrews and Mudd, 1984, 1985). Similarly to the plastidial enzyme (Andrews and
Mudd, 1985), as well as that of cauliflower mitochondria (Doucoure and Dupont, 1969), but unlike that of spinach leaf microsomes (Marshall and Kates, 1972), 0.5 mm HgCl₂ completely inhibited the PGPase activity of mung bean mitochondria but hardly affected the PGPS activity (Table I).

With regard to its pH optimum, its dependence on Mg²⁺, and its stimulation by Triton X-100, the PGPase of mung bean mitochondria resembles more closely the respective enzyme of E. coli (Chang and Kennedy, 1967b) than those of yeast and mammalian mitochondria (MacDonald and McMurray, 1980; Kelly and Greenberg, 1990).

The dephosphorylation rates of palmitoyl2GroPGroP by mung bean mitochondria increased with increasing substrate concentrations up to 60 μm giving maximal rates of 12 pKat mg⁻¹ mitochondrial protein. The apparent Kₘ for palmitoyl₂GroPGroP was 12 μm, which is closer to the value reported for the enzyme from yeast mitochondria (Kelly and Greenberg, 1990) than to that for the enzyme from E. coli (Chang and Kennedy, 1967b) and mammalian mitochondria (MacDonald and McMurray, 1980). To save substrate, PGPase assays were routinely carried out with 20 μm palmitoyl₂GroPGroP, giving dephosphorylation rates that were proportional to the amount of protein added up to 25 μg and constant for at least 15 min.

In summary, optimal assay conditions for PGPase from mung bean mitochondria differ in certain aspects from those for PGPS. The two enzymatic activities can be affected differently, especially by divalent cations and pH of the reaction mixture (Figs. 2 and 3). Under optimal conditions PGPase displayed distinctly higher activities than PGPS. This appears to be also true under in vivo conditions since acyl₂GroPGroP, giving dephosphorylation rates that were proportional to the amount of protein added up to 25 μg and constant for at least 15 min.

A comparison of the presented results with those reported for PGPS and PGPase of other organisms reveals that both enzymatic activities from mung bean mitochondria resemble more closely the respective enzymes of E. coli (Chang and Kennedy, 1967a, 1967b) than those of mitochondria from mammalian and yeast cells (MacDonald and McMurray, 1980; Carman and Belunis, 1983; Daum, 1985; Kelly and Greenberg, 1990; Karkhoff-Schweizer et al., 1991).

### Table II. Substrate specificity of PGPase from mung bean mitochondria

<table>
<thead>
<tr>
<th>Substrate</th>
<th>PGPase Activity</th>
<th>%</th>
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<tbody>
<tr>
<td>Palmitoyl₂GroP[U⁻¹⁴C]GroP</td>
<td>6.1</td>
<td>100</td>
</tr>
<tr>
<td>Oleoyl₂GroP[U⁻¹⁴C]GroP</td>
<td>3.1</td>
<td>51</td>
</tr>
<tr>
<td>Oleoyl₂[U⁻¹⁴C]GroP</td>
<td>0.09</td>
<td>1</td>
</tr>
<tr>
<td>Palmitoyl[U⁻¹⁴C]GroP</td>
<td>0.06</td>
<td>1</td>
</tr>
<tr>
<td>Oleoyl[U⁻¹⁴C]GroP</td>
<td>0.11</td>
<td>2</td>
</tr>
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### Substrate Specificities of PGPase and PGPS

Under conditions giving high PGPase activity, mitochondrial fractions of mung bean seedlings hardly dephosphorylated acyl₂GroP or acyl₂GroP (Table II). Hence, the PGPase from plant mitochondria shows the same substrate specificity as the phosphatase of E. coli encoded by the pgpA gene but differs from the E. coli phosphatase encoded by the pgpB gene (Icho and Raetz, 1983; Icho, 1988).

PGPase of mung bean mitochondria dephosphorylated palmitoyl₂GroPGroP with distinctly higher rates than oleoyl₂GroPGroP (Table II; Fig. 4B). This substrate species specificity of the enzyme was due to differences in the apparent Vₘₐₓ values. As shown in Figure 4A, the PGPS from mung bean mitochondria displayed the same, but even more pronounced, species specificity than the PGPase. Highest incorporation rates of [U⁻¹⁴C]GroP into acyl₂GroPGroP and acyl₂GroPGro were obtained with CDP-palmitoyl₂Gro, lower ones with CDP-acyl₂Gro from egg lecithin, which predominantly consists of CDP-1-palmitoyl-2-oleoyl₂Gro (Hostetler et al., 1975), and very low ones with CDP-oleoyl₂Gro and CDP-decanoyl₂Gro (Fig. 4A). Again, the differences in enzymic activities determined with the various substrate species were predominantly based on differences in the apparent Vₘₐₓ values. With regard to its specificities, the PGPS from mung bean mitochondria differs from the respective enzyme from

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**Figure 3.** PGPase activity in isolated mung bean mitochondria in dependence on pH (A), divalent cation concentrations (B), and Triton X-100 concentrations (C). Dephosphorylation rates of palmitoyl₂GroP[U⁻¹⁴C]GroP were measured in A, the buffer mixture given in Figure 2; in B, the presence of the given concentration of MgCl₂ (□) and MnCl₂ ( ○); and in C, the presence of the given concentration of Triton X-100. All other conditions were the standard assay conditions.
CDP-palmitoylGro (O), CDP-oleoyl2Gro (O), and CDP-decanoylGro (O), and CDP-acyl2Gro from egg lecithin. 

Figure 4: Substrate species specificities of the mitochondrial PGPS (A) and PGPase (B) from mung bean mitochondria. A, Total incorporation rates of [U-14C]GroP into the lipophilic products (acylGroPGro plus acylGroPGroP) are given as a function of the concentrations of CDP-palmitoylGro (O), CDP-oleoyl2Gro (■), CDP-decanoylGro (□), and CDP-acyl2Gro from egg lecithin (△). B, Dephosphorylation rates are given as a function of the concentrations of palmitoylGroP[U-14C]GroP (O) and oleoyl2GroP[U-14C]GroP (■).

mammalian cells (Hostetler et al., 1975; Stuhne-Sekalec and Stanacev, 1989). The results shown in Figure 4 correlate very well with the fatty acid composition of acyl2GroPGro from mung bean mitochondria, which is predominantly esterified with palmitoyl groups (Bligny and Douce, 1980). They, however, do not correspond to the fatty acid composition of cardiolipin, which is formed from acylGroPGro (Schlame et al., 1993) and which contained almost exclusively unsaturated C18 acyl groups (Bligny and Douce, 1980). To approach the problem via which reaction sequences the typical fatty acid pattern of cardiolipin is established, experiments are in progress to determine the properties of the cardiolipin synthase, especially its substrate specificities.

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