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Phosphatidylglycerol Synthesis in Pea Chloroplasts

PATHWAY AND LOCALIZATION

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ABSTRACT

Isolated intact pea chloroplasts synthesized phosphatidylglycerol from either [^{14}C]acetate or [^{14}C]glycerol 3-phosphate. Both time-course and pulse-chase labeling studies demonstrated a precursor-product relationship between newly synthesized phosphatidic acid and newly synthesized phosphatidylglycerol.

The synthesis both of CDP-diacylglycerol from exogenous phosphatidic acid and CTP, and of phosphatidylglycerol from exogenous CDP-diacylglycerol and glycerol 3-phosphate, could be assayed in fractions obtained from disrupted chloroplasts. Moreover, the enzymes catalyzing these reactions were localized in the inner envelope membrane. Exogenous phosphatidic acid was incorporated into phosphatidylglycerol, but only following its incorporation into CDP-diacylglycerol. Finally, radioactive phosphatidic acid synthesized in the envelope membranes from [^{14}C]palmitoyl-ACP and 1-oleoyl-glycerol 3-phosphate was sequentially incorporated into labeled CDP-diacylglycerol and phosphatidylglycerol upon the addition of appropriate substrates and cofactors. Thus, we have demonstrated that (a) the synthesis of phosphatidylglycerol in chloroplasts occurs by the pathway: phosphatidic acid → CDP-diacylglycerol →→ phosphatidylglycerol, and (b) phosphatidylglycerol synthesis is located in the inner envelope membrane.

Phosphatidylglycerol (PG)[1] is the major phospholipid in chloroplast membranes (15). Although its functions have not yet been clearly defined, it has been implicated in granal stacking (14), in the oligomeric structure of the light-harvesting Chl protein complex (11), and in chilling sensitivity (26). The lipid is characterized by the presence of *trans*-3 hexadecenoic acid, which is found only in plastids (16), and by its 'procaryotic' fatty acid composition and distribution within the DG moiety (27).

It has been clearly demonstrated recently that isolated intact spinach chloroplasts can synthesize PG *de novo* from either acetate or glycerol 3-P under conditions of fatty acid synthesis (25, 28). We have now utilized pea chloroplasts to investigate the pathway and localization of PG synthesis. Although pea chloroplasts can synthesize PA from glycerol 3-P and activated fatty acids, this lipid is only very slowly incorporated into galactolipids, even upon the addition of UDP-Gal (17). Thus, it seemed reasonable that newly synthesized PA might give rise predominantly to PG. Pea chloroplasts, therefore, represented a potentially simpler system than spinach chloroplasts in which to examine PG synthesis.

We report here that pea chloroplasts can synthesize PG from either [^{14}C]acetate or [^{14}C]glycerol 3-P. This synthesis has been demonstrated by several lines of evidence to occur via PA and CDP-DG. Finally, the enzymes which catalyze the synthesis of PG from PA have been shown to be localized in the inner envelope membrane.

[1] Abbreviations: PG, phosphatidylglycerol; ACP, acyl carrier protein; CDP-DG, CDP-diacylglycerol; DG, diacylglycerol; FFA, free fatty acids; glycerol 3-P, glycerol 3-phosphate; LPA, lysophosphatidic acid; MG, monoacylglycerol; PA, phosphatidic acid; PGP, phosphatidylglycerophosphate.

MATERIALS AND METHODS

Materials. [2-^{14}C]Acetic acid, L-[U-^{14}C]glycerol 3-P, and [U-^{14}C]cytidine 5'triphosphate, were purchased from Amersham. [1-^{14}C]Palmitoyl-ACP was a generous gift from Drs. J. B.Ohlrogge and T.-M. Kuo, USDA Northern Regional Research Center, Peoria, IL. CDP-DG and LPA (1-oleoyl-glycerol 3-P) were purchased from Serdary Research Laboratories, Inc.; PA was purchased from Sigma Chemical Co.; lipid standards were purchased from either Serdary Research Laboratories, Inc. or Sigma Chemical Co., or prepared in our laboratory. Acetate, DL-glycerol 3-P, CTP, ATP, CoA, DTT, Triton X-100, BSA, and the buffers Hepes, Tricine, MOPS, and Tris were obtained from Sigma Chemical Co.; all other chemicals were reagent grade.

Purification and Fractionation of Chloroplasts. Intact chloroplasts were obtained from homogenates of 14- to 17-d-old pea seedlings (*Pisum sativum* var Laxton's Progress No. 9) by differential centrifugation followed by Percoll density gradient centrifugation as described (8), with the following exceptions: 1. the pH of the buffer was adjusted with KOH instead of NaOH; 2. the centrifugation speeds were increased and the times decreased, as modified from the protocol described by Heinz and Roughan (17) to: (a) first sedimentation, 3300*g*, 90 s; (b) Percoll gradient sedimentation, 13,000*g*, 5 min; (c) dilution and recovery, and wash sedimentations, 3300*g*, 90 and 75 s, respectively. Those chloroplasts used directly for lipid synthesizing assays were resuspended in 0.3 M sorbitol in 33.4 mM Tricine-NaOH (pH 7.9).

