Biosynthesis of Cardiolipin in Plant Mitochondria[1]

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The properties of cardiolipin synthase were investigated in mitochondria and submitochondrial fractions from etiolated mung bean (*Vigna radiata* L.) seedlings. Direct evidence is presented that the enzyme utilizes CDP-diacylglycerol in addition to phosphatidylglycerol for the synthesis of cardiolipin. Cardiolipin synthase had an alkaline pH optimum of about 9 and required divalent cations for activity. Maximal activity was obtained in the presence of 16 mM $MnCl_2$. The apparent K_m values for CDP-diacylglycerol and phosphatidylglycerol were 0.8 and 50 µM, respectively. Cardiolipin synthase was localized predominantly in the inner membrane of mung bean mitochondria and displayed a substrate species specificity. Highest activities were measured with the dioleoyl species of both CDP-diacylglycerol and phosphatidylglycerol, and somewhat lower activities were measured with mixed species of the two substrates containing a palmitoyl and an oleoyl group. On the other hand, the cardiolipin synthase hardly used the dipalmitoyl species and strongly discriminated against CDP-dipalmitoylglycerol from a mixture with CDP-dioleoylglycerol.

Cardiolipin (diphosphatidylglycerol) is an anionic phospholipid with a unique tetraacyl structure found in mitochondria of eukaryotes as well as in membranes of various prokaryotes. It is generally accepted that cardiolipin is a characteristic component of the inner mitochondrial membrane, but recent reports have shown that it is also present in the outer membrane (Hovious et al., 1990, 1993) and is even enriched in the contact sites between the two membrane systems (Ardail et al., 1990; Simbeni et al., 1991). There is a growing body of evidence that cardiolipin is of vital importance for mitochondria by interacting with several enzymes and substrate carriers in such a way that makes it an essential membrane component for respiration and oxidative phosphorylation (Daum, 1985; Hoch, 1992; Robinson, 1993). Moreover, it appears to play a decisive role in targeting cytoplasmically synthesized preproteins to the mitochondria (Leenhouts et al., 1993; Robinson, 1993).

Cardiolipin can be synthesized by two alternative pathways (Fig. 1). In bacteria a membrane-bound condensing enzyme, the cardiolipin synthase, utilizes two molecules of $acyl_2GroPGro$ for cardiolipin synthesis (Fig. 1; Shibuya, 1992). On the other hand, the mitochondrial cardiolipin synthase from diverse eukaryotes catalyzes the transfer of a phosphatidyl residue from CDP-$acyl_2Gro$ to $acyl_2GroPGro$ (Fig. 1; Daum, 1985; Tamai and Greenberg, 1990; Schlame et al., 1993). Substantial information on cardiolipin synthesis

in yeast, rat liver, and *Escherichia coli* has been obtained (Daum, 1985; Schlame and Rüstow, 1990; Stuhne-Sekalec and Stanacev, 1990; Tamai and Greenberg, 1990; Schlame and Hostetler, 1991; Shibuya, 1992; Schlame and Haldar, 1993; Schlame et al., 1993). So far, cardiolipin synthase of both *E. coli* and rat liver have been purified (Hiraoka et al., 1991; Schlame and Hostetler, 1991) and the gene of *E. coli* encoding the enzyme has been cloned (Ohta et al., 1985), whereas little is known about cardiolipin biosynthesis in plants (Kinney, 1993). Recently, cardiolipin synthase activity has been clearly demonstrated for the first time by Schlame et al. (1993) in isolated plant mitochondria. The authors, however, failed to detect this enzymic activity when radioactively labeled CDP-$acyl_2Gro$ instead of $acyl_2GroPGro$ was used as the tracer molecule. Thus, it is still unclear whether the plant enzyme follows the prokaryotic or the eukaryotic pathway (Fig. 1). Furthermore, the mechanisms that cause the different fatty acid patterns of $acyl_2GroPGro$ and cardiolipin from plant mitochondria as well as the origin of polyunsaturated C_{18} fatty acids, which are esterified in a distinctly higher proportion in cardiolipin than in its precursor $acyl_2GroPGro$ (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 started to characterize cardiolipin synthase in plant mitochondria. In this paper the properties of the enzyme from mung bean (*Vigna radiata* L.) mitochondria are presented that clearly show that in plants, as in other eukaryotes, the committed step of cardiolipin biosynthesis involves the transfer of a phosphatidyl group from CDP-$acyl_2Gro$ to $acyl_2GroPGro$ (Fig. 1). Furthermore, the observed substrate species specificities and selectivities of the synthase are discussed with regard to the typical fatty acid pattern of cardiolipin.

