Phosphatidylglycerol Biosynthesis in *Bacillus licheniformis*. Resolution of Membrane-Bound Enzymes by Affinity Chromatography on Cytidinediphospho-*sn*-1,2-diacylglycerol Sepharose†

Timothy J. Larson, Takashi Hirabayashi, and William Dowhan*

ABSTRACT: Cytidinediphospho-*sn*-1,2-diacylglycerol (CDP-diglyceride) has been covalently linked to Sepharose 4B via an adipic acid dihydrazide spacer arm forming an effective affinity chromatography column. This liponucleotide ligand and *sn*-glycero-3-phosphate are substrates for the formation of 3-*sn*-phosphatidyl-1′-*sn*-glycero-3′-phosphate (PGP) catalyzed in both eukaryotic and prokaryotic organisms by *sn*-glycero-3-phosphate:CMP phosphatidyltransferase (PGP synthetase). Using this CDP-diglyceride Sepharose affinity column we were able to resolve the membrane associated 3-*sn*-phosphatidyl-1′-*sn*-glycerol (PG) synthesizing system present in *Bacillus licheniformis* into two activities. A PGP synthetase activity was adsorbed to the affinity column and was eluted using buffer containing CDP-diglyceride; a PGP phosphatase activity had no affinity for the column. Both the PGP synthetase and PGP phosphatase of *B. licheniformis* were associated with a membrane component of the cell as evidenced by sucrose gradient centrifugation, differential centrifugation, and solubilization by buffers containing detergent. The synthetase and phosphatase appeared to be associated with different membrane components of the cell since the synthetase activity sediments slightly slower than the phosphatase activity on sucrose gradients. The solubilized and partially purified PGP synthetase required magnesium ion and a nonionic detergent such as Triton X-100 for the stoichiometric conversion of CDP-diglyceride and *sn*-glycero-3-phosphate to PGP and CMP. The solubilized PGP phosphatase required a nonionic detergent and little, if any, magnesium for the conversion of PGP to PG and inorganic phosphate.

Detailed information is not available concerning the physical, chemical, and enzymological properties of enzymes involved in phospholipid biosynthesis in both eukaryotic and prokaryotic organisms. Of this group of enzymes, several have been partially purified and studied (Chang and Kennedy, 1967a,b; Raetz et al., 1972; Raetz and Kennedy, 1974; Rao and Strickland, 1974) while only the phosphatidylserine decarboxylase from *Escherichia coli* has been purified to homogeneity (Dowhan et al., 1974). The major obstacles to obtaining homogeneous enzyme preparations for study are the low amounts of these enzymes present in various organisms and the requirement for detergents to solubilize most of these enzymes from the membrane; these detergents generally interfere to some extent with all normally employed purification techniques (Helenius and Simons, 1975).

We report herein the preparation of an affinity chromatography column to which CDP-diglyceride[1] has been covalently bound. Since CDP-diglyceride is a substrate for several enzymes involved in phospholipid biosynthesis in both eukaryotic and prokaryotic organisms, such an affinity column should be useful in purifying a number of enzymes. The presence of nonionic detergents used to solubilize many of these membrane associated enzymes should not interfere with chromatography since these same detergents are usually required for maximal enzymatic activity.

As an example of the resolving power of the CDP-diglyceride Sepharose column we report the separation of the previously unresolved membrane associated system for the synthesis of PG in *Bacillus licheniformis* into two enzymes which catalyze the following reactions:

$$\text{CDP-diglyceride} + \textit{sn}\text{-glycero-3-phosphate} \rightarrow \text{CMP} + \text{PGP} \quad (1)$$

$$\text{PGP} \rightarrow \text{P}_i + \text{PG} \quad (2)$$

Reaction 1 is catalyzed by *sn*-glycero-3-phosphate:CMP phosphatidyltransferase (PGP synthetase) and reaction 2 by PGP phosphatase. Chang and Kennedy (1967a,b) and Kiyasu et al. (1963) have demonstrated the requirement for these two enzymes for the synthesis of PG from CDP-diglyceride in *E. coli* and liver mitochondria, respectively. Patterson and Lennarz (1971) have shown the in vitro synthesis of PG from CDP-diglyceride and *sn*-glycero-3-phosphate in *Bacillus* PP but were unable to establish the intermediate formation of PGP.