Those chloroplasts which were fractionated were suspended in 0.6 M sucrose (2 mg Chl/ml), and broken by slow freezing (−20°C, about 45 min) and thawing (22–24°C, about 30 min) (8). The suspension was diluted with buffer to 0.36 M sucrose, and 5 ml were pipetted over the following sucrose step-density gradients: 1.3 M, 2 ml; 1.2 M, 1 ml; 1.1 M, 4 ml; 0.5 M, 5 ml (as modified from Joyard and Douce [18]). The gradients were then centrifuged at 116,000g_{max} for 90 min. The soluble stromal components remained within the 0.36 M layer, the envelopes sedimented to the 0.5/1.1 M interface, while the thylakoids sedimented to the 1.3 M layer. A small green pellet, which accounted for 12 to 40% of the total Chl, was assumed to be unbroken chloroplasts. The envelope membranes were collected with a Pasteur pipette, diluted 4- to 5-fold with buffer, and collected by centrifugation (90,000g_{max}, 60 min); they were then resuspended in either buffer only or 0.2 M sucrose (usually to about 0.5–1 mg protein/ml). All solutions used in this and the following procedure were buffered with 10 mM Tricine-KOH

(pH 7.5) + 2 mM EDTA.

Inner and outer envelope membrane fractions were prepared by a modification of the procedure reported first by Cline *et al.* (8). In this procedure, the solution of chloroplasts broken by freeze-thaw lysis was diluted to 0.2 M sucrose, and the thylakoids removed by centrifugation at $4500g_{max}$ for 15 min. The envelopes were recovered from the supernatant by centrifugation at $40,000g_{max}$ for 45 min, resuspended in 0.2 M sucrose, and fractionated by sedimentation through linear density sucrose gradient (0.5–1.2 M sucrose). The gradient fractions were pooled as follows (10): Outer envelope membranes, the entire light membrane band ($p = 1.08$ g/cm^3); mixed envelope membranes, the upper portion of the heavy membrane band ($p = 1.125$ g/cm^3); inner envelope membranes, the lower portion of the heavy membrane band. Membranes were recovered by centrifugation ($90,000g_{max}$, 90 min) and resuspended in 0.2 M sucrose. The outer envelope membrane fraction contains highly purified outer envelope membranes, while the inner envelope membrane fraction is greatly enriched in inner envelope membranes. The mixed envelope membrane fraction contains both inner and outer envelope membranes which cannot be separated by linear density sucrose centrifugation (10).

Lipid Synthesis from Acetate and Glycerol 3-P in Intact Pea Chloroplasts. Chloroplasts were incubated under the lipid synthesizing conditions described by Mudd and DeZacks (25) for spinach chloroplasts. In this system, fatty acids which are synthesized from acetate may be incorporated into glycerolipids by esterification to glycerol 3-P. Thus, lipids may be labeled from either [^{14}C]acetate or [^{14}C]glycerol 3-P.

CDP-DG Synthesis. The synthesis of CDP-DG was measured by using a modification of the procedures described by McCaman and Finnerty (24) and Ganong *et al.* (13) for measuring the activity in bacterial homogenates. The reaction mixture contained in a total volume of 150 μl: MOPS-KOH, 250 mM (pH 7.4); PA, 1 or 2.5 mM; [^{14}C]CTP, 1 mM, 0.1 to 0.32 μCi/assay; and $MgCl_2$, 30 mM. The reaction was initiated by the addition of envelope membranes (15–40 μg protein) followed in 15 s by the addition of $MgCl_2$. Incubations were carried out for 5 to 60 min at 30°C, and terminated by the addition of 2.3 ml of methanol:$CHCl_3$, 1:1 (v/v). After addition of 20 μg of cold CDP-DG to act as a carrier, the single phase was broken by the addition of 1 ml 1 M KCl in 0.2 M H_3PO_4. The $CHCl_3$ layer was removed and dried under N_2; the residue was resuspended in 1 ml $CHCl_3$. A known volume was removed for determination of total radioactivity; this was placed in a scintillation vial, dried under air, and the radioactivity assayed in 5 ml Aquamix (West-Chem). Lipid synthesis was calculated as nmol CTP incorporated into $CHCl_3$-soluble products. The remaining $CHCl_3$ suspension was utilized for product analysis as described below. Variations from this procedure are noted in the text and figure legends.

PG Synthesis. The synthesis of PG was measured by using a slight modification of the procedure described by Marshall and Kates (23) for measuring the activity in spinach leaf homogenates. The reaction contained in a total volume of 150 μl: Tris-HCl, 250 mM (pH 7.3); CDP-DG, 0.1 mM; [^{14}C]-DL-glycerol 3-P, 1 mM, 0.4 to 0.8 μCi/assay; $MgCl_2$, 30 mM; and Triton X-100, 0.15% (w/v). The reaction was initiated by the addition of envelope membrane proteins (15–80 μg). Incubations were carried out for 5 to 60 min at 37°C, and terminated by the addition of 1.88 ml of methanol: $CHCl_3$, 2:1 (v/v). The single phase was broken by the addition of 0.63 ml of $CHCl_3$ and 0.63 ml of H_2O; the $CHCl_3$ layer was removed and treated as described above. Lipid synthesis was calculated as nmol L-glycerol 3-P incorporated into $CHCl_3$-soluble products, from the information that 49% of the DL-glycerol 3-P was L-glycerol 3-P. Variations from this procedure are noted in the text and figure legends.

PA Synthesis. The procedure for synthesizing labeled PA in envelope membranes from 1-oleoyl-glycerol 3-P (LPA) and [^{14}C] palmitoyl-ACP was that described by Frentzen *et al.* (12). The concentration of LPA was 50 μM, while that of palmitoyl-ACP was 2.5 to 3 μM. Incubations were carried out for 5 min at 22 to 24°C. This resulted in 80 to 95% of the palmitate offered as palmitoyl-ACP incorporated into lipid products; of these, PA was predominant, containing 95% or more of the incorporated radioactivity.

