Membrane-Associated Phosphatidylglycerophosphate Synthetase from *Escherichia coli:* Purification by Substrate Affinity Chromatography on Cytidine 5′-Diphospho-1,2-diacyl-*sn*-glycerol Sepharose†

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ABSTRACT: The membrane-associated cytidine 5′-diphospho-1,2-diacyl-*sn*-glycerol (CDPdiglyceride):*sn*-glycerol-3-phosphate phosphatidyltransferase (EC 2.7.8.5) from *Escherichia coli* has been solubilized with Triton X-100 and purified 6000-fold to 85% of homogeneity. The major purification was attained using several modifications of the CDP-diglyceride Sepharose affinity chromatography system described by Larson et al. (Larson, T. J., Hirabayashi, T., and Dowhan, W. (1976), *Biochemistry 15,* 974). The native enzyme in Triton X-100 had an apparent molecular weight of over 200 000, as judged by Sepharose 6B gel filtration. The apparent size of the native enzyme appeared to be due to its association with Triton X-100, as judged by sucrose gradient centrifugation, polyacrylamide gel electrophoresis, and the lack of affinity for ion-exchange resins. The minimum subunit molecular weight of the enzyme, determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis, was 24 000. This low molecular weight is consistent with the stability of the enzyme to heat, urea, or sodium dodecyl sulfate denaturation. The purified enzyme had an absolute requirement for magnesium ion (K_M = 50 mM) and Triton X-100 (0.5–6%) for activity when either CDP-diglyceride or dCDP-diglyceride was used as substrate. Kinetic analysis of the enzymatic reaction indicated an ordered sequential Bi-Bi reaction with the liponucleotide forming a dead-end complex at high concentration, which inhibited both the forward and reverse reactions. The enzyme would not hydrolyze the pyrophosphate bond of its lipid substrate or the phosphate esters of its lipid product but would catalyze a cytidine 5′-monophosphate dependent exchange reaction between glycero-3-phosphate and phosphatidylglycerophosphate.

The enzymes responsible for phospholipid biosynthesis are involved in the processes of membrane biogenesis and cell growth. Due to the particulate nature of most of these enzymes, they are also important functional components of many membranes. Currently, little is known concerning the control of this important group of enzymes or their physical, chemical, and enzymological properties. These types of studies have been hampered by the lack of rapid procedures for obtaining significant amounts of highly purified enzymes for investigation. Within this group of enzymes, only the phosphatidylserine decarboxylase of *Escherichia coli* has been purified to near homogeneity (Dowhan et al., 1974).

This and the following paper in this issue (Larson and Dowhan, 1976) describe the purification and properties of the CDP-diglyceride[1]:*sn*-glycerol-3-phosphate phosphatidyltransferase (EC 2.7.8.5, PGP synthetase) and CDP-diglyceride:L-serine *O*-phosphatidyltransferase (EC 2.7.8.8, phosphatidylserine synthetase), respectively, from *E. coli.* Since these two enzymes form the branch point in phospholipid metabolism which leads to the formation of either a zwitterionic phospholipid (phosphatidylethanolamine) or acid phospholipids (phosphatidylglycerol and cardiolipin), they may be involved in controlling the charge density of the membrane surface, as well as the absolute levels of these lipids. The phosphatidylserine synthetase is a cytoplasmic or possibly a ribosomal-associated enzyme (Raetz and Kennedy, 1972 and 1974), which may play an important role in the coordination of phospholipid metabolism with other cellular processes. The PGP synthetase in both *E. coli* (Chang and Kennedy, 1967a) and *Bacillus licheniformis* (Larson et al., 1976) is associated tightly with the membrane. The PGP synthetases can be solubilized from the membrane using nonionic detergents but are resistant to purification by ion-exchange chromatography in the presence of these detergents. Larson et al. (1976) were able to partially purify the PGP synthetase from *B. licheniformis* by CDP-diglyceride Sepharose affinity chromatography. Using a similar affinity chromatography technique, we have purified the membrane-associated PGP synthetase from *E. coli* 6000-fold to near homogeneity. We report the minimum molecular weight of the enzyme and discuss the interaction of the native enzyme with detergent. The effect of substrate, detergent, and divalent metal ion on enzymatic activity is also reported.

Materials and Methods

Reagents. All chemicals were reagent grade or better. *sn*-Glycero-3-phosphate was purchased from Calbiochem. DEAE-Sephadex (A-50) and Sepharose 4B and 6B were products of Pharmacia. Radiochemicals were purchased from Amersham/Searle. Triton X-100 was a product of Rohm and Haas. Sodium dodecyl sulfate (99% dodecyl) was purchased from Bio-Rad. Ovalbumin and hemoglobin were supplied by Sigma. Bovine serum albumin and erythrocuprein were purchased from Miles Laboratories. *Escherichia coli* B (¾ log), grown on rich medium, was purchased as the frozen cell paste from Grain Processing, Muscatine, Iowa.