Materials and Methods

Reagents. All chemicals used were reagent grade or better quality. Radiochemicals were purchased from Amersham/Searle. *sn*-Glycero-3-phosphate and phospholipase D (cabbage) were purchased from Calbiochem. Glycerokinase and CMP-morpholidate as the dicyclohexylammonium salt were purchased from Sigma. Precoated silica gel thin-layer chromatography plates were obtained from E. Merck, Darmstadt, Germany. Sephadex G-75 and Sepharose 4B

† From the Department of Biochemistry and Molecular Biology, University of Texas Medical School at Houston, Houston, Texas 77025. *Received September 12, 1975.* This work was supported by grants from the National Institutes of Health (GM 20478) and the Robert A. Welch Foundation (AU-599). Material for this manuscript will be used for partial fulfillment of the requirements for the Ph.D. of T.J.L.

[1] Abbreviations used are: PGP, 3-*sn*-phosphatidyl-1′-*sn*-glycero-3′-phosphate; PG, 3-*sn*-phosphatidyl-1′-*sn*-glycerol; CDP-diglyceride, cytidinediphospho-*sn*-1,2-diacylglycerol.

were purchased from Pharmacia, Uppsala, Sweden. Triton X-100 (*p-tert*-octylphenoxypolyethoxyethanol) was a product of Rohm and Haas. Standard cytidinediphospho-*sn*-1,2-dipalmitoylglycerol (Tris salt) was the gift of Dr. E. P. Kennedy.

Analytical Methods. All protein determinations were done by the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard. The method was modified to accommodate samples containing Triton X-100 by adding 10 mg of sodium dodecyl sulfate to each assay tube. Organic and inorganic phosphates were quantitatively determined by the method of Bartlette (1959). The water-soluble deacylation products of phospholipids were formed by mild alkaline hydrolysis as described by Kates (1972). Radioactivity was determined using a Beckman LS-233 liquid scintillation counter. Aqueous samples (1 ml or less) were counted in 10 ml of Beckman Ready-Solv 1A; samples soluble in chloroform were counted in 10 ml of toluene-based scintillation fluid. Radioactive profiles on silica gel thin-layer plates and paper chromatograms were determined by counting 1-cm strips in scintillation fluid. Phospholipids were identified by chromatography on activated (110 °C, 30 min) silica gel thin-layer plates. Water-soluble compounds were identified using ascending chromatography on Whatman No. 1 paper. Phosphate-containing compounds on silica gel thin-layer plates and paper chromatograms were visualized by the specific sprays of Dittmer and Lester (1964) and Vorbeck and Marinetti (1965), respectively. The following solvent systems (v/v) were used in this work: (1) chloroform–methanol–glacial acetic acid–water (50:28:4:8); (2) saturated ammonium sulfate in 0.1 M potassium phosphate (pH 6.8)–2-propanol (100:2); (3) 1 M ammonium acetate (pH 7.5)–ethanol (35:65); (4) chloroform–methanol–acetic acid (65:25:8); (5) methanol–88% formic acid–water (80:13:7); (6) 1-butanol–acetic acid–water (5:4:1); (7) 1 M ammonium acetate (pH 7.4)–ethanol (3:7).

Growth of Organisms. Bacillus licheniformis (ATCC No. 14580) was grown at 32 °C with high aeration in a medium consisting of 1% peptone, 0.5% yeast extract, and 0.5% sodium chloride in a 150-l. New Brunswick Scientific Co. Fermacel. The cells were collected during late log phase of growth and stored as the frozen cell paste at −20 °C.

Preparation of Substrates. 3-*sn*-Phosphatidylcholine, isolated from fresh chicken eggs by the method of Wells and Hanahan (1969), was hydrolyzed by phospholipase D as described by Kates and Sastry (1969) to form phosphatidic acid. CDP-diglyceride was prepared from phosphatidic acid and CMP-morpholidate as described by Raetz and Kennedy (1973). The product had an ester to phosphate to cytidine ratio of 1.8:1.9:1.0 and the expected A_{280}:A_{260} ratio of 2.1 in 0.01 M HCl. In addition, the product cochromatographed on silica gel thin-layer plates with authentic CDP-dipalmitin in solvents 1 and 4 and showed a single fluorescent spot under ultraviolet light which was positive to phosphate-specific spray. The concentration of CDP-diglyceride was determined by its molar extinction coefficient in 0.01 N HCl (12.8×10^3) and by its quantitative reaction with *sn*-[2-^{3}H]glycero-3-phosphate in the presence of either *B. licheniformis* PGP synthetase or a preparation of *E. coli* PGP synthetase free of CDP-diglyceride hydrolase activity.