Analysis of Labeled Lipid Products. Lipids were analyzed by one-dimensional TLC on Whatman K-6 silica gel plates with the following solvent systems:

1. Double solvent system; acetone:acetic acid:H_2O (100:2:1) to the top; $CHCl_3$:methanol:NH_4OH:H_2OH (65:25:2:2) in the same direction to 13 cm from the bottom. The plates were dried in a hood for 25 min after development in the first system.
2. $CHCl_3$:methanol:acetic acid:H_2O (75:25:7:3).
3. $CHCl_3$:methanol:NH_4OH:H_2OH (65:25:2:2).
4. $CHCl_3$:acetone:methanol:acetic acid:H_2O (10:4:2:2:1).

The lipids were located by lightly staining with iodine and identified by co-chromatography with standards. Radioactive lipid products were located by autoradiography, and the radioactivity in each lipid measured by scraping and assaying the radioactivity in the silica which contained it.

Protein was determined by the Bradford procedure (6) using BSA as the standard. Chl was determined by the method of Arnon (4).

RESULTS AND DISCUSSION

Lipid Synthesis in Isolated Intact Pea Chloroplasts. To determine whether pea chloroplasts can synthesize PG, isolated intact chloroplasts were suspended in the fatty acid synthesizing media used for lipid synthesis in spinach chloroplasts (25). PG synthesis was observed when either acetate (Fig. 1) or glycerol 3-P (Fig. 2) was supplied as the radioactive precursor. The addition of ATP, CoA, and DTT to the incubation medium increased total lipid synthesis from [^{14}C]acetate (Fig. 1A); the proportion of total label in PG and PC was also increased, while the proportion in MG/FFA was decreased. The addition of ATP alone had only a very slight stimulatory effect on total lipid synthesis (Fig. 1A), while the distribution of label within the lipid products was not different from that when all three components were absent (data not shown). Thus, it appeared that ATP, CoA, and DTT stimulated PG synthesis. Acyl-CoA is probably not the physiological acyl donor for glycerolipid synthesis in intact chloroplasts (12). However, its synthesis upon the addition of CoA would decrease the concentration of FFA which might otherwise be inhibitory.

The addition of $MnCl_2$ was reported to stimulate PG synthesis in spinach chloroplasts (28). However, the addition of $MnCl_2$ (1–4 mM) to pea chloroplasts had no significant effect on the proportion of label incorporated into PG, although the proportion of label present in PA and LPA appeared to increase slightly (data not shown). The second set of observations is consistent with the report that cations inhibit the PA phosphate in spinach chloroplast envelopes (20). However, the level of this enzyme is reportedly very low in pea chloroplasts (12); and surprisingly, we observed that the proportion of label appearing in DG did not change upon the addition of $MnCl_2$. Thus, although pea chloroplasts can synthesize PG, the conditions necessary for this synthesis, at least during *in vitro* assays, may be different from those required in spinach chloroplasts.

The proportion of label in LPA and PA decreased during a 1-h incubation of pea chloroplasts with either [^{14}C]acetate (Fig. 1) or [^{14}C]glycerol 3-P (Fig. 2), while the proportion of radioactivity in PG increased. This suggested that newly synthesized PG was derived from newly synthesized PA. Such a precursor-product relationship was examined by first incubating chloroplasts with [^{14}C]glycerol 3-P in lipid synthesizing medium for 15 min. The

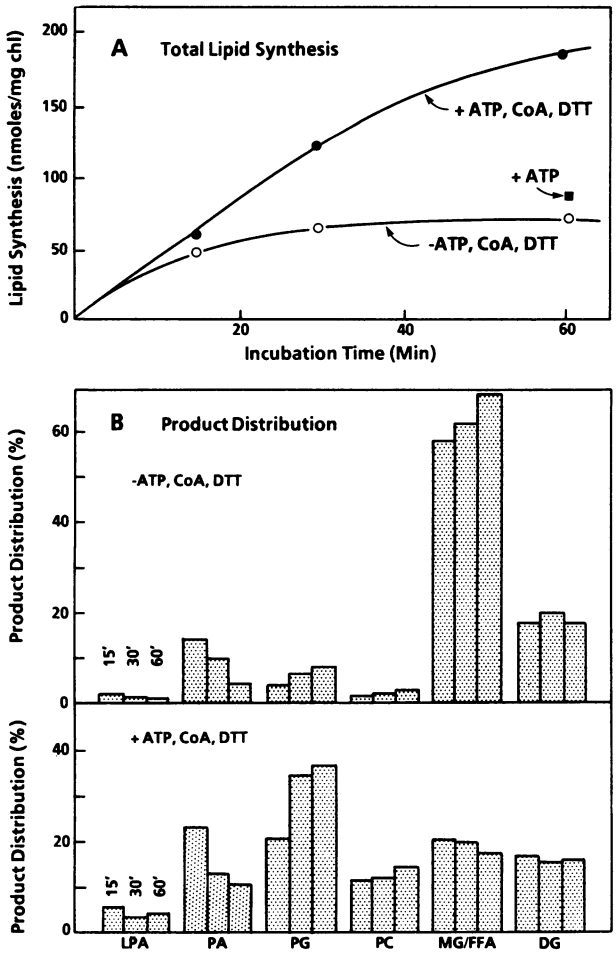

FIG. 1. Lipid synthesis from [^{14}C]acetate by isolated pea chloroplasts. Chloroplasts (149 μg Chl/assay) were incubated in lipid-synthesizing medium (25) for the indicated times in the absence (O) or presence (●) of ATP, CoA, and DTT. Lipid synthesis is expressed as nmol acetate incorporated into $CHCl_3$-soluble products/mg Chl. Lipid products were separated by one dimensional TLC in solvent system No. 1 and analyzed as described. A, Total lipid synthesis; B, distribution of label among the lipid products.