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[1] Abbreviations used are: CDP-diglyceride and dCDP-diglyceride, cytidine and deoxycytidine 5′-diphospho-1,2-diacyl-*sn*-glycerol, respectively; PGP, 3-*sn*-phosphatidyl-1′-*sn*-glycero-3′-phosphate; DEAE, diethylaminoethyl.

TABLE I: Purification of PGP Synthetase.[a]

Step	Total Vol. (ml)	Total Protein (mg)	Sp Act. (Units/mg)	Yield (%)
(1) Broken Cells	1300	89 000	3.1	100
(2) Cell envelope	840	40 600	5.1	74
(3) Triton X-100 extract	690	13 800	14	67
(4) Oxidized affinity resin	200			47
(5) Reduced affinity resin	470			30
(6) DEAE-Sephadex	4.7	3.0	18 600	20

[a] Data based on starting with 1 lb of frozen cell paste. See Results for details.

Analytical Methods. Protein was determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard. The method was modified as described earlier (Larson et al., 1976) when samples contained Triton X-100.

Preparation of Substrates. CDP-diglyceride and dCDP-diglyceride (both derived from egg lecithin) were prepared as described earlier (Larson et al., 1976). [5-^{3}H]CDP-diglyceride was prepared as described by Raetz and Kennedy (1974). *sn*-[2-^{3}H]Glycero-3-phosphate, 3-*sn*-phosphatidyl-1′-*sn*-[2′-^{3}H]glycero-3′-phosphate, and 3-*sn*-phosphatidyl-1′-*sn*-glycero-3′-[^{32}P]phosphate were prepared as described by Chang and Kennedy (1967a,b).

Enzyme Assays. PGP synthetase activity was determined in a 12-ml polypropylene tube at 30 °C by a modification of the method of Chang and Kennedy (1967a). The assay mixture consisted of the standard assay buffer (0.25 M Tris-HCl (pH 8.0), 1% Triton X-100 (w/v), 0.1 M $MgCl_2$), supplemented with 0.2 mM CDP-diglyceride, 0.5 mM *sn*-[2-^{3}H]glycero-3-phosphate (150 cpm per nmol), and enzyme in a final volume of 0.1 ml. The reaction was stopped after 10 min by addition of 0.5 ml of methanol (0.1 N in HCl), followed by the addition of chloroform (1.5 ml) and 1 M $MgCl_2$ (3 ml). After brief centrifugation, an aliquot (1.0 ml) of the chloroform phase was removed, evaporated to dryness, and counted for radioactivity. One unit of PGP synthetase activity was 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. Phosphatidylserine synthetase activity (Raetz and Kennedy, 1972), phosphatidylserine decarboxylase activity (Dowhan et al., 1974), and CDP-diglyceride hydrolase activity (Raetz et al., 1972) were determined as previously described.

Polyacrylamide Disc Gel Electrophoresis. Electrophoresis under nondenaturing conditions was carried out at 25 °C using the pH 8.12 running buffer system described by Davis (1964). The gels contained 5% acrylamide, 0.16% *N,N′*-methylenebisacrylamide, and 0.1% Triton X-100. Prior to electrophoresis, samples were dialyzed vs. upper gel buffer. Enzyme was extracted from the gels by crushing 2-mm segments in standard assay buffer. Protein was visualized by staining the gels with Coomassie blue.

Electrophoresis in the presence of sodium dodecyl sulfate was carried out as described by Weber and Osborn (1969) using an acrylamide to *N,N′*-methylenebisacrylamide ratio of 37:1. Samples for electrophoresis were first treated at 100 °C for 5 min in the electrophoresis buffer containing 8 M urea, 1% sodium dodecyl sulfate, and 10 mM dithiothreitol, followed by dialysis against the electrophoresis buffer. After staining with Coomassie blue, the gels were scanned at 550 nm using a Gilford recording spectrophotometer equipped with a linear transporter and a No. 2412 cuvette.

Synthesis of Affinity Resins. Two types of affinity resins were employed in this work. One resin (oxidized affinity resin) was prepared as described by Larson et al. (1976) and had a $NaIO_4$ oxidized derivative of CDP-diglyceride covalently attached to Sepharose 4B via an adipic acid dihydrazide spacer arm. The second resin (reduced affinity resin) was prepared by reducing the oxidized affinity resin (100-ml settled volume) at 4 °C in 2 resin volumes of 0.5 M Tris-HCl (pH 8.0) by three successive additions of $NaBH_4$ (0.7 g) at 1-h intervals. The reduced affinity resin was extensively washed with 1 M KCl before use.