MATERIALS AND METHODS

Chemicals

sn-[U-^{14}C]GroP (5.88 kBq nmol^{-1}) was purchased from Amersham Buchler and the various CDP-$acyl_2Gro$ and $acyl_2GroPGro$ species were obtained from Serdary Research

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Abbreviations: acylGroP, 1-acyl-sn-glycerol-3-phosphate; $acyl_2Gro$, 1,2-diacyl-sn-glycerol-3-phosphate; $acyl_2GroPGro$, phosphatidylglycerol; $acyl_2GroPGroP$, phosphatidylglycerophosphate; Ampso, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid; Capso, 3-[cyclohexylamino]-2-hydroxy-1-propane-sulfonic acid; CDP-$acyl_2Gro$, CDP-diacylglycerol; Ches, 2-[N-cyclohexylamino]ethanesulfonic acid; GroP, sn-glycerol-3-phosphate.
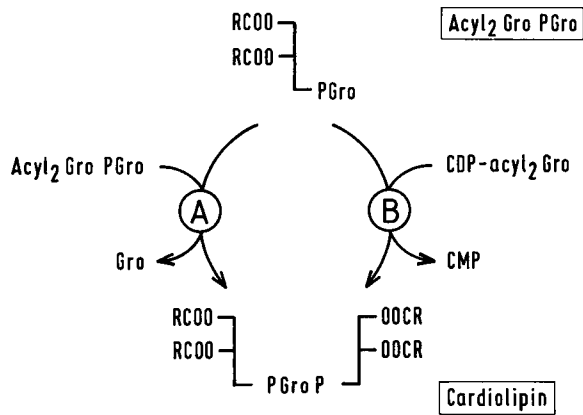
Figure 1. Biosynthesis of cardiolipin via the prokaryotic (A) and the eukaryotic pathway (B).

Laboratories (London, Ontario, Canada) or Sigma. CDP-acyl$_2$[U-^{14}C]Gro (5.88 kBq nmol^{-1}) was synthesized enzymatically from [U-^{14}C]GroP, acyl-CoA, and CTP in three consecutive reactions. At first the 1-oleoyl and 1-palmitoyl derivatives of [U-^{14}C]GroP were produced with purified GroP acyltransferase from pea chloroplasts according to Bertrams and Heinz (1981). Next, labeled acylGroP was converted to the 1,2-diacyl derivative in an acylGroP acyltransferase assay consisting of 0.1 M Tris-HCl, pH 8, 12 μM acyl[U-^{14}C]GroP, 30 μM oleoyl-CoA, and about 30 μg of crude membrane protein from the *E. coli* strain JC201/pPLSC, which overproduces acylGroP acyltransferase (Coleman, 1992), in a total volume of 2 mL. After an incubation of 60 min at 37°C, lipids were extracted according to Hajra (1974), dried in a stream of argon, and resuspended in 50 mM phosphate buffer, pH 7.4. Finally, CDP-acyl$_2$-[U-^{14}C]Gro was synthesized from acyl$_2$-[U-^{14}C]GroP and CTP by membrane fractions of *E. coli* harboring pCD100 and subsequently purified by TLC as described by Tamai and Greenberg (1990). [U-^{14}C]GroP was converted to the final product with a yield of 50 to 60%.

Purification and Fractionation of Mitochondria

Mitochondria were isolated from etiolated mung bean (*Vigna radiata* L.) seedlings according to the procedure of Neuburger et al. (1982) as described previously (Griebau and Frentzen, 1994). Purified mitochondria were washed and resuspended in 20 mM Pipes-KOH, pH 7.2, 5 mM DTT. To isolate mitochondrial membranes, purified organelles corresponding to about 4 mg of protein were diluted in 20 mL of hypotonic medium containing 2 mM Pipes-NaOH, pH 7.3, and 0.25 mM EDTA. The suspension was stirred for 20 min, briefly sonicated (twice for 5 s), and then spun for 30 min at 140,000g. The resulting pellet of outer and inner membranes was resuspended in 20 mM Pipes-NaOH, pH 7.3, containing 5 mM DTT and 50% (v/v) glycerol and stored at −20°C.