CDP-diglyceride with tritium in the cytosine moiety was prepared enzymatically using the CMP exchange reaction catalyzed by *E. coli* phosphatidylserine synthetase as described by Raetz and Kennedy (1974). *sn*-[2-^{3}H]Glycero-3-phosphate, *sn*-glycero-3-[^{32}P]phosphate, *sn*-[2-^{3}H]glycero-3-[^{32}P]phosphate, and 3-*sn*-phosphatidyl-1′-*sn*-glycero-3′-[^{32}P]phosphate were prepared enzymatically as described by Chang and Kennedy (1967a,b).

Enzyme Assays. PGP synthetase activity was determined at 30 °C by a modification of the method of Chang and Kennedy (1967a) in a 40-ml glass centrifuge tube (12-ml polypropylene tube after step 5, Table I). The assay mixture consisted of 0.5 mM CDP-diglyceride, 0.5 mM *sn*-[2-^{3}H]glycero-3-phosphate (150 cpm per nmol), 0.1 M Tris-maleate (pH 7), 2% Triton X-100, 50 mM $MgCl_2$, 0.1 M KCl, 1 mg per ml of bovine serum albumin, and enzyme in a final volume of 0.1 ml. The reaction was stopped after 20 min by the addition of 1 ml of methanol (0.1 N in HCl). Chloroform (1.5 ml) and 1 M $MgCl_2$ (3 ml) were added, the system was mixed thoroughly, and the phases were separated by a brief centrifugation. An aliquot (1.0 ml) of the chloroform phase was removed and taken to dryness on a 60 °C water bath and the radioactivity determined. One unit of PGP synthetase activity is defined as the amount of enzyme required to convert 1 nmol of *sn*-glycero-3-phosphate to chloroform soluble product in 1 min under the above conditions. Blank reaction mixtures containing either no enzyme, boiled enzyme, or a complete reaction mixture stopped at time zero were incubated with each assay.

PGP phosphatase activity was determined by a modification of the procedure of Chang and Kennedy (1967b) and was carried out as described above for the PGP synthetase with the following changes. The reaction mixture consisted of 0.2 mM 3-*sn*-phosphatidyl-1′-*sn*-glycero-3′-[^{32}P]phosphate (200–1000 cpm per nmol), 0.1 M Tris-maleate (pH 7), 1% Triton X-100, 50 mM $MgCl_2$, 0.1 M KCl, and enzyme in a final volume of 0.11 ml. The reaction was stopped by the addition of 0.5 ml of methanol (0.1 N in HCl) followed by 1.5 ml of chloroform and 1 ml of 0.2 M $MgCl_2$ containing 0.2 μmol of carrier potassium phosphate. After separation of the phases, a 1.0-ml aliquot of the aqueous phase was counted for radioactivity. One unit of PGP phosphatase activity is defined as the amount of enzyme required to catalyze the release of 1 nmol of inorganic phosphate from PGP per minute under the above conditions.

Results

Sucrose Gradient Centrifugation of PGP Synthetase and PGP Phosphatase. Sucrose gradient centrifugation was used to determine the subcellular localization of the PGP synthetase and PGP phosphatase. The distribution of nucleic acid and protein throughout the sucrose gradient was determined by the absorbance at 260 nm (Figure 1). The absorbance near the top of the gradient (fractions 22–25) was due primarily to protein as indicated by the A_{280}:A_{260} ratio (Warburg and Christian, 1941). The absorbance peak near the bottom of the gradient also was due primarily to protein (A_{280}:A_{260} indicated less than 10% nucleic acid) as well as light scattering caused by the very cloudy nature of the membrane containing fractions. When 3-*sn*-phosphatidyl-1′-*sn*-3′-[^{32}P]phosphate was briefly sonicated with the sample before centrifugation (data not shown), 85% of the radioactivity was found associated with the peak of absorbance near the bottom of the gradient indicating the membrane fraction had concentrated on top of the 70% sucrose shelf. The peak of the absorbance in fractions 13–17 was due primarily to ribosomes since the A_{280}:A_{260} ratio indicated a content of over 95% nucleic acid.

The PGP phosphatase activity (70%) was found associated with the membrane fraction located near the bottom of

Table I: Separation of PGP Synthetase and PGP Phosphatase.

