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Articles

Purification and Characterization of a Membrane-Associated Phosphatidylserine Synthase from *Bacillus licheniformis*†

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ABSTRACT: A CDP-diacylglycerol-dependent phosphatidylserine synthase was solubilized from *Bacillus licheniformis* membranes and purified to near homogeneity. The purification procedure consisted of CDP-diacylglycerol–Sepharose affinity chromatography followed by substrate elution from blue dextran–Sepharose. The purified preparation showed a single band with an apparent relative molecular mass of 53 000 daltons when subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteolytic digestion of the enzyme yielded a smaller (41 000 daltons) active form. The preparation was free of any phosphatidylglycerophosphate synthase, phosphatidylserine decarboxylase, CDP-diacylglycerol hydrolase, and phosphatidylserine hydrolase activities. The utilization of substrates and the formation of products occurred with the expected stoichiometry. Radioisotopic exchange patterns between related substrate and product pairs suggest a sequential Bi-Bi reaction as opposed to the ping-pong mechanism exhibited by the well-studied phosphatidylserine synthase of *Escherichia coli* [Larson, T. J., & Dowhan, W. (1976) *Biochemistry 15*, 5212–5218]. The *B. licheniformis* enzyme was also found to be markedly dissimilar to the *E. coli* enzyme with regard to association with detergent micelles, affinity for ribosomes, and antigenicity.

Investigations to date on phospholipid biosynthetic pathways have revealed two modes of phosphatidylethanolamine formation. A CDP-diacylglycerol-dependent phosphatidyl transfer to L-serine takes place in bacteria to form phosphatidylserine, which is subsequently decarboxylated to form phosphatidylethanolamine (Raetz, 1978; Dutt & Dowhan, 1981). In mammalian and plant systems, however, CDP-ethanolamine and diacylglycerol react directly to form phosphatidylethanolamine (Bell & Coleman, 1980). In yeast, both the mammalian and bacterial pathways of phosphatidylethanolamine formation (Steiner & Lester, 1972) coexist; the latter pathway can apparently suffice in cells grown in the absence of ethanolamine. Therefore, the synthesis of phosphatidylserine is the first committed step in the biosynthetic route to the formation of a major phospholipid component of the membranes of both yeast and bacteria.

There appears to be two subclasses within the CDP-diacylglycerol:L-serine *O*-phosphatidyltransferases (EC 2.7.8.8). The enzyme in Gram-negative bacteria is found associated with ribosomes in broken cells (Raetz & Kennedy, 1972; Dutt & Dowhan, 1977); this nonmembrane localization is observed even under conditions where the enzyme is overproduced 200-fold as reported in *Escherichia coli* (Ohta et al., 1981). In contrast, the CDP-diacylglycerol-dependent phosphatidylserine synthase from bacilli is membrane associated in cell-free extracts (Dutt & Dowhan, 1981; Langley et al., 1979). Studies in yeast using detergent extracts of membranes (Carson et al., 1982; Carman & Matas, 1982; Letts et al., 1983) and in *Clostridium perfringens* using a partially purified enzyme preparation (Cousminer et al., 1982) have shown the phosphatidylserine synthases of these organisms to be very similar in their properties to the enzyme found in bacilli. When the properties of the partially purified membrane-associated forms of the enzyme are compared to those of the purified

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enzyme from *E. coli*, there are also marked differences in catalytic properties as well as physical properties.

In view of the above differences between the well-characterized enzyme from *E. coli* and the enzymes from crude extracts of yeast and Gram-positive bacteria, we have undertaken the purification of the membrane-associated phosphatidylserine synthase from *Bacillus licheniformis*. Purification to homogeneity and characterization of the latter activity would establish whether two types of phosphatidylserine synthases exist which are distinct in their physical and catalytic properties.

Materials and Methods

Chemicals. All chemicals were reagent grade or better. Radiochemicals and scintillation counting supplies were obtained from Amersham/Searle and New England Nuclear. Phosphatidylserine, *p*-aminobenzamidine hydrochloride, and CMP-morpholidate (dicyclohexylammonium salt) were products of Sigma. Triton X-100 was obtained from New England Nuclear. Sepharose 4B and blue dextran 2000 were purchased from Pharmacia. Precoated silica gel thin-layer chromatography plates were obtained from E. Merck. Acrylamide, *N,N'*-methylenebis(acrylamide), ammonium persulfate, and sodium dodecyl sulfate (SDS) were purchased from Bio-Rad, and urea was from Schwarz/Mann. Bacterial growth media were supplied by Difco. Homogeneous *E. coli* phosphatidylserine synthase (Ohta et al., 1981) was obtained from K. Louie. Rabbit antiserum directed against purified phosphatidylserine synthase from *E. coli* was obtained from Y. C. Chen.

Bacterial Strains and Media. Bacillus licheniformis (ATCC 14580) was obtained from the American Type Culture Collection, and *Escherichia coli* strain RA80 (*pss*$^-$, defective in phosphatidylserine synthase activity) was obtained from C. R. H. Raetz (University of Wisconsin); the growth conditions for both strains were as previously described (Dutt & Dowhan, 1981; Ohta et al., 1981).