Results

Purification of PGP Synthetase. All procedures were carried out at 4 °C. The results are summarized in Table I. Frozen *E. coli* B cell paste (450 g) was suspended in 950 ml of buffer A (20 mM potassium phosphate, pH 7.0, 10 mM $MgCl_2$, and 10 mM 2-mercaptoethanol) using a Waring blender. The cells were broken by passing the suspension through a French pressure cell. The cell envelope fraction was collected by centrifugation at 100 000*g* for 2 h in a Beckman Type 35 rotor. The supernatant was discarded and the pellet was suspended in 750 ml of buffer A, containing 4% Triton X-100 (w/v), using a Waring blender. The suspension was allowed to stand for 12 h before insoluble material was removed by centrifugation at 100 000*g* for 60 min. The supernatant (Triton X-100 extract) was retained and the pellet was discarded.

PGP synthetase activity in the Triton X-100 extract was adsorbed to the oxidized affinity resin (50-ml settled volume) by mixing the extract in two successive batches (250 ml each) with resin for 5 h. The supernatant, which contained between 5 and 10% of the original PGP synthetase activity and 100% of both the phosphatidylserine synthetase and CDP-diglyceride hydrolase activities present in the original extract, was discarded. The affinity resin was packed into a 2 × 16 cm column and an additional 130 ml of the Triton X-100 extract was passed through the column; application of the extract was stopped when only 70% of the applied enzymatic activity was retained by the column. The column was then washed at 50 ml/h with 250 ml of buffer A, containing 0.1% Triton X-100 and 0.5 M KCl, then 400 ml of buffer A containing 0.1% Triton X-100 and 1.0 M KCl. The PGP synthetase activity was then eluted at 10 ml/h from the oxidized affinity resin using 300 ml of buffer A, containing 0.1% Triton X-100, 0.5 M KCl, and 0.8 M hydroxylamine-HCl (readjusted to pH 7 with KOH).

The eluate fractions containing the synthetase activity were pooled (step 4) and dialyzed extensively against buffer B (20 mM potassium phosphate, pH 7.0, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.1% Triton X-100). The dialyzed enzyme preparation was applied at 50 ml/h to a 1.4 × 16 cm column of reduced affinity resin in buffer B; over 95% of the PGP

synthetase activity was retained by the column. The resin was washed (50 ml/h) with 300-ml portions of buffer B containing, first, 0.1 M KCl, then 0.4 mM CDP-diglyceride and 0.1 M KCl, and finally only 0.1 M KCl. The fractions from both the second and third washes, which contained PGP synthetase activity, were pooled (step 5).

The following procedure was used to concentrate the enzyme. The pooled sample from step 5 was diluted with 2 volumes of buffer B and applied at 50 ml/h to a column (1.4 × 9.5 cm) of oxidized affinity resin. The column was washed with 150 ml of buffer B, containing 1 M KCl, and the enzyme was eluted with buffer B, containing 0.8 M hydroxylamine and 0.5 M KCl, as described previously. The fractions containing PGP synthetase activity were pooled (140 ml) and exhaustively dialyzed against buffer B. The dialyzed sample was then concentrated to 10 ml using an Amicon ultrafiltration device equipped with a PM10 filter. The concentrated enzyme was passed at 50 ml/h through a 2 × 16 cm DEAE-Sephadex (A-50) column equilibrated and eluted with buffer B. The PGP synthetase emerged in the void volume of the column while yellow material was adsorbed to the resin. The fractions containing enzymatic activity were pooled (35 ml) and concentrated to 4.7 ml (step 6) using ultrafiltration as described above.

The PGP synthetase was purified 6000-fold by the above procedure. The overall recovery at each step was always better than 70%, indicating the enzyme was not significantly altered by the purification procedure. The outlined procedure is rapid and can be easily scaled up to produce larger amounts of enzyme.

The difference in chromatographic properties of the enzyme on the oxidized vs. the reduced affinity resin is still unclear. The enzyme adsorbs to both resins with high affinity and can be eluted from both resins by splitting the linkage between CDP-diglyceride and the spacer arm with hydroxylamine, indicating a common linkage for both types of resins. On the other hand, the enzyme could be eluted with CDP-diglyceride only from the reduced resin in good yield (60–80%); the yield from the oxidized resin was always less than 20% when substrate elution was used. The low yield from the oxidized resin using CDP diglyceride as eluent could be due to the presence of other reactive groups on $NaIO_4$-oxidized CDP-diglyceride. These groups might have been removed by $NaBH_4$ treatment during the synthesis of the reduced resin.

Enzyme Purity. Polyacrylamide disc gel electrophoresis, in the presence of sodium dodecyl sulfate (Figure 1), indicated a highly purified preparation with the major protein peak accounting for 85% of the total absorption profile. Similar electrophoretic analysis of the Triton X-100 extract showed no band corresponding to the major band found in the purified preparation. The specific activity, after the oxidized affinity column (not measured in this particular preparation), was generally around 10 000; the major protein component after this step accounted for about 50% of the total protein, as determined by Coomassie blue staining of sodium dodecyl sulfate gels. Although Coomassie blue staining is not necessarily proportional to the amount of protein present, the increase in specific activity attained by the last steps of the purification procedure was in good agreement with the increase in proportion of the major protein band, indicating this band to be the PGP synthetase. In addition, the method of purification employed should select for enzymes which have affinity for CDP-diglyceride; there were no other known CDP-diglyceride dependent activities in the preparation.