Step	Total Protein (g)	Total Vol (ml)	PGP Synthetase Sp Act. U/mg	PGP Synthetase Yield (%)	PGP Phosphatase Sp Act. U/mg	PGP Phosphatase Yield (%)	Synthetase: Phosphatase
1. Cell-free extract	12.8	435	0.69	100	0.72	100	0.95
2. Membrane fraction	8.1	350	0.87	82	0.83	74	1.05
3. Triton X-100 extract	2.3	280	2.8	71	2.3	56	1.22
4. Column run through and wash	2.9	760	0.26	9	1.7	55	0.15
5. CDP-diglyceride eluate[a]	0.0075	60	360	26	0		

[a] Yield was based on the activity in the pooled peak. Protein and specific activity were measured on the concentrated, dialyzed, Sephadex G-75 peak.

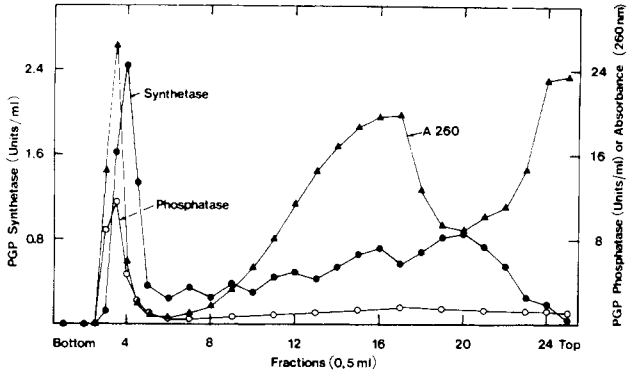

FIGURE 1: Sucrose gradient sedimentation. Frozen cells of *B. licheniformis* were suspended in the gradient buffer and broken using a French pressure cell; whole cells were removed by low-speed centrifugation. A 0.5-ml aliquot of broken cells containing 15 mg of protein was layered on top of a 5–20% sucrose gradient (10.4 ml) with a 70% sucrose shelf (1.5 ml) at the bottom. The buffer system used throughout was 50 mM Tris-HCl (pH 8.0) containing 10 mM $MgCl_2$. After centrifugation (Spinco L2-65B preparative ultracentrifuge equipped with a SW 41 rotor) at 200 000*g* for 90 min at 4 °C, fractions were collected and the absorbance at 260 nm, the PGP synthetase activity, and the PGP phosphatase activity were determined as indicated. In the region of the 70% sucrose shelf, half-fractions (0.25 ml) were collected and analyzed.

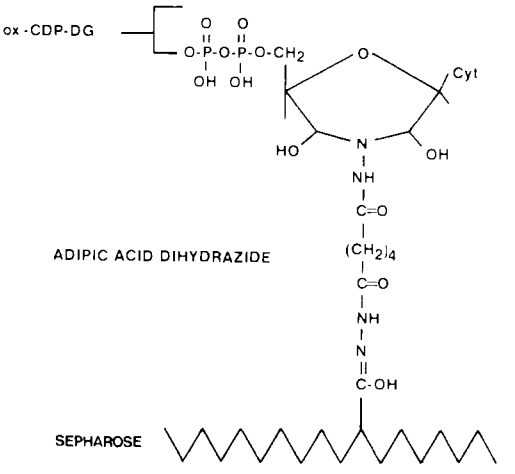

FIGURE 2: Structure of CDP-diglyceride Sepharose affinity resin.

the gradient; therefore, the phosphatase appears to be associated with the major membrane component of the cell, i.e. the cytoplasmic membrane. The PGP synthetase, on the other hand, was found associated with a slightly slower sedimenting peak; this activity was also spread to a greater extent throughout the gradient. This result may indicate that the synthetase is associated with a smaller sized membrane fraction of the cell such as the mesosome fraction present in various gram-positive organisms (Reusch and Burger, 1974; Patch and Landman, 1971).

Preparation of CDP-Diglyceride Affinity Column. A modification of a method used originally for the coupling of water-soluble nucleotides to Sepharose (Lamed et al., 1973) was used to covalently link an oxidized derivative of CDP-diglyceride to Sepharose 4B. Sepharose 4B (100 ml) was activated by CNBr (20 g) as described by Cuatrecasas (1970). The activated resin was suspended in 100 ml of 0.1 M Na_2CO_3 (pH 9.5) containing 9 g of adipic acid dihydrazide and mixed for 17 h at 4 °C. After washing extensively with first 1 M NaCl and then water, the resin gave a positive test for the presence of covalently bound unsubstituted hydrazide (Cuatrecasas, 1970).