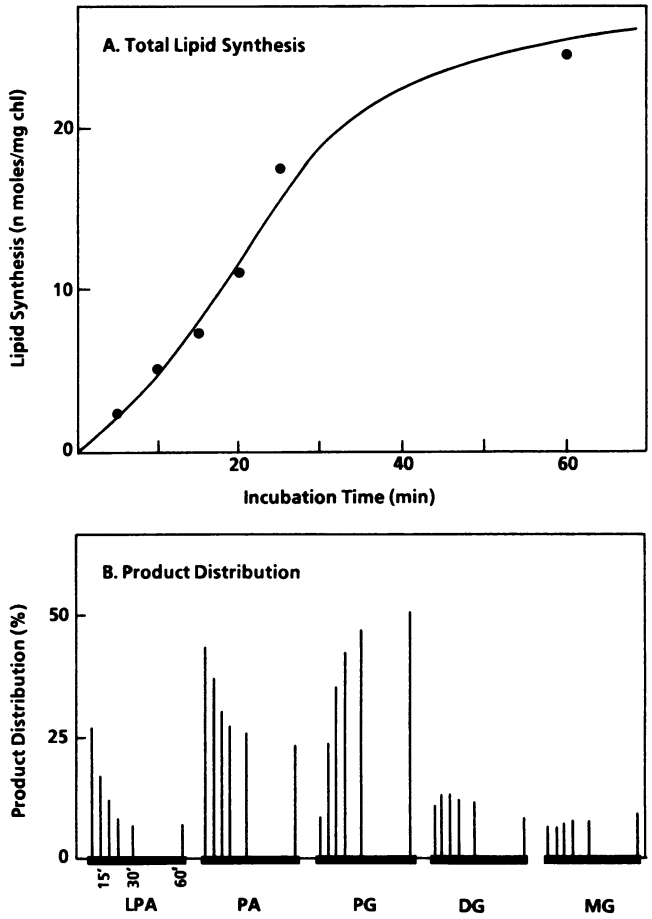

FIG. 2. Lipid synthesis from [^{14}C]glycerol 3-P by isolated pea chloroplasts. Chloroplasts (175 μg Chl/assay) were incubated in lipid-synthesizing medium (25) with 0.4 mM [^{14}C]-DL-glycerol 3-P (1 μCi/assay) for the indicated times. Lipid synthesis, calculated on the basis that only 49% of the DL-glycerol 3-P was available as the L-form, is expressed as nmol L-glycerol 3-P incorporated into $CHCl_3$-soluble products/mg Chl. Lipid products were analyzed as described in Figure 1. A, Total lipid synthesis; B, distribution of label among lipid products.

chloroplasts were then sedimented, resuspended in fresh cold reaction medium, and re-incubated for the indicated times (Fig. 3). The amount of labeled PA decreased during the chase period, while the amount of labeled PG increased (Fig. 3). Since the label in PA was located in its glycerol backbone, the disappearance of this label from PA and its appearance in PG implicated PA as a direct precursor for PG synthesis. However, only a portion of the PA gave rise to PG, as indicated by the decrease in transfer of label from PA to PG with time (Fig. 3). This could be due either to a decrease in the capability of isolated chloroplasts to synthesize lipids with time (see, *e.g.* Figs. 1 and 2), or to the synthesis of some molecular species of PA which do not act as precursors to PG.

In conclusion, both time-course and pulse-chase labeling studies with isolated intact pea chloroplasts demonstrated a precursor-product relationship between newly synthesized PA and newly synthesized PG. The synthesis of PG from PA has been well established in several systems to occur by the following pathway:

$$\text{PA} \xrightarrow{+\text{CTP}} \text{CDP-DG} \xrightarrow{+\text{glycerol 3-P}} \text{phosphatidylglycerol-P} \rightarrow \text{PG}$$

It seemed reasonable to assume that the synthesis of PG in chloroplasts occurred by the same set of steps. Because plastidial PA synthesis occurs in the envelope membranes (19), we decided to assay directly the envelopes for both CDP-DG and PG synthesis from added glycerolipid substrates. The addition of glycerolipid substrates would also overcome the dependence of PG synthesis in intact chloroplasts on active fatty acid synthesis; such dependence probably explains the loss of PG synthesis observed in spinach chloroplasts upon disruption of the chloroplasts (27).

CDP-DG Synthesis in Pea Chloroplast Envelope Membranes. The first enzyme in the postulated scheme of PG synthesis, phosphatidate cytidyltransferase (EC 2.7.7.41) was assayed in pea chloroplast envelope membranes by incubating the membranes with exogenous PA and [^{14}C]CTP in the presence of $MgCl_2$ ("Materials and Methods"). Radioactivity was incorporated into chloroform-soluble products. Analysis of these products by one dimensional TLC in two different solvent systems (solvent systems Nos. 1 and 2, "Materials and Methods") indicated that 86 to 94% co-migrated with authentic CDP-DG, while the rest remained at the origin. No other radioactive spots were detected. Omitting PA from the incubation, or adding boiled envelopes, resulted in essentially blank levels of activity (2). The activity was also fairly stable for at least 1 d after envelope isolation,