Enzyme Assays. All assays were carried out at 37 °C according to the method previously described (Raetz & Kennedy, 1972). Reactions were carried out for 10 min or longer, but always under conditions where initial rates were being measured. One unit of enzymatic activity was defined as the amount of enzyme required to form 1 nmol of product in 1 min under optimal conditions. Specific activity was based on the units per milligram of protein.

Phosphatidylserine synthase activity from *B. licheniformis* was measured in the presence of CDP-diacylglycerol, Triton X-100, $MnCl_2$, and phosphatidylserine; the standard assay conditions were as previously described (Dutt & Dowhan, 1981). When phosphatidylserine synthase activity was monitored via serine-dependent CMP formation, the release of water-soluble [5-^{3}H]CMP from [5-^{3}H]CDP-diacylglycerol was measured under the same conditions. Phosphatidylserine hydrolase was measured under the conditions described for phosphatidylserine synthase with the omission of L-serine and CDP-diacylglycerol and the substitution of phosphatidyl-L-[U-^{14}C]serine for phosphatidylserine; release of water-soluble serine was measured in this case.

Phosphatidylglycerophosphate synthase (Larson et al., 1976), CDP-diacylglycerol hydrolase (Raetz et al., 1972), and *E. coli* phosphatidylserine synthase (Raetz & Kennedy, 1972) were assayed under the conditions previously described.

Preparation of Substrates and Affinity Resins. CDP-diacylglycerol (Agranoff & Suomi, 1963), [5-^{3}H]CDP-diacylglycerol (Raetz & Kennedy, 1972, 1974), and [^{32}P]CDP-1,2-diacyl-*sn*-glycerol (Dutt & Dowhan, 1981; Raetz & Kennedy, 1972) were synthesized as previously described.

CDP-diacylglycerol–Sepharose affinity resin was made by the procedure described by Larson et al. (1976), except that the initial step was modified by activating the Sepharose 4B with CNBr dissolved in acetonitrile (March et al., 1974); blue dextran–Sepharose was prepared by the method of Ryan & Vestling (1974).

Separation and Analysis of Lipids. After enzymatic reactions, appropriate carrier lipids (at 1 mg/mL) were added prior to partitioning the reaction mixture between chloroform and acidic (0.1 N in HCl) methanol–water. The chloroform-soluble material was analyzed by two-dimensional silica gel thin-layer chromatography as previously described (Nishijima & Raetz, 1979). Carrier lipid and standards were detected by phosphate-positive spray (Dittmer & Lester, 1964) or brief exposure to I_2 vapor. Autoradiography was used to locate ^{32}P-labeled lipid which was quantitated as previously described (Dutt & Dowhan, 1981).

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of SDS was performed as described by Laemmli (1970). Samples were pretreated at 100 °C for 2 min with buffer containing 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5, 10 mM disodium ethylenediaminetetraacetic acid (Na_2EDTA), 1 mM dithiothreitol, 1% SDS, 10% glycerol, and 8 M urea. The gels were either stained with Coomassie blue or silver stained (Merril et al., 1981).

Electrophoresis in two dimensions was carried out as follows. Polyacrylamide gel electrophoresis under nondenaturing conditions was carried out in an apparatus equipped with a circulating water bath to maintain the temperature at 4 °C. CDP-diacylglycerol (1 mM) and Triton X-100 (0.2%) were included in the gel composition as described (Blattler et al., 1972). Entire lanes into which phosphatidylserine synthase had been loaded were cut out of the slab gel and incubated in buffer, as described by McHenry & Crow (1979). The gel strip was then sealed into the stacking gel of a standard SDS gel and electrophoresed in the second dimension.

Miscellaneous Procedures. Polyacrylamide gel electrophoresis of purified phosphatidylserine synthase from *E. coli* and *B. licheniformis* followed by immune blotting (Bowen et al., 1980; Towbin et al., 1979) using antibody to the *E. coli* enzyme was used to determine antigenic similarity.

Glycerol density gradient centrifugation of purified enzyme preparations was carried out for 15 h as described by Carman & Dowhan (1979). Similar centrifugation of purified enzyme added to crude cell-free extracts was done for 3 h as described by Louie & Dowhan (1980). Protein amounts were estimated by either the Lowry et al. (1951) method or the dye binding method of Schaffner & Weissmann (1973) with bovine serum albumin as standard. Both methods were comparable; the latter method was used to determine protein in samples with less than 0.05 mg/mL protein. Phosphorus was assayed by the method of Bartlett (1959).

Results

Purification of Phosphatidylserine Synthase. All the following procedures (Table I) were carried out between 4 and 5 °C. Frozen *B. licheniformis* cell paste (125 g) was suspended in 350 mL of 50 mM Tris–maleate buffer (pH 7.0) containing 10 mM $MnCl_2$, 1 mM dithiothreitol, and 1 mM *p*-aminobenzamidine hydrochloride with the aid of a Brinkman polytron. The cells were broken by passing the suspension through a French pressure cell. Unbroken cells were removed by centrifugation at 3000*g*; the supernatant constituted the cell-free extract.