Outer mitochondrial membranes were separated from the inner ones by the swelling contraction method (Douce et al., 1973) as described previously (Frentzen et al., 1990). The

protein concentration in mitochondrial fractions was determined according to Bradford (1976).

Enzyme Assays

Unless otherwise stated, cardiolipin synthase was measured at 30°C in isolated mung bean mitochondria or mitochondrial membrane fractions (up to 10 μg of protein) in the presence of 50 mM Bis-Tris-propane-HCl, pH 9, 16 mM MnCl$_2$, 0.5 mM oleoyl$_2$GroPGro, and 3 μM CDP-oleoyl$_2$[U-^{14}C]Gro in a total volume of 50 μL. Reaction was started by the addition of MnCl$_2$ and stopped after 20 min with 240 μL of chloroform:methanol (1:1, v/v) containing 50 μg of cardiolipin and 100 μL of 0.2 M H$_3$PO$_4$, 1 M KCl. After phase separation, 100 μL of the chloroform layer were routinely analyzed 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 and their water-soluble products obtained by deacylation were analyzed as described previously (Griebau and Frentzen, 1994).

Acyl$_2$GroPGroP synthase activity was measured by determining the incorporation of [U-^{14}C]GroP into lipophilic products as described previously (Griebau and Frentzen, 1994). Acyl$_2$GroPGroP phosphatase activity was assayed and the reaction product was separated by TLC according to Griebau and Frentzen (1994). The activity of CDP-acyl$_2$Gro synthase was measured by monitoring the incorporation of [5-^{3}H]CTP
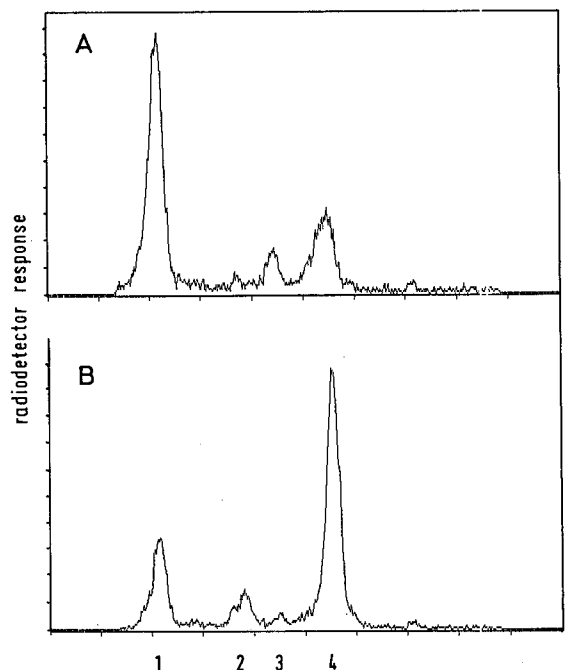

Figure 2. Biosynthesis of cardiolipin in mung bean mitochondria from labeled CDP-oleoyl$_2$Gro. The radioscans show the labeling pattern of the reaction products formed by isolated mung bean mitochondria (30 μg of protein) from CDP-oleoyl$_2$[U-^{4}C]Gro without (A) and with (B) exogenously added oleoyl$_2$GroPGro. 1, CDP-acyl$_2$Gro; 2, phosphatidylethanolamine; 3, acyl$_2$GroPGro; 4, cardiolipin.

into chloroform-soluble products under conditions similar to those reported by Carman et al. (1980). In brief, the reaction mixture was composed of 50 mM Bis-Tris-propane-HCl, pH 7.5, 10 mM MgCl$_2$, 0.5 mM oleoyl$_2$GroP, 5 mM Triton X-100, up to 20 μg of mitochondrial protein, and 1.2 mM [5-^{3}H]CTP (310 Bq nmol^{-1}) in a total volume of 50 μL. Reactions were terminated and lipophilic products were extracted (Hajra, 1974) after 20 min at 30°C. AcylGroP acyltransferase and Cyt c oxidase were determined as outlined by Frentzen et al. (1990) and Jesaitis et al. (1977), respectively.