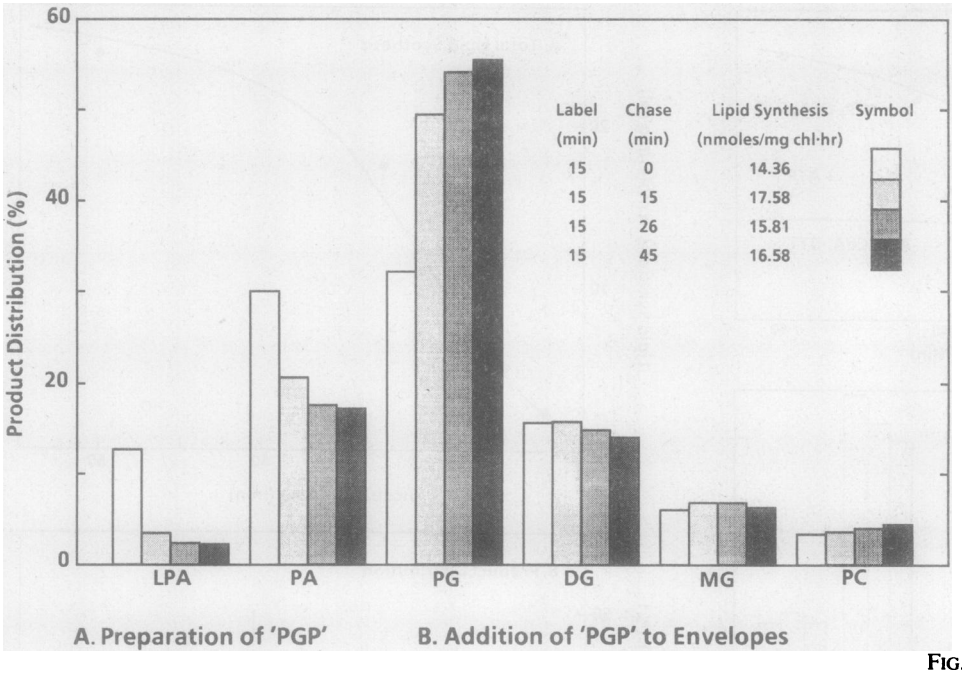

FIG. 3. Pulse-chase study of lipid synthesis from [^{14}C]glycerol 3-P by pea chloroplasts. Chloroplasts (194 μg Chl/sample) were incubated in 1 ml of lipid synthesizing medium which contained [^{14}C]-DL-glycerol 3-P (25) for 15 min (Pulse). The samples were then diluted 1:1 with buffer (0.3 M sorbitol in 33.4 mM Tricine, pH 7.9), and centrifuged at 3300g for 1 min. The chloroplasts were resuspended in 1 ml fresh cold lipid-synthesizing medium and re-incubated for the indicated times (Chase). Lipid synthesis was calculated and expressed as indicated in Figure 2; lipid products were analyzed as described in Figure 1.

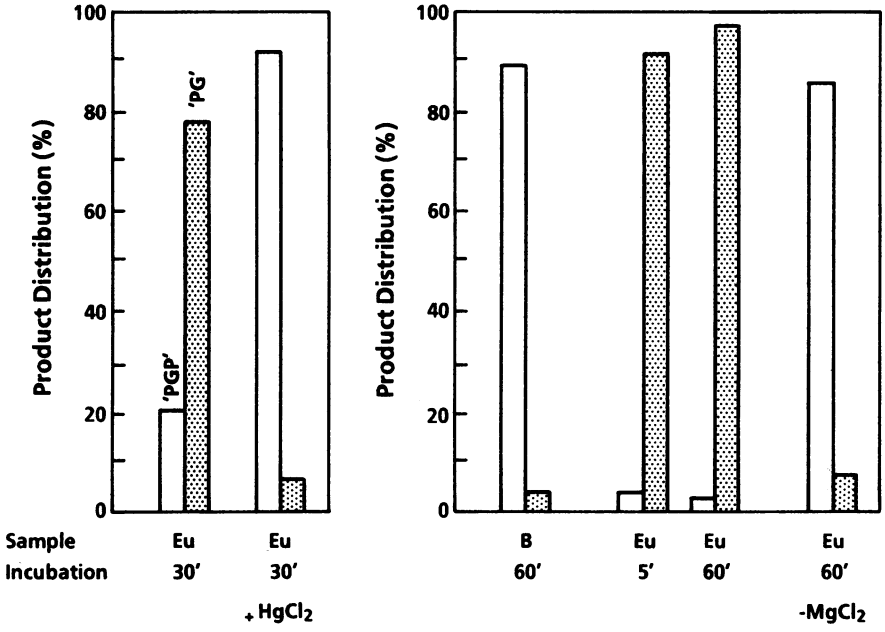

FIG. 4. Conversion of 'PGP' to PG by pea chloroplast envelopes. A, 'PGP' of high specific activity was prepared by incubating envelope membranes (36 μg protein/assay) with CDP-DG and [^{14}C]-DL-glycerol 3-P (0.1 mM, 0.4 μCi/assay) in the presence of $HgCl_2$ (0.5 mM) for 30 min as described for assaying PG synthesis. This resulted in 92.3% of the $CHCl_3$-soluble radioactivity migrating in solvent system No. 4 with an R_F value similar to that reported for PGP in the same solvent system (5). B, About 3.5% of this $CHCl_3$-soluble material (~14,000 cpm, or 0.22 nmol) was then added to each of several clean tubes, along with Triton X-100 dissolved in $CHCl_3$ to give a final reaction mixture concentration of 0.15% (w/v). These samples were dried under N_2, and dissolved in Tris-HCl with and without $MgCl_2$ (final reaction mixture concentrations: 250 mM (pH 7.3) and 30 mM, respectively). Reactions were initiated by adding either 0.2 M sucrose in buffer (B) or envelope membranes (E_u, 18 μg protein/assay), and the incubations continued and terminated as described for measuring PG synthesis. Lipids were separated by one dimensional TLC in solvent system 4, and analyzed as described.

although it was more stable when the membranes were stored frozen (80.3–90.6% control levels) than when stored refrigerated (40.2–73.9% control levels).