The ribosyl hydroxyls of CDP-diglyceride (0.36 mmol) were converted to aldehydes by oxidation with $NaIO_4$ (0.47 mmol) in 70 ml of sodium acetate (pH 4.5) in the dark by first incubating at room temperature for 1 h and then overnight at 0 °C; excess periodate was destroyed by adding 0.1 ml of glycerol. Oxidation was approximately 90% complete as determined by silica gel thin-layer chromatography in solvent 1; the oxidized product had an increased R_f relative to CDP-diglyceride.

The oxidized CDP-diglyceride (0.36 mmol) was covalently attached to the Sepharose 4B–adipic acid dihydrazide column (90 ml setted volume) by stirring the two reactants overnight at 4 °C in 180 ml of 0.1 M sodium acetate (pH 5.0) containing 0.5% Triton X-100. Unreacted CDP-diglyceride was removed by extensive washing with 0.1 M sodium acetate (pH 5.0) containing 0.5% Triton X-100 and 0.5 M KCl followed by deionized water and finally with the buffer to be used for chromatography. Analysis of the resin phosphate content (Bartlette, 1959) in various preparations indicated about 1–2 μmol of covalently bound oxidized CDP-diglyceride per ml of Sepharose 4B. Based on the studies done by Hansske et al. (1974) on the structure of the products of the reaction between periodate oxidized AMP and carboxylic acid hydrazides, Figure 2 appears to be the best representation of the structure of the affinity resin.

Separation of PGP Synthetase from PGP Phosphatase. The following procedures (Table I) were carried out at 0–4 °C. *B. licheniformis* frozen cell paste (90 g) was suspended in 350 ml of buffer A (50 mM Tris-maleate (pH 7), 10 mM $MgCl_2$, and 5 mM 2-mercaptoethanol) with the aid of a Waring blender. The cells were broken by passing the suspension through a French pressure cell. The membrane fraction was collected by centrifugation at 100 000*g* for 2 h. The resulting pellet was suspended in a final volume of 350 ml of buffer A which was 0.2 M in KCl and 4% in Triton X-100 (membrane fraction). After stirring for 1 h the sus-

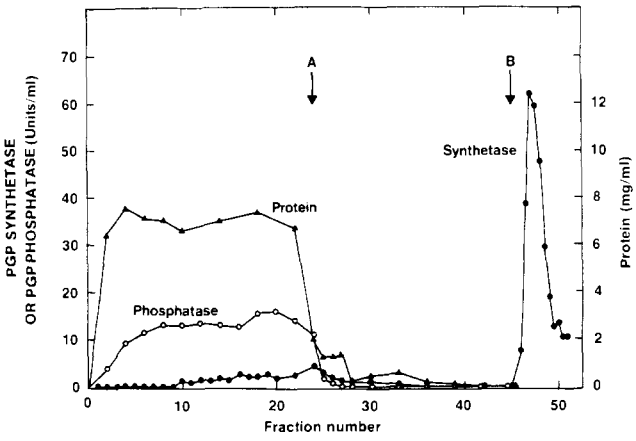

FIGURE 3: Affinity column chromatography of PGP synthetase. A Triton X-100 extract of *B. licheniformis* membranes (Table I) was applied to a CDP-diglyceride affinity column and the eluate was collected in 17-ml fractions. At point A, a Triton–KCl wash was begun, followed by elution at point B with buffer containing CDP-diglyceride.

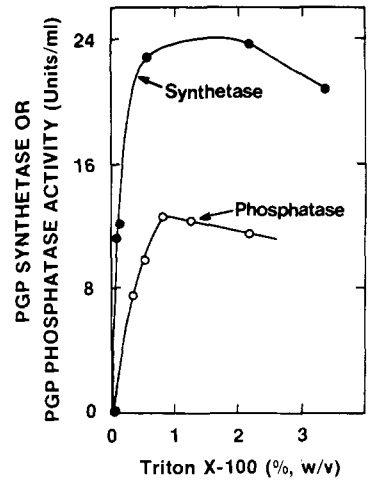

FIGURE 4: Dependence of PGP synthetase and PGP phosphatase activities on Triton X-100. Standard assay conditions were used except the Triton concentration was varied as indicated.

pension was centrifuged at 100 000*g* for 2 h as above. The supernatant (Triton X-100 extract) was saved and the pellet discarded. As indicated in Table I, both the PGP synthetase and PGP phosphatase activities were associated with the membrane fraction and could be extracted by disruption of the membrane structure with a detergent such as Triton X-100. Similar results were obtained when ionic detergents such as cholate or deoxycholate were used. The yields do not represent either an activation or inactivation of either enzyme since the remaining activity could be accounted for in the supernatant of step 2 and the pellet of step 3, respectively.