RESULTS AND DISCUSSION

Assay Conditions

To elucidate the reaction sequence resulting in the formation of cardiolipin in plant mitochondria, we have optimized an in vitro assay for cardiolipin synthase. Since hardly any cardiolipin was formed via in situ-synthesized acyl$_2$GroPGro, CDP-oleoyl$_2$[U-^{14}C]Gro was prepared and incubated with mung bean mitochondria. The analysis of the reaction products revealed that a lipid was labeled as the main product and its formation rate was appreciably stimulated by exogenously added oleoyl$_2$GroPGro (Fig. 2). This product was identified as cardiolipin by rechromatography in different solvent systems and analysis of its deacylation product. Cardiolipin synthesis in mung bean mitochondria has been reported previously (Schlame et al., 1993). In contrast to the results shown in Figure 2, Schlame et al. (1993) did not detect cardiolipin synthase activity when they used labeled CDP-acyl$_2$Gro instead of acyl$_2$GroPGro as a tracer molecule. These differences are presumably due to the different assay conditions used. Hence, direct evidence is provided that in plant mitochondria cardiolipin biosynthesis requires not only acyl$_2$GroPGro but also CDP-acyl$_2$Gro and is, thus, catalyzed by an acyl$_2$GroPGro:CDP-acyl$_2$Gro phosphatidyltransferase as in the organelles of other organisms (Fig. 1) (Daum, 1985; Tamai and Greenberg, 1990; Schlame et al., 1993).

Cardiolipin synthase from mung bean mitochondria exhibited an alkaline pH optimum of about 9 (Fig. 3A), similar to the respective enzyme from yeast (Tamai and Greenberg,

Table I. *Enzymic activities in outer (OM) and inner (IM) membrane fractions from mung bean mitochondria*

Enzyme	Total Activity		Specific Activity	
	OM	IM	OM	IM
	pmol		*pmol min^{-1} mg^{-1}*	
AcylGroP acyltransferase	11	91	92	20
CDP-acyl$_2$Gro synthase	36	3400	300	740
Acyl$_2$GroPGroP synthase	6	330	53	73
Acyl$_2$GroPGroP phosphatase	64	4200	540	920
Cardiolipin synthase	12	700	97	150
Cyt c oxidase[a]	6	6400	51	1400

[a] 10^{-3} values are given.

1990) and rat liver mitochondria (Schlame and Hostetler, 1991). The activity of the plant enzyme varied appreciably in dependence on the buffer substance. Highest activities were measured in Bis-Tris-propane-HCl, but 2- to 5-fold lower activities were measured in Ampso-NaOH, Ches-NaOH, Tris-HCl, and Capso-NaOH.

As depicted in Figure 3B, cardiolipin synthase requires divalent cations for its activity. Maximal activities were achieved by addition of MnCl$_2$ to a final concentration of about 16 mM. Stimulation of cardiolipin synthase by Mg^{2+} was distinctly lower than by Mn^{2+}, whereas Co^{2+} or Ca^{2+} showed no effect (Fig. 3B). Due to the low solubility and stability of Mn^{2+} at alkaline pH values, the enzyme assays were started by the addition of MnCl$_2$. In this way reaction rates were independent of whether or not oxidation of Mn^{2+} was largely prevented during the incubation. A requirement of the cardiolipin synthase activity from mung bean mitochondria for divalent cations has also been described by Schlame et al. (1993). An absolute dependence on such cations appears to be typical of the mitochondrial cardiolipin synthase from various organisms, although they differ in their cation specificities (Daum, 1985; Tamai and Greenberg, 1990; Schlame et al., 1993). Unlike the enzyme of mung beans, those of other eukaryotes displayed a specificity for
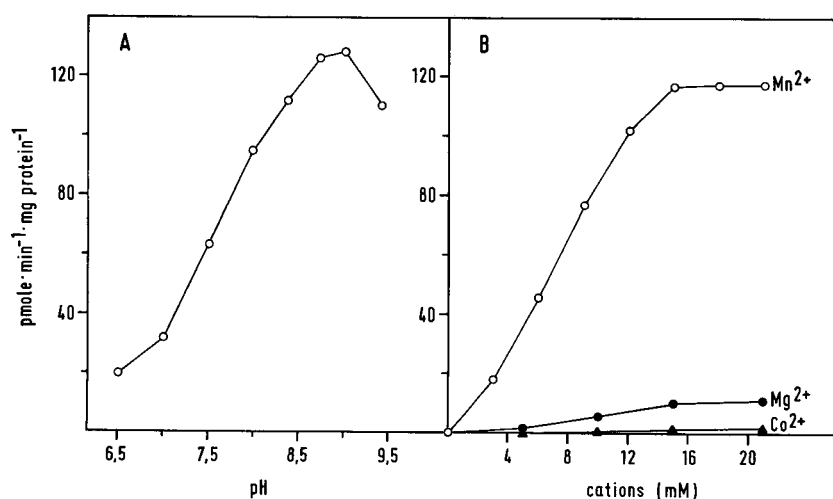