Thus, we have demonstrated that phosphatidate cytidyltransferase activity was present in the envelope membranes in pea chloroplasts. The activity was not affected by the slow freezing and thawing used to break the chloroplasts, as nearly identical levels of activity were observed in envelopes obtained from chloroplasts broken by hypotonic lysis (data not shown). The levels of activity were fairly high when compared to those reported for other plant systems (22, 29). This may be due to the fact that the envelope membrane preparation is highly purified with respect to other membranes, and contains relatively low amounts of protein.

PG Synthesis in Pea Chloroplast Envelope Membranes. The second and third enzymes in the postulated scheme of PG synthesis, glycerophosphate phosphatidyltransferase (CDPdiacylglycerol:*sn*-glycerol-3-phosphate phosphatidyltransferase, EC 2.7.8.5) and phosphatidylglycerophosphatase (phosphatidylglycerophosphate phosphohydrolase, EC 3.1.3.27), were assayed together in pea chloroplast envelope membranes by incubation of the membranes with exogenous CDP-DG and [^{14}C]glycerol 3-P in the presence of $MgCl_2$ and Triton X-100 ("Materials and Methods"). Radioactivity was incorporated into chloroform-soluble products. Analysis of these products by one dimensional TLC (solvent systems Nos. 1–4, "Materials and Methods") indicated that several labeled compounds were present. The predominant product, which contained from 50 to 85% of the total radioactivity, co-migrated with PG. A second compound, which contained up to 45% (but usually less than 20%) of the total radioactivity remained at the origin in the double solvent system (No. 1, "Materials and Methods"). At least some of this material

Table I. *Incorporation of Exogenous PA into PG by Pea Chloroplast Envelopes*

Envelope proteins (45 μg) were incubated with PA (1 mM), $MgCl_2$ (30 mM), and either labeled or unlabeled CTP (0.5 mM) in MOPS-KOH (pH 7.4) for the times indicated in the table. DL-Glycerol 3-P (labeled or unlabeled) was then added to a final concentration of 0.5 mM and the incubations continued as indicated. The reactions were terminated, and the lipids analyzed in solvent system No. 2, as described in "Materials and Methods."

Experiment	Labeled Substrate Incorporated	Labeled Product
	nmol	
PA + [^{14}C]CTP		
20′	2.78	CDP-DG
50′	4.25	CDP-DG
PA + [^{14}C]CTP, 20′		
+ Glycerol 3-P, 10′	1.51	CDP-DG
+ Glycerol 3-P, 30′	0.26	CDP-DG
PA + CTP, 20		
+ [^{14}C]Glycerol 3-P, 10′	1.93	PGP,[a] PG
+ [^{14}C]Glycerol 3-P, 30′	3.55	PGP, PG
PA + [^{14}C]Glycerol 3-P, 10′	0	None
+ [^{14}C]Glycerol 3-P, 30′	0	None

[a] PGP identification is tentative.

has been tentatively identified as PGP on the basis of the following observations.

During time-course studies of PG synthesis, the proportion of label in PGP, which was very high at short times, decreased with a concomitant increase in the proportion of label in PG. A final low level of radioactivity which remained at the origin in solvent system No. 1 may represent PGP which cannot act as a substrate for the phosphatase.

Both $HgCl_2$ and *N*-ethylmaleimide are known to inhibit the phosphatidylglycerophosphatase in other systems (7). When either was added to the incubation mixture for PG synthesis, the appearance of labeled PG was either completely inhibited ($HgCl_2$) or greatly reduced (*N*-ethylmaleimide), while a large amount of label appeared in PGP (2). When the products from these assays were chromatographed with different solvent systems, the PGP moved off the origin in two of them (Nos. 2 and 4, "Materials and Methods"). The resulting R_F values of PGP were similar in both systems, and similar to that reported for PGP when chromatographed in solvent system No. 4 (5). The amount of label which moved with PGP accounted for 91 to 99% of that originally present at the origin when the double solvent system (No. 1) was used.

Finally, the addition of labeled PGP to freshly prepared envelope membranes resulted in the conversion of greater than 90% to PG, indicating that this material was indeed a precursor to PG synthesis (Fig. 4). This conversion proceeded rapidly, with very little additional metabolism occurring after 5 min. The presence of $MgCl_2$ was required for the conversion of PGP to PG (Fig. 4); thus, this ion appears necessary for both the synthesis of PGP from CDP-DG (2), and its dephosphorylation to PG.

These observations suggest that PG synthesis from CDP-DG occurred via the intermediate PGP. The synthesis of PG was dependent upon both the addition of exogenous lipid substrate (CDP-DG) and the presence of active enzyme (2), and was stable for at least 1 d after envelope isolation. The activity was the same when the membranes were stored either refrigerated (73.7–93.7% control levels) or frozen (75.0–93.4% control levels).