Interaction of the Enzyme with Detergent. Several pieces

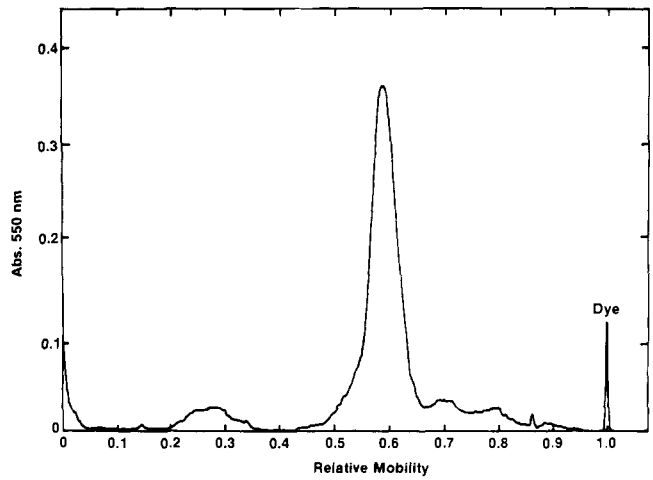

FIGURE 1: Sodium dodecyl sulfate polyacrylamide disc gel electrophoresis of PGP synthetase. PGP synthetase (30 μg) after step 6, Table I, was prepared for sodium dodecyl sulfate disc gel electrophoresis and electrophoresed in 10% acrylamide disc gels. After staining the gels with Coomassie blue, the protein profile was determined by scanning the gels at 550 nm.

of evidence indicate the enzyme associates with large amounts of detergent. The purified enzymatic activity, as well as its associated protein, did not enter 5% polyacrylamide gels under nondenaturing conditions. When CDP-diglyceride was added to the sample, the enzyme migrated as a broad protein and activity band; the mobility of the sample was proportional to the amount of CDP-diglyceride added. Such results indicate the enzyme may be associated with a Triton X-100 micelle, resulting in a high size to charge ratio for the complex. Added CDP-diglyceride would be expected to associate with this micelle and would increase its negative charge and, consequently, its mobility.

The enzyme did not adsorb to either anionic or cationic exchange resins in the presence of Triton X-100. On the other hand, the enzyme tenaciously bound to DEAE-cellulose in the presence of deoxycholate and could be eluted only with Triton X-100 containing buffers. Tight association of the enzyme with an anionic detergent would result in strong binding to DEAE-cellulose. The displacement of deoxycholate from the enzyme by Triton X-100 would result in elution of the enzyme from the resin.

In the presence of 0.1% Triton X-100, the enzyme eluted in the included volume of Sepharose 6B as a sharp peak with an apparent molecular weight on the order of 200 000 (K_{av} = 0.38). On the other hand, sedimentation of the purified enzyme (2 μg) through a 5–20% sucrose gradient with a 70% sucrose shelf on the bottom, in the presence of 0.1% Triton X-100, at 120 000g for 17 h at 4 °C, indicated an apparent molecular weight for the enzyme slightly higher than hemoglobin. In a similar gradient containing no Triton X-100, the enzyme collected at the bottom of the gradient, indicating aggregation in the absence of detergent. Association of the enzyme with detergent would result in an increased Stokes radius, but a decrease in the apparent density of the enzyme.

All of these data are consistent with the interaction of the enzyme with detergent micelles through a hydrophobic region on the protein. Similar results have been reported for cytochrome b_5 (Robinson and Tanford, 1975), phosphatidylserine decarboxylase (Dowhan et al., 1974), the cholinergic receptor (Meunier et al., 1972), and CDP-diglyceride hydrolase (Raetz et al., 1976).

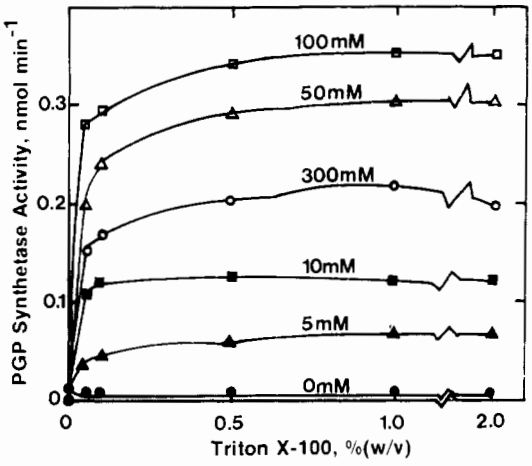

FIGURE 2: Dependence of PGP synthetase activity on Triton X-100 and magnesium concentrations. Standard assay conditions were employed, except the concentrations of $MgCl_2$ and Triton X-100 were varied as indicated.