Figure 3. Effect of pH (A) and divalent cation (B) on cardiolipin synthase activity in mung bean mitochondria. Incorporation rates of CDP-oleoyl$_2$[U-^{14}C]Gro into cardiolipin were measured in a reaction mixture buffered with Bis-Tris-propane-HCl (A) and containing the given cation concentrations (B) (Ca^{2+} gave the same results as Co^{2+}).
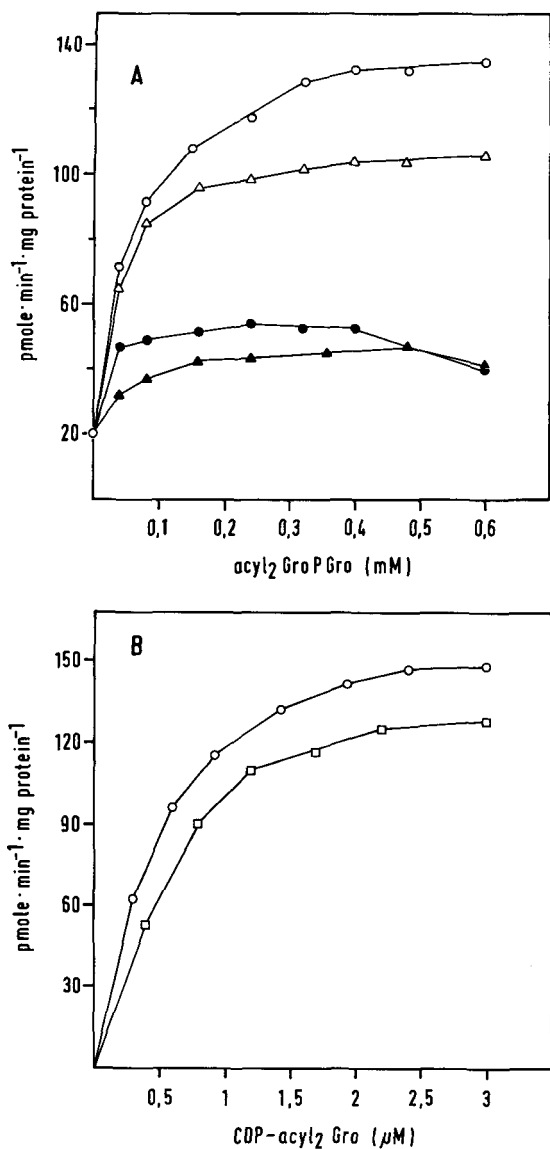
Figure 4. Substrate species specificities of the cardiolipin synthase from mung bean mitochondria. Formation rates of cardiolipin by inner membrane fractions are given as a function of the concentrations of species of acyl₂GroPGro (O, dioleoyl; ●, dipalmitoyl; ▲, dimyristoyl; and △, the mixture from egg lecithin) (A) and CDP-oleoyl₂[U-¹⁴C]Gro (O) and CDP-1-palmitoyl-2-oleoyl[U-¹⁴C]Gro (□) (B).

either Co^{2+} or Mg^{2+} (Schlame et al., 1993; Tamai and Greenberg, 1990). On the other hand, the activity of the *E. coli* enzyme, which catalyzes the condensation of two molecules of acyl₂GroPGro, is independent of metal ions (Hiraoka et al., 1991).