The Triton X-100 extract was brought to final concentrations of 2% Triton, 50 mM $MgCl_2$, and 20% glycerol. The sample was then applied at a flow rate of 17 ml/h to a CDP-diglyceride affinity column (1 × 7 cm) equilibrated with the sample buffer. The sample-loaded column was washed extensively with buffer A containing 2% Triton X-100, 0.5 M KCl, and 20% glycerol. The applied protein and PGP phosphatase activity (Figure 3) were accounted for in the column run through and washing step. Finally, the bound PGP synthetase was eluted using buffer A containing 0.5% Triton X-100, 0.2 M KCl, 50 mM $MgCl_2$, and 1 mM CDP-diglyceride.

In order to determine the specific activity of the purified synthetase, the CDP-diglyceride eluate was pooled and concentrated by Amicon pressure dialysis (XM 50 filter). CDP-diglyceride and Triton X-100 which interfere with the Lowry protein determination were removed by filtration through Sephadex G-75 in the presence of sodium cholate. The synthetase emerged at the void volume (30% yield) while CDP-diglyceride and Triton X-100 were retained by the column. The enzyme was then concentrated (30% yield) and dialyzed to remove Tris buffer. The dialyzed enzyme preparation was used to determine the specific activity of the purified PGP synthetase.

Table I summarizes the partial purification of the PGP synthetase. CDP-diglyceride eluted 42% of the synthetase activity which was adsorbed to the affinity column. The yield of activity from such columns varied from 30 to 100% depending on the stability of the PGP synthetase activity in Triton extracts (discsssed below). In this particular case, if the yield were corrected for the decay in activity in the Triton extract over the time interval of the experiment, the enzymatic activity eluted would account for over 95% of the activity originally adsorbed to the column. Without correcting for losses, an overall purification for the PGP synthetase of 550-fold was obtained by the outlined procedure, and the synthetase was completely separated from the phosphatase by the affinity chromatography step. The final specific activity of the synthetase represents a minimum value since considerable enzymatic acitivity was lost in concentrating the enzyme and in removing detergent and lipid.

Properties of the Detergent Extracted PGP Synthetase and PGP Phosphatase. To establish optimal assay conditions, several parameters affecting enzymatic activity were studied. Both enzymatic activities were stimulated by the addition of Triton X-100 to the assay mixture (Figure 4); inhibition of activity was observed at high Triton concentrations. Magnesium ion (80 mM) was required for maximal PGP synthetase activity; other divalent cations such as manganese and calcium could not substitute for magnesium ions. The PGP phosphatase did not require the addition of high concentrations of divalent cations since maximal response was obtained with the amount of $MgCl_2$ (1 mM) added to the assay with the enzyme aliquot. PGP phosphatase activity was maximal in the pH range from 6.0 to 8.5. The PGP synthetase showed increasing activity as the pH was increased through this range. Neither of the enzymatic activities was significantly affected by ionic strength.

The PGP synthetase exhibited normal saturation kinetics when the CDP-diglyceride concentration was held constant (0.2 mM) and *sn*-glycero-3-phosphate (K_m of 0.15 mM) was varied. Anamolous kinetics were observed when the CDP-diglyceride concentration was varied while holding *sn*-glycero-3-phosphate concentration constant (0.5 mM). The apparent K_m at low substrate concentrations was 5 μM; concentrations above 0.1 mM CDP-diglyceride were inhibitory. Similar anomalous saturation curves were observed at various Triton (0.15–2.1%) concentrations. The PGP phosphatase showed normal saturation kinetics with an apparent K_m for PGP of 0.18 mM.