Enzyme Stability. The PGP synthetase was unusually stable to heat, chaotropic agents, and ionic detergents at neutral pH in the presence of 10 mM $MgCl_2$ and reducing agents (10 mM 2-mercaptoethanol). Triton X-100 extracts (step 3, Table I) retained full activity after 5 min at 55 °C, about half-activity after 5 min at 60 °C, and lost all activity at 65 °C. A similar heat stability has been reported by Raetz (1975) for lysed-cell preparations. In the presence of 0.1% Triton X-100, the purified enzyme retained full activity in 8 M urea after 2 h at 30 °C and retained about half its activity after 2 h at 30 °C in 1% sodium dodecyl sulfate. The combination of both 4 M urea and 1% sodium dodecyl sulfate at 30 °C completely inactivated the enzyme.

Molecular Weight. Since the PGP synthetase appears to interact strongly with detergents and is quite stable to denaturation, the enzyme poses several problems in molecular weight determination. The molecular weight of the native enzyme in Triton X-100 cannot be determined until the interaction with detergent has been quantitated (Tanford et al., 1974). To determine the minimum subunit molecular weight by polyacrylamide disc gel electrophoresis in sodium dodecyl sulfate, the free mobility in sodium dodecyl sulfate of both the PGP synthetase and the marker proteins used to calibrate the gel system must be independent of molecular weight. This criterion is met if the proteins bind a constant amount of sodium dodecyl sulfate per unit weight and the resulting complex behaves as a free draining structure (Neville, 1971).

The fully reduced and denatured PGP synthetase showed a linear relationship between % gel concentration (between 5 and 12%) and relative mobility in the presence of sodium dodecyl sulfate (Ferguson, 1964). Extrapolation to 0% gel indicated the PGP synthetase had the same relative free mobility as the standard proteins used to calibrate the gel system (hemoglobin, erythrocuprein, egg ovalbumin, bovine serum albumin). The similarity in electrophoretic behavior between the synthetase and the marker proteins indicates that this method of molecular weight determination should be reliable for the PGP synthetase. The average minimum molecular weight for the enzyme, determined from electrophoresis at four gel concentrations, was 24 000 ± 500. This low molecular weight is consistent with the stability properties of the enzyme.

Effect of Detergent and Metal Ions on Activity. The purified PGP synthetase showed an absolute requirement (Figure 2) for a nonionic detergent, such as Triton X-100, and a divalent metal ion, such as magnesium, for enzymatic activity; these results are similar to those reported by Chang and Kennedy (1967a) for the partially purified enzyme, as well as for the enzyme from *B. licheniformis* (Larson et al., 1976). The apparent K_M for magnesium at 1% Triton X-100 was 50 mM. Manganese was found to be much less effective than magnesium and was inhibitory at concentrations above 10 mM. Calcium would not support enzymatic activity and strongly inhibited the magnesium-dependent activity. The metal ion requirement for this enzyme, therefore, is unique among CDP-diglyceride dependent enzymes in *E. coli,* since the CDP-diglyceride hydrolase (Raetz et al., 1972) and the phosphatidylserine synthetase (Kanfer and Kennedy, 1964) show no requirement for divalent metal ions.

The PGP synthetase also differed markedly from other enzymes which have phospholipid substrates in its dependence on detergent. The *E. coli* phosphatidylserine synthetase (Raetz and Kennedy, 1972), CDP-diglyceride hydrolase (Raetz et al., 1972), and phosphatidylserine decarboxylase (Dowhan et al., 1974; Warner and Dennis, 1975), as well as the phospholipase A_2 from *Naja naja naja* (Deems et al., 1975), all require Triton X-100 at low concentrations (0.1–0.2%) in molar ratios of Triton X-100 to lipid substrate from 2:1 to 6:1 for maximal activity. At higher molar ratios the enzymes are inhibited by Triton X-100. These enzymes fit a model proposed by Dennis (1973) and Warner and Dennis (1975) in which enzyme first binds to the surface of the micelle. The interaction of enzyme with its lipid substrate then becomes dependent on the mole fraction of substrate within the mixed micelle and not the absolute concentration of substrate in solution. Inhibition by Triton X-100 is interpreted as a dilution of substrate within the mixed micelle as the mole fraction of detergent is increased. No such inhibition of the PGP synthetase by Triton X-100 was observed between 0.2 and 6% Triton X-100 or up to a molar ratio of Triton X-100 to lipid substrate of 420:1. The minimum le of Triton X-100 ((0.2%) ratio of 15:1) required for m...um activity was most likely that necessary for the proper physical state of magnesium CDP-diglyceride in a mixed micelle. Once a proper mixed micelle of substrate and detergent was formed, the enzyme behaved in a classical manner with respect to lipid substrate at all higher Triton X-100 levels. Such results indicate a possible difference in the mode of interaction of this enzyme with its micellar substrate.