In summary, these results demonstrated that PG synthesis from CDP-DG and glycerol 3-P could occur in pea chloroplast envelopes. The levels of activity were unaffected by the slow freezing and thawing used to break the chloroplasts (data not shown).

Utilization of PA for PG Synthesis in Pea Chloroplast Envelopes. The synthesis of CDP-DG from PA, and of PG from CDP-DG, by chloroplast envelope membranes supports the hypothesis that PA is a precursor to PG. More conclusive evidence that PA was incorporated into PG was obtained by two additional experiments.

In the first, envelopes were incubated with PA and [^{14}C]CTP, giving rise to [^{14}C]CDP-DG. The subsequent addition of glycerol 3-P resulted in a rapid decrease in the amount of [^{14}C]CDP-DG (Table I), suggesting that this product was being utilized as a substrate for PG synthesis, releasing water-soluble [^{14}C]CMP. The incubation of envelopes with PA and cold CTP, which was followed by the addition of [^{14}C]glycerol 3-P, gave rise to [^{14}C] PG (Table I). Since the synthesis of PG was dependent upon the presence of CDP-DG (2), these results indicated that the CDP-DG synthesized from the added PA and CTP was subsequently utilized in PG synthesis. The incubation of PA alone with [^{14}C] glycerol 3-P did not result in any labeled lipid products (Table I). This indicated that PA must first be incorporated into CDP-DG for use in PG synthesis. The results (not shown) from the

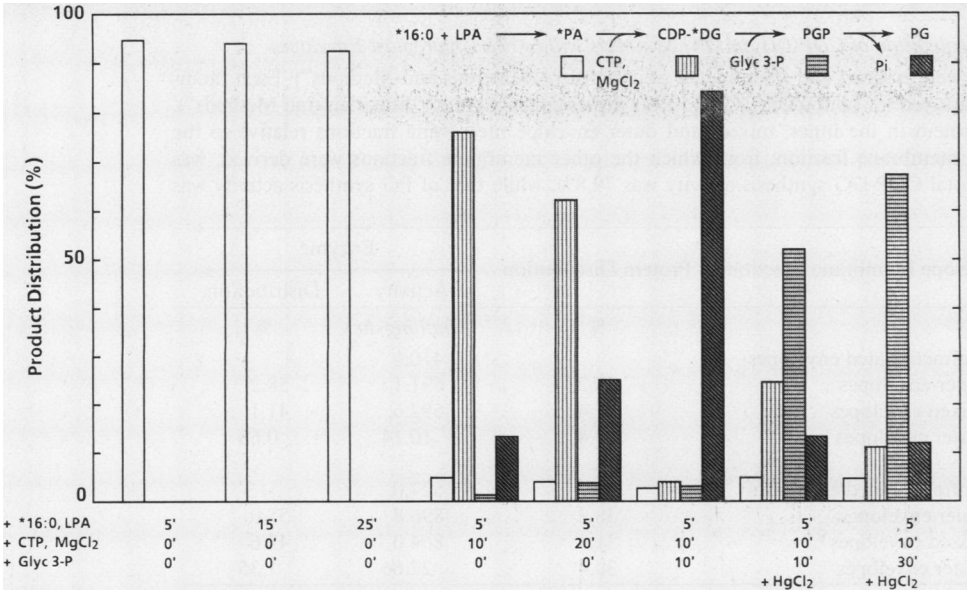

FIG. 5. Synthesis of PA and subsequent incorporation into CDP-DG and PG by envelope membranes. LPA (1-oleoyl-glycerol 3-P) and [^{14}C]palmitoyl-ACP were added to envelope membranes (30 μg/assay). This was followed in 5 min by the addition of CTP (5 mM) with $MgCl_2$ (20 mM), then 10 min later by the addition of glycerol 3-P (1 mM). When present, $HgCl_2$ (1 mM) was added with glycerol 3-P. The numbers under each set of bar graphs indicate the incubation period (min) for each set of additions. Lipid products were analyzed as described in Figure 1.

Table II. *Localization of CDP-DG and PG Synthesis within Pea Chloroplasts*

Chloroplasts broken by freeze-thaw lysis were fractionated by step density gradient centrifugation ("Materials and Methods"). Envelope and thylakoid membranes were pelleted and resuspended before they were assayed for CDP-DG and PG synthesis ("Materials and Methods").

	Chloroplast Fraction	Protein Distribution	Enzyme	
			Activity	Distribution
		%	*nmol mg·hr*	%
CDP-DG	Envelope	0.84	390	27.8
	Thylakoid			
Synthesis	stroma	25.5	26.5	72.2
		73.6	0	0
PG	Envelope	0.84	123	32.6
	Thylakoid			
Synthesis	stroma	25.5	10.5	67.4
		73.6	0	0

addition of Triton X-100 with glycerol 3-P were in agreement with earlier observations that the detergent stimulates the synthesis of PG but almost completely inhibits the synthesis of CDP-PG (2).

The second experiment was a more direct proof that PA was a precursor to PG. Incubation of envelope membranes with 1-oleoyl-glycerol 3-P (LPA) and [^{14}C]palmitoyl-ACP gave rise to labeled PA (Fig. 5). The addition of CTP (with $MgCl_2$) resulted in a rapid disappearance of labeled PA with concomitant appearance of labeled CDP-DG (Fig. 5). The subsequent addition of glycerol 3-P resulted in a transfer of the label from CDP-DG to PG (Fig. 5). The addition of glycerol 3-P alone, without CTP, to envelopes containing labeled PA, did not result in the transfer of label from PA to PG (data not shown). These results demonstrated that the DG moiety of PA, which is labeled at the 2 position with [^{14}C]palmitate (12), was transferred intact into PG via CDP-DG. In addition, the entire sequence of PG synthesis, from LPA and palmitoyl-ACP, has now been demonstrated to occur in the envelope membranes of pea chloroplasts.