The solubilized PGP phosphatase was stable at 4 °C for weeks. The PGP synthetase lost activity slowly in broken cell preparations but exhibited variable stability in different preparations when solubilized by Triton. In general, about 50% of the activity was lost in 12 h at 4 °C after solubilization with Triton. Once the synthetase was purified by affini-

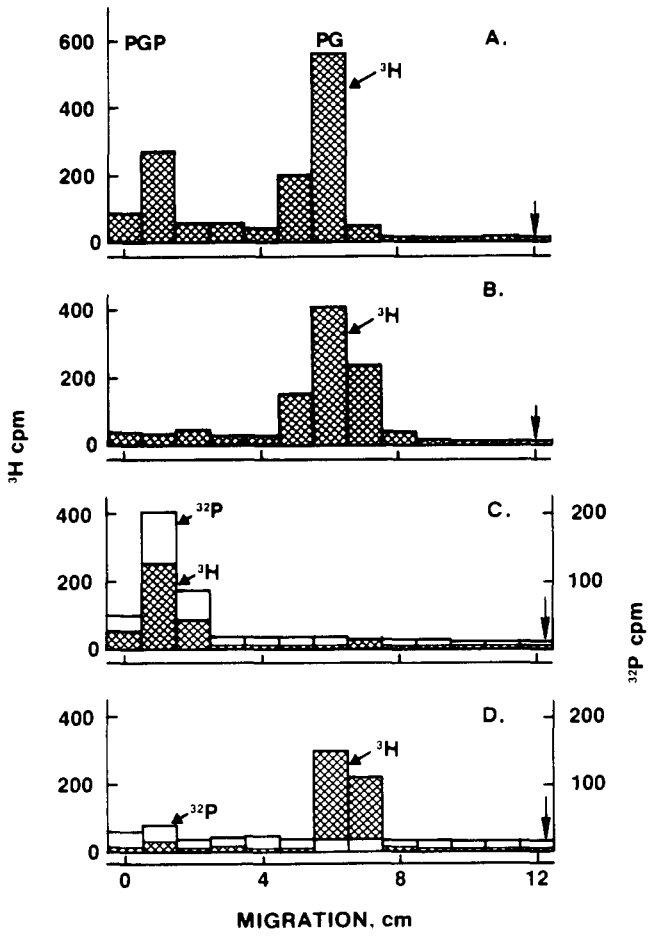

FIGURE 5: Reaction products of PGP synthetase and PGP phosphatase. The standard assays were scaled up fivefold in each case using the labeled substrates indicated. The chloroform-soluble reaction products from each assay were separated on silica gel thin-layer plates in solvent 4. No correction was made for background levels of radioactivity. The arrow marks the position of the solvent front: (A) CDP-diglyceride and *sn*-[2-^{3}H]glycero-3-phosphate were allowed to react in the presence of an *E. coli* B broken cell preparation; (B) same as A except that a Triton X-100 extract of *B. licheniformis* membranes was used; (C) *B. licheniformis* PGP synthetase from step 5, Table I, was used with CDP-diglyceride and *sn*-[2-^{3}H]glycero-3-[^{32}P]phosphate as substrates; (D) an aliquot of the chloroform-soluble reaction products from C was dried down under nitrogen and dissolved in the PGP phosphatase assay buffer minus substrate. An aliquot of a Triton X-100 extract of *B. licheniformis* membranes was added, and the reaction was allowed to proceed for 40 min. After partitioning the reaction mixture, the chloroform phase was processed as described above and the aqueous phase was analyzed as described in the text.

ty chromatography, the enzyme was stable for months at 4 °C.

Identification of the Reaction Products of PGP Synthetase and PGP Phosphatase. The standards (Figure 5A) used for PGP and PG were the reaction products of the PGP synthetase and PGP phosphatase, respectively, from *E. coli* B (Chang and Kennedy, 1967a). The product of the PGP synthetase assay using a Triton X-100 extract of *B. licheniformis* (Figure 5B) was solely PG; a similar profile was obtained using aliquots from steps 1–4 of Table I. After purification of the PGP synthetase by affinity chromatography (step 5, Table I), the product of the assay (Figure 5C) was over 95% PGP. The ^{3}H:^{32}P ratio (1.05) in this product was identical with that of the doubly labeled substrate (1.05).

The lipid product formed by PGP synthetase in Figure 5C was further characterized after mild alkaline hydrolysis. The water-soluble deacylation product was chromatographed on paper and had the expected R_f for 1-(3-glycerophosphoryl)-glycero-3-phosphate (Chang and Kennedy, 1967a) of 0.21 in solvent 3 and 0.12 in solvent 6. The deacylation product also had a ^{3}H:^{32}P ratio of 1.05.