Other Enzymatic Activities. The purified PGP synthetase was analyzed for the presence of other known enzymes of phospholipid metabolism in order to establish enzyme purity, as well as to determine if other reactions are catalyzed by this enzyme. Assays were carried out under both the published assay conditions for the respective activity being tested and the assay conditions of the PGP synthetase. Labeled substrates employed had a fivefold higher specific activity than normally used. The reactions were allowed to proceed for 1 h in the presence of a fourfold excess of PGP synthetase over that normally used for assay. Under all assay conditions tested, the purified PGP synthetase contained no CDP-diglyceride hydrolase activity, phosphatidylserine synthetase activity, or phosphatidylserine decarboxylase activity. In addition, the preparation did not release water-soluble radioactivity from either 3-*sn*-phosphatidyl-1′-*sn*-[2′-^{3}H]glycero-3′-phosphate or 3-*sn*-phosphatidyl-1′-*sn*-glycero-3′-[^{32}P]phosphate; therefore, the preparation contained no PGP phosphatase activity (Chang and Kennedy, 1967b) nor was the enzyme capable of hydrolyzing any of the phosphate ester bonds of PGP.

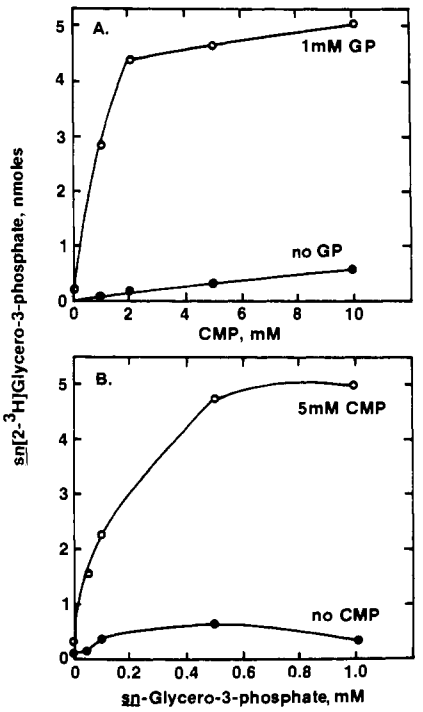

FIGURE 3: Release of glycero-3-phosphate from PGP. Purified PGP synthetase (2.5 units) was incubated for 30 min at 30 °C in 0.1 ml of the standard assay buffer, containing 0.2 mM 3-*sn*-phosphatidyl-1′-*sn*-[2′-^{3}H]glycero-3′-phosphate (335 cpm/nmol) and varying amounts of CMP and *sn*-glycero-3′-phosphate. The reaction was stopped and the mixture was partitioned as described under Materials and Methods. Glycero-3-phosphate release was determined by counting the upper phase for radioactivity.

In addition to the synthesis of PGP, the purified enzyme catalyzed the reverse reaction, i.e., the formation of glycero-3-phosphate (Figure 3A) and CDP-diglyceride ([5-^{3}H]CMP and PGP used as substrates) from CMP and PGP; glycero-3-phosphate (0.1 mM) or CDP-diglyceride (0.1 mM) completely inhibited the reverse reaction, as would be expected, since the equilibrium lies far in the direction of PGP formation. The enzyme did not catalyze an exchange reaction between [5-^{3}H]CMP (0.83 mM) and CDP-diglyceride (0.13 mM) in either the presence or absence of *sn*-glycero-3-phosphate. The presence or absence of magnesium, the lowering of the Triton X-100 concentration to 0.1%, or the addition of another 100 units of enzyme had no effect on this result. The enzyme, also, did not catalyze an exchange reaction between PGP and glycero-3-phosphate, except in the presence of CMP (Figure 3); the apparent K_M values for CMP and glycero-3-phosphate for the exchange reaction were 1.2 and 0.13 mM, respectively. The absence of hydrolytic activities toward the lipid substrate and product, along with the isotope exchange pattern of the enzyme, are consistent with a sequential reaction mechanism for the PGP synthetase, as opposed to a ping-pong reaction mechanism (Cleland, 1970).

Kinetic Properties of the Enzyme. When the concentration of CDP-diglyceride was varied at constant concentration of glycero-3-phosphate, double-reciprocal plots at various concentrations of glycero-3-phosphate (Figure 4A) extrapolated to a common point; such results are consistent with a sequential reaction mechanism for the enzyme (Cleland, 1970). In an analogous plot at various CDP-diglyceride concentrations, the extrapolated lines did not have a common intercept on the abscissa (Figure 4B) and a secondary plot of the intercepts on the ordinate vs. CDP-diglyceride went through a minimum.