It is interesting to note that in this experiment, the addition of CTP to envelopes containing labeled PA resulted in the appearance not only of labeled CDP-PG, but also of a low proportion of labeled PG (Fig. 5). Similar results were observed in several separate experiments. This suggests either that the envelope membrane preparation contained glycerol 3-P, which seems unlikely, or that a headgroup transfer occurred between endogenous PG and newly synthesized CDP-DG. If this were the case, then the levels of CDP-DG synthesis may be higher than those actually calculated.

Localization of PG Synthesis within Pea Chloroplasts. When whole chloroplast preparations were analyzed for both CDP-DG synthesis from PA and [^{14}C]CTP, and PG synthesis from CDP-DG and [^{14}C]glycerol 3-P, $CHCl_3$-soluble radioactive material was synthesized in both assays. The levels of activity were comparable to those expected if the envelope membranes were assumed to contain about 1% of the total chloroplast protein, and were the same for both intact and broken chloroplasts (data not shown). However, an analysis of the products indicated that whereas [^{14}C]CDP-DG contained 94% of the radioactivity incorporated from [^{14}C]CTP, [^{14}C]PG contained only 13% of the incorporated label from [^{14}C]glycerol 3-P, with 76% of the label behaving chromatographically like PGP. This may indicate that chloroplasts contained an inhibitor of phosphatidylglycerophosphatase, which was removed to varying degrees from the envelope membranes during isolation. Alternatively, as stated earlier, some of this material may not be a precursor to PG. Pea chloroplasts rapidly incorporated [^{14}C]glycerol 3-P into certain lipids even under conditions in which fatty acid synthesis from [^{14}C]acetate was not occurring (*e.g.* when chloroplasts were broken and incubated under the conditions described for glycerolipid synthesis in "Materials and Methods," data not shown). It is possible that glycerol 3-P may also be incorporated into other products by chloroplasts. Thus, it was difficult to use whole chloroplast preparations to examine PG synthesis from [^{14}C] glycerol 3-P and CDP-DG.

Pea chloroplasts were fractionated into stromal, thylakoid, and envelope fractions ("Materials and Methods"); each fraction was then assayed for the presence of CDP-DG and PG synthesizing activities. For both CDP-DG and PG synthesis, the specific activities were greatest in the envelope fraction (Table II), indicating that both synthetic capabilities were localized to the envelope membranes. The major product formed from [^{14}C]CTP and PA in both the envelope and thylakoid membrane fractions was CDP-DG (94%). However, when thylakoid membranes were incubated with [^{14}C]glycerol 3-P and CDP-DG, PG contained only 38% of the total product label, while 57% remained at the origin. Thus, thylakoid membranes presented problems similar to whole chloroplasts in determining levels of PG synthesis.

Envelope membranes were isolated and fractionated by linear

Table III. *Localization of CDP-DG and PG Synthesis within Pea Chloroplast Envelopes*

Envelope membranes were isolated and fractionated as described ("Materials and Methods"). Each membrane fraction was then assayed for both CDP-DG and PG synthesizing activities ("Materials and Methods"). The recovery of total protein in the inner, mixed, and outer envelope membrane fractions relative to the unfractionated envelope membrane fraction, from which the other membrane fractions were derived, was 72.3%; the recovery of total CDP-DG synthesis activity was 79.8%, while that of PG synthesis activity was 85.4%.

	Envelope Membrane Fraction	Protein Distribution	Enzyme	
			Activity	Distribution
		%	*nmol mg·hr*	%
CDP-DG	Unfractionated envelopes		410.6	
Synthesis	Inner envelopes	38.2	691.1	58.2
	Mixed envelopes	31.4	592.6	41.1
	Outer envelopes	30.4	10.14	0.68
PG	Unfractionated envelopes		527.0	
Synthesis	Inner envelopes	38.2	896.8	55.0
	Mixed envelopes	31.4	864.0	43.6
	Outer envelopes	30.4	27.66	1.35

density sucrose centrifugation as described ("Materials and Methods"); each membrane fraction was then assayed for the presence of CDP-DG and PG synthesizing activities (Table III). The specific activity for the synthesis of these lipids was 70 and 30 times greater, respectively, in the inner envelope membrane fraction than in the outer envelope membrane fraction. This indicated that the synthesis of both lipids was located in the inner envelope membrane. As the synthesis of PA from LPA also occurs in this membrane (3), the entire synthesis of PG from LPA occurs in the inner envelope membrane in pea chloroplasts.

Upon chloroplast fractionation, the majority of the total activity for the synthesis of both CDP-DG and PG was associated with the thylakoid fraction (Table II). Although it is not possible to rule out a dual localization of CDP-DG and PG synthesis, the observed distributions may represent a preferential contamination of the thylakoid fraction by inner envelope membranes. In support of this hypothesis are the observations that two other inner envelope membrane enzymes show a similar distribution of total activity among chloroplast fractions (1-acylglycerophosphate acyltransferase and acyl-CoA thioesterase, references [3] and [1], respectively). This is in contrast to the distributions observed for two outer envelope membrane enzymes, in which the greatest proportion of total activity is associated with the envelope membrane fraction (galactosyltransferase and acyl-CoA synthetase, references [9] and [1], respectively).

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