In mung bean mitochondria maximal cardiolipin formation rates were attained at 3 μM CDP-oleoyl₂Gro and 500 μM oleoyl₂GroPGro. Under these conditions cardiolipin formation was linear with respect to time for at least 20 min and with respect to protein up to at least 10 μg. Specific activities of cardiolipin synthase of about 0.1 nmol min⁻¹ mg⁻¹ protein

were determined. Apparent K_m values for CDP-oleoyl₂Gro and oleoyl₂GroPGro were 0.8 and 50 μM, respectively. These values are very close to those reported for the cardiolipin synthase of rat liver mitochondria (Schlame and Hostetler, 1991), whereas about 20-fold higher values have been described for the respective enzyme from yeast mitochondria (Tamai and Greenberg, 1990).

Cardiolipin synthase of mung beans was found to be sensitive to detergents such as Triton X-100 just like the enzyme of rat liver (Hostetler and van den Bosch, 1972), but in contrast to those of yeast and *E. coli*, the activities of which were stimulated by Triton X-100 (Tamai and Greenberg, 1990; Hiraoka et al., 1991). Consequently, apart from its cation specificity the cardiolipin synthase of mung bean mitochondria displayed properties very similar to those of the respective synthase from rat liver. Thus, it differs from the acyl₂GroPGroP synthase and phosphatase that catalyze the biosynthesis of acyl₂GroPGro in mung bean mitochondria and that resemble the respective enzymes of *E. coli* more closely than those of mitochondria from mammalian and yeast cells (Griebau and Frentzen, 1994).

Submitochondrial Localization

In mitochondria from mung bean seedlings cardiolipin synthase is firmly bound to the membrane, since its total activity measured in mitochondria could be recovered in the membrane fractions even after washing the membranes with buffer containing 0.6 M KCl. To localize the enzyme more precisely, the two membrane systems were separated on Suc step gradients. As shown in Table I, most of the cardiolipin synthase activity was recovered in the inner membrane frac-
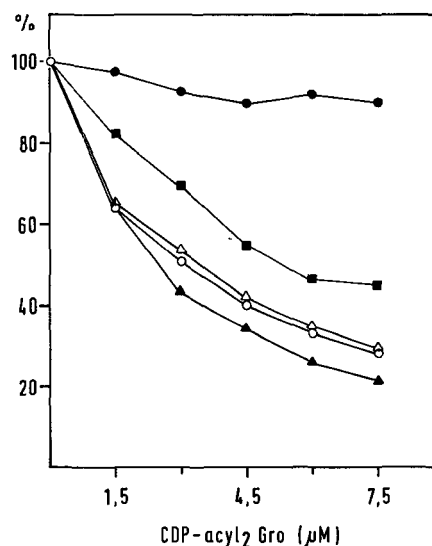

Figure 5. CDP-acyl₂Gro selectivity of cardiolipin synthase from mung bean mitochondria. Effect of increasing concentrations of various unlabeled CDP-acyl₂Gro on the incorporation rates of 1.5 μM CDP-oleoyl₂[U-¹⁴C]Gro into cardiolipin by inner membrane fractions is given. (●, Dipalmitoyl; ■, didecanoyl; △, the mixture from egg lecithin; O, dioleoyl; ▲, dimyristoyl. One hundred percent corresponds to 127 pmol min⁻¹ mg⁻¹ protein).

tions, and in these fractions the specific activity of the enzyme was about 2-fold higher than in the outer fractions. The distribution of the cardiolipin synthase corresponded to those of CDP-acyl$_2$Gro synthase, acyl$_2$GroPGroP synthase, and phosphatase, but differed from that of acylGroP acyltransferase (Table I). Furthermore, results very similar to those given in Table I were obtained when the experiments were carried out with mitochondrial membrane fractions isolated from etiolated cucumber seedlings.