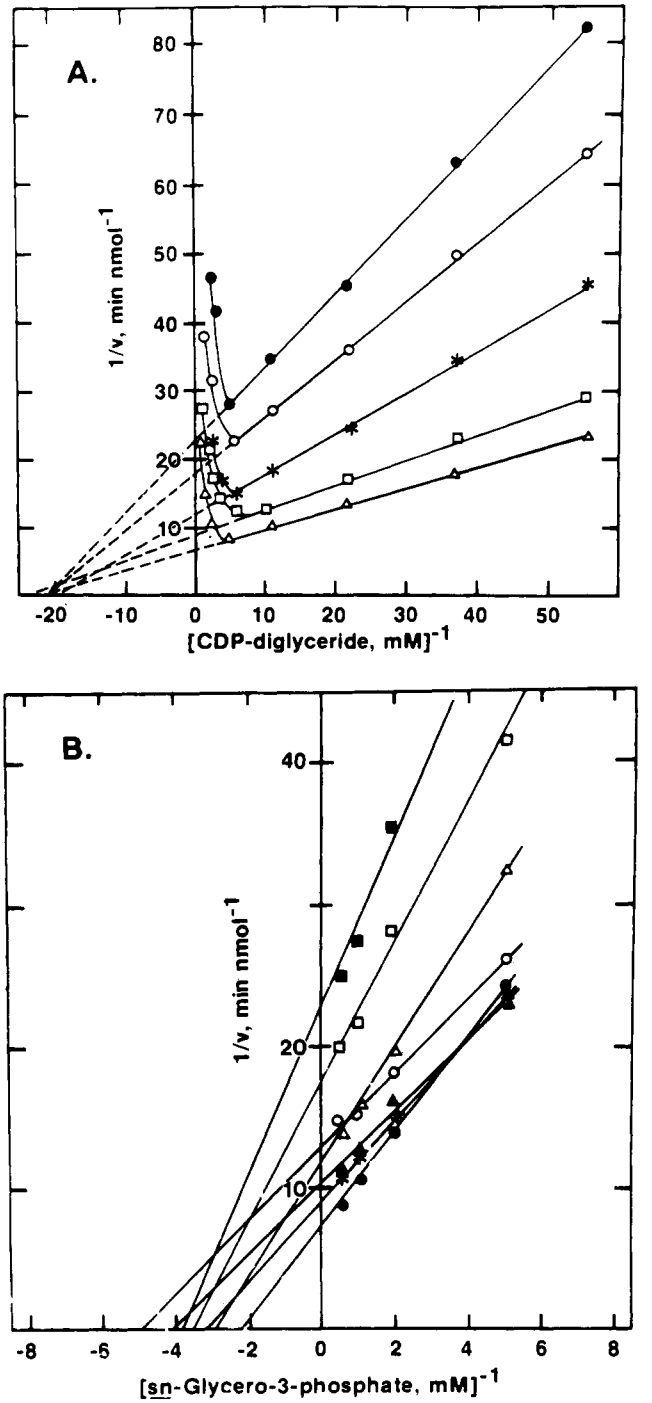

FIGURE 4: Effect of glycero-3-phosphate and CDP-diglyceride on activity. (A) Double-reciprocal plots of initial velocity of PGP synthetase at fixed concentrations of glycero-3-phosphate and variable concentrations of CDP-diglyceride. Standard assay buffer was employed, containing 0.15 unit of purified enzyme and the concentrations of substrates were varied as indicated. Concentrations of *sn*-glycero-3-phosphate were 0.1 (●), 0.2 (○), 0.5 (*), 1.0 (□), and 2 (△) mM. (B) Double-reciprocal plots of the initial velocity of PGP synthetase at fixed concentrations of CDP-diglyceride and variable concentrations of *sn*-glycero-3-phosphate. Standard assay buffer was employed containing 0.15 unit of purified enzyme, and the concentrations of substrates were varied as indicated. Concentrations of CDP-diglyceride were 18 (■), 27 (□), 45 (△), 180 (●), 270 (*), 450 (▲), and 980 (○) μM.

This pattern was the same at both 1 and 3% Triton X-100, indicating the inhibition by high CDP-diglyceride concentration was not due to the lack of sufficient Triton X-100 to form a proper mixed micelle with substrate.

An uncompetitive substrate inhibition pattern suggests an ordered reaction mechanism in which the liponucleotide forms a dead-end complex with the enzyme before the release of the last product. Although inhibition by binding of a second molecule of liponucleotide to the enzyme before reaction takes place cannot be ruled out kinetically, the lack of isotope exchange between [5-^{3}H]CMP and CDP-diglyceride, in the presence of PGP, is consistent with the liponucleotide competing for either the CMP or PGP binding site.

The apparent K_M values for CDP-diglyceride and *sn*-glycero-3-phosphate were 46 and 320 μM, respectively. dCDP-diglyceride behaved in a very similar manner to CDP-diglyceride with an apparent K_M of 34 μM. The extrapolated V_{max} was the same for both liponucleotides, but dCDP-diglyceride appeared to be a better inhibitor at high concentration. The K_M values determined for CDP-diglyceride and glycero-3-phosphate were in good agreement with those reported by Chang and Kennedy (1967a) for the crude PGP synthetase.