To verify the release of CMP from CDP-diglyceride by PGP synthetase, a typical assay was scaled up fivefold and run for 40 min using CDP-diglyceride containing tritium in the cytosine moiety and *sn*-glycero-3-[^{32}P]phosphate as substrates. Formation of PGP was confirmed by analyzing the chloroform phase for ^{32}P after partitioning the reaction products. Carrier CMP was added to the aqueous phase and aliquots were chromatographed on paper. Carrier CMP was visualized under ultraviolet light. All of the radioactivity was coincident with CMP and had the expected R_f values (Raetz et al., 1972) of 0.12 and 0.73 in solvents 2 and 7, respectively.

The PGP phosphatase converted doubly labeled PGP (Figure 5D) to tritium labeled PG with the release of ^{32}P to the aqueous phase. The water-soluble ^{32}P cochromatographed on paper with authentic inorganic phosphate (R_f of 0.62) in solvent 5; authentic glycerophosphate had an R_f of 0.73 in this system. The ^{32}P released was further characterized by using a modification of the method of Bartlette (1959) for phosphate determination. Digestion with perchloric acid was omitted so that phosphate esters in the aqueous phase would remain intact and only inorganic phosphate would show a positive reaction. Using this method, all of the ^{32}P released could be accounted for as inorganic phosphate.

Stoichiometry of PGP Synthetase Reaction. Typical reaction mixtures were set up with [^{3}H]CDP-diglyceride (0.2 mM, 660 cpm per mmol) and *sn*-glycero-3-[^{32}P]phosphate (0.46 mM, 150 cpm per nmol) as substrates. The PGP synthetase used in this experiment was from the peak of enzymatic activity eluted from the Sephadex G-75 column (step 5, Table I) and was, therefore, free of CDP-diglyceride. After partitioning the reaction mixture, aliquots of the chloroform and aqueous phases were counted. The aqueous phase should have contained [^{3}H]CMP as product and unreacted *sn*-glycero-3-[^{32}P]phosphate. The chloroform phase should have contained unreacted [^{3}H]CDP-diglyceride and 3-*sn*-phosphatidyl-1′-*sn*-glycero-3′-[^{32}P]phosphate as product. The average of six determinations showed 11.4 ± 0.7 nmol of glycerophosphate was consumed while 10.5 ± 0.5 nmol of CMP was released and 9.9 ± 0.6 nmol of PGP was formed; therefore, CDP-diglyceride and *sn*-glycero-3-phosphate was stoichiometrically converted to PGP and CMP by the partially purified PGP synthetase.

Discussion

The phosphatidylglycerol synthesizing system is associated with the membrane fraction of *B. licheniformis* as has been reported for *E. coli* (Chang and Kennedy, 1967a,b) and *Bacillus* PP (Patterson and Lennarz, 1971). This conclusion is based on differential sedimentation of the PGP synthetase and PGP phosphatase at 100 000*g*, their distribution on sucrose gradients, and their solubilization by detergents. Once solubilized by Triton X-100, the PGP synthetase and PGP phosphatase activities were difficult to separate by standard purification techniques (data not shown). The synthetase and phosphatase had little or no affinity for ion exchange resins when nonionic detergents were present. They could not be separated by disc gel electrophoresis or Sepharose gel filtration in the presence of

Triton X-100. The difficulties encountered in separating these two activities may be due to the binding of large amounts of Triton to the hydrophobic regions of these enzymes. Such binding, which appears to be a general property of membrane proteins (Robinson and Tanford, 1975; Dowhan et al., 1974; Meuniger et al., 1972; Makino et al., 1975), would tend to mask differences in ionic and hydrodynamic properties making separation difficult.

Since Triton X-100 is necessary for enzymatic activity of many bacterial enzymes of phospholipid biosynthesis, our approach to the problem of detergent interference with purification was to develop substrate affinity chromatography columns. The first column we have developed utilizes an oxidized derivative of CDP-diglyceride covalently linked to Sepharose via adipic acid dihydrazide. This column successfully separated the *B. licheniformis* PGP phosphatase from PGP synthetase with a 140-fold purification of the latter enzyme. We have also been able to purify the *E. coli* PGP synthetase 2000-fold utilizing CDP-diglyceride Sepharose (T. Hirabayashi and W. Dowhan, unpublished data). Since CDP-diglyceride plays a central role in both eukaryotic and prokaryotic lipid metabolism, such a substrate affinity column technique provides a method for obtaining purified enzymes of phospholipid metabolism. The success of this affinity column along with those columns used to purify water-soluble phospholipases (Rock and Snyder, 1975) and lipases (Verine et al., 1974) indicates the general feasibility of using lipid ligands covalently bound to solid supports for purifying both membrane associated and cytoplasmic proteins.

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