The observed submitochondrial localization of the plant enzymes involved in acyl$_2$GroP, CDP-acyl$_2$Gro, and acyl$_2$GroPGro biosynthesis is in agreement with previous results (Douce et al., 1972; Sparace and Moore, 1979; Frentzen et al., 1990). Hence, in plant mitochondria all enzymic activities that catalyze the reactions from acyl$_2$GroP to acyl$_2$GroPGro are located in the inner membrane, and within this membrane CDP-acyl$_2$Gro and acyl$_2$GroPGro serve as substrates for the biosynthesis of cardiolipin. A comparison of the activity patterns of these enzymes with that of Cyt c oxidase, however, revealed that the activities determined in the outer membranes could not be attributed merely to contamination of these fractions with inner membranes (Table I). Rather, the results indicate that the biosynthesis of cardiolipin occurs predominantly but not exclusively in the inner membrane. In this respect mitochondria of mung bean and cucumber seedlings resemble more closely the organelles of guinea pig liver (Stuhne-Sekalic and Stanacev, 1990) than those of rat liver, in which cardiolipin synthase is located exclusively in the inner membranes (Hostetler and van den Bosch, 1972; Stuhne-Sekalic and Stanacev, 1990; Schlame and Haldar, 1993).

Substrate Specificities and Selectivities

To understand by which reaction sequences the typical fatty acid composition of cardiolipin is established, the substrate specificities of the cardiolipin synthase of mung bean mitochondria have been investigated. Figure 4A shows results of cardiolipin synthesis from CDP-oleoyl$_2$[U-^{14}C]Gro and various species of acyl$_2$GroPGro exogenously added as sonicated micellar solutions. Highest activities of cardiolipin synthase were obtained with oleoyl$_2$GroPGro, and lower activities were obtained with acyl$_2$GroPGro from egg lecithin, which consists predominantly of the 1-palmitoyl-2-oleoyl species (Hostetler et al., 1975). The dipalmitoyl and dimyristoyl species, however, stimulated the formation rates of cardiolipin only slightly (Fig. 4A).

Furthermore, cardiolipin synthase not only showed a substrate specificity with regard to acyl$_2$GroPGro but also with regard to CDP-acyl$_2$Gro. As depicted in Figure 4B, cardiolipin synthase activity was slightly higher with CPD-oleoyl$_2$Gro than with CDP-1-palmitoyl-2-oleoylGro. The 1-oleoyl-2-palmitoyl isomer was incorporated into cardiolipin at the same rates as CDP-1-palmitoyl-2-oleoylGro. These results were confirmed and extended by competition experiments in which the incorporation rates of CDP-oleoyl$_2$[U-^{14}C]Gro into cardiolipin were determined in the presence of increasing concentrations of various unlabeled CDP-acyl$_2$Gro species (Fig. 5). CDP-myristoyl$_2$Gro as well as the species mixture from egg lecithin efficiently competed with the dioleoyl spe-

cies for cardiolipin synthesis, whereas CDP-decanoyl$_2$Gro was less effective. On the other hand, CDP-palmitoyl$_2$Gro hardly altered the incorporation rates of the dioleoyl species (Fig. 5). With regard to its CDP-acyl$_2$Gro species specificity, cardiolipin synthase from mung bean mitochondria differs from the respective enzyme from mammalian cells (Hostetler et al., 1975; Stuhne-Sekalec and Stanacev, 1989). Furthermore, it has a substrate specificity quite different from those of acyl$_2$GroPGroP synthase and phosphatase from mung bean mitochondria (Griebau and Frentzen, 1994). Unlike these enzymes, which showed higher activities with the dipalmitoyl species of their substrates than with the dioleoyl species, cardiolipin synthase specifically used the dioleoyl species of both CDP-acyl$_2$Gro and acyl$_2$GroPGro and strongly discriminated against the dipalmitoyl species of both its substrates. These results correlate with the fatty acid composition of cardiolipin consisting of more than 90% unsaturated C_{18} acyl groups. However, the results obtained with the substrate species containing both a palmitoyl and an oleoyl group strongly suggest that the incorporation of such mixed species into cardiolipin cannot be prevented by the properties of the synthase. Perhaps de- and reacylation reactions are also involved in establishing the typical fatty acid pattern of cardiolipin, as has been described for cardiolipin of mammalian cells (Schlame and Rüstow, 1990), but this awaits clarification.

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