The kinetic data, including the uncompetitive inhibition by the liponucleotides and the isotope exchange data, are consistent with an ordered sequential Bi-Bi reaction mechanism (Cleland, 1970) for the PGP synthetase. The data are insufficient to establish the order of the reaction, but the binding of the enzyme to CDP-diglyceride Sepharose in the absence of glycero-3-phosphate would indicate the liponucleotide binds to the enzyme first. Although a classical kinetic analysis of this enzyme appears possible at saturating levels of Triton X-100, these conclusions concerning the reaction mechanism may be an oversimplification of the complex mixed micellar substrate system.

Discussion

The use of detergents to "solubilize" membrane enzymes in an active form has been successful in a large number of cases (Helenius and Simons, 1975). Purification to homogeneity in the presence of detergents has been successful in the cases of cytochrome b_5 (Spatz and Strittmatter, 1971), phosphatidylserine decarboxylase (Dowhan et al., 1974), phospholipase A1 (Scandella and Kornberg, 1971), cytochrome P_{450} reductase (Dignam and Strobel, 1975), and penicillinase (Sawai and Lampen, 1974). On the other hand, there are many membrane proteins which apparently cannot be purified by standard techniques due to the interference by the detergents necessary to solubilize the proteins. The inability to resolve these proteins appears to be due to their association with detergent. Several "native" membrane proteins, such as cytochrome b_5 (Robinson and Tanford, 1975) and the cholinergic receptor (Meunier et al., 1972), appear to undergo a comicellization with detergent molecules. This process is presumably mediated by a hydrophobic region of the membrane protein. Such an interaction increases the apparent size and lowers the apparent density of the proteins (Helenius and Simons, 1975) which affects their physical properties, as measured by gel filtration and sucrose gradient centrifugation. Water-soluble proteins (Helenius and Simons, 1972) and the polar peptide produced by trypsin treatment of cytochrome b_5 (Robinson and Tanford, 1975) do not interact with detergent micelles. Membrane proteins, then, can associate with variable amounts of a nonionic detergent, depending on the size and distribution of their respective hydrophobic regions. Those proteins, which still have considerable ionic character even when associated with nonionic detergents, can be purified by ion-exchange chromatography and electrophoresis.

The PGP synthetases of *E. coli* and *B. licheniformis* (Larson et al., 1976) appear to bind significant amounts of Triton X-100 based on their hydrodynamic properties in detergent. The *E. coli* synthetase has a minimum molecular weight which is 50% greater than cytochrome b_5, yet does not have sufficient ionic character in Triton X-100 to bind to ion-exchange resins or to be displayed on native polyacrylamide gel electrophoresis. This particular protein must have a large proportion of its surface covered by detergent, which effectively reduces its charge character and gives it a high size to charge ratio. The degree of hydrophobicity and the distribution of hydrophobic regions on this protein will have to be determined by detergent binding studies, amino acid analysis, and sequence studies.

Proteins, such as the PGP synthetase, pose difficult problems when their purification is attempted. Affinity for substrate and catalytic activity are two properties of many membrane enzymes which are not adversely affected by nonionic detergents. Therefore, the use of affinity chromatography may be one of the few ways of obtaining homogeneous preparations of such enzymes. In the case of the *E. coli* PGP synthetase, affinity chromatography on CDP-diglyceride Sepharose has proven to be very effective. This method should have general applicability to other PGP synthetases (Larson et al., 1976).

The amount of total phospholipid in *E. coli* is controlled primarily at the level of the acyltransferases, which are responsible for the synthesis of phosphatidic acid (Cronan and Vagelos, 1972, Bell, 1974, and Cronan and Bell, 1974). The regulation of the relative levels of phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin probably lies beyond the formation of the liponucleotide pool. Neither PGP synthetase nor phosphatidylserine synthetase (Larson and Dowhan, 1976) has a differential affinity or activity toward either dCDP-diglyceride or CDP-diglyceride. This observation suggests the relative levels of the two liponucleotides have no controlling effect at this branch point in metabolism, as has been suggested by Raetz and Kennedy (1973) based on experiments with crude enzyme preparations.

Part of the control of phospholipid metabolism beyond the formation of the liponucleotide pool may be through a response to the intracellular levels of guanosine tetraphosphate and pentaphosphate, which have been shown in crude extracts to affect the activity of the PGP synthetase (Merlie and Pizer, 1973). In addition, the phosphatidylserine synthetase may respond to changes in the net charge on the membrane surface where its lipid substrate is located; there is some evidence that this enzyme has affinity for micellar surfaces (Larson and Dowhan, 1976), as well as phosphate moieties (Raetz and Kennedy, 1974). The PGP synthetase, which is apparently embedded in the inner membrane (White et al., 1971; Bell et al., 1971), may respond not only to the net charge of the surrounding head groups, but also to the state of the hydrophobic portions of the lipids in the membrane. Since we have obtained purified preparations of both the PGP synthetase and the phosphatidylserine synthetase (Larson and Dowhan, 1976), we are now in a position to elucidate the factors which control the relative proportions of the various phospholipids in the membrane.

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