Table I: Purification of Phosphatidylserine Synthase from *B. licheniformis*

step	total units	total volume (mL)	sp act. [nmol min^{-1} (mg of protein)$^{-1}$]	yield (%)
(1) cell-free extract	38 000	320	2.7	100
(2) membranes	34 200	300	4.2	89
(3) Triton X-100 extract	31 800	265	7.5	83
(4) CDP-diacylglycerol–Sepharose column eluate	15 700	225	320	41
(5) blue dextran–Sepharose column eluate	4 600	50	2900	12

The membrane fraction (step 2) was collected by centrifugation at 186000*g* for 2 h in a Ti 45 rotor. The resulting membrane pellet was suspended in 250 mL of 50 mM Tris–maleate (pH 7.0) containing 10 mM $MnCl_2$, 0.2 M KCl, 1 mM dithiothreitol, 1 mM *p*-aminobenzamidine hydrochloride, and 4% Triton X-100. After being stirred for 90 min, the suspension was again centrifuged at 186000*g* for 2 h. The supernatant constituted the Triton X-100 extract (step 3). A high fraction of phosphatidylserine synthase activity was recovered up to this point; the remainder of the actvity in steps 2 and 3 could be accounted for in the discarded supernatant and pellet fractions, respectively.

The Triton X-100 extract was brought to a final concentration of 2% Triton X-100 and 5 mM $MnCl_2$ by 1:1 dilution with buffer consisting of 50 mM Tris–maleate (pH 7.0), 1 mM dithiothreitol, and 1 mM *p*-aminobenzamidine hydrochloride. The phosphatidylserine synthase activity in the extract was then allowed to adsorb to 200 mL (settled volume) of CDP-diacylglycerol–Sepharose preequilibrated with 50 mM Tris–maleate (pH 7.0) buffer containing 2 mM $MnCl_2$, 2% Triton X-100, 1 mM dithiothreitol, and 1 mM *p*-aminobenzamidine hydrochloride by a batch procedure. The resin and Triton X-100 extract were stirred together for 1 h; the resin–extract suspension was then poured into a 5 × 40 cm column and allowed to flow-pack. The flow-through supernatant contained 9% of the phosphatidylserine synthase activity applied to the resin. The column was then washed with 400 mL of the buffer used to equilibrate the resin. This was followed by washes with 400 mL of the loading buffer containing 1.5 M NaCl and 800 mL of the same buffer containing 3.0 M NaCl. After the column was washed again with 2 column volumes of the original equilibrating buffer, it was washed with 2 column volumes of 50 mM Tris–maleate (pH 7.0) buffer containing 0.5% Triton X-100, 2 mM $MnCl_2$, 1 mM dithiothreitol, 1 mM *p*-aminobenzamidine hydrochloride, and 0.8 M hydroxylamine hydrochloride. The resin was incubated for 15 h in the hydroxylamine-containing buffer and then eluted with the same buffer; the peak fractions of enzymatic activity constituted the affinity column eluate.

The affinity column eluate was next diluted with buffer and adjusted to a final concentration of 20 mM Tris–maleate (pH 7.0), 0.25% Triton X-100, 1 mM $MnCl_2$, 1 mM dithiothreitol, 1 mM *p*-aminobenzamidine hydrochloride, and 0.2 M hydroxylamine hydrochloride. The eluate was then applied to a column packed with 50 mL of blue dextran–Sepharose equilibrated with buffer of composition identical with that of the sample buffer; the phosphatidylserine synthase activity was completely bound to the column. After the column was washed with 100 mL of the loading buffer (excluding hydroxylamine), it was washed extensively with the same buffer containing 0.8 M NaCl until the wash fractions contained no more detectable phosphatidylglycerophosphate synthase activity; 25% of the phosphatidylserine synthase activity applied

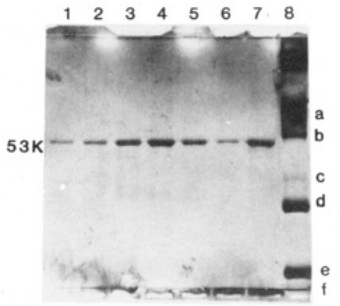

FIGURE 1: SDS–polyacrylamide gels of phosphatidylserine synthase samples taken across the elution peak from the blue dextran–Sepharose column. Lanes 1–6, sequential fractions across the activity peak; lane 7, peak fraction 3; lane 8, protein standards. Equal volumes (25 μL) of each fraction were applied to the gel. The total number of units in each sample applied was as follows: lane 1, 1.3 units; lane 2, 1.4 units; lane 3, 1.6 units; lane 4, 1.5 units; lane 5, 1.1 units; lane 6, 0.6 unit; lane 7, 1.6 units. The proteins used as molecular weight standards were bovine serum albumin (a), *E. coli* phosphatidylserine synthase (b), ovalbumin (c), carbonic anhydrase (d), and soybean trypsin inhibitor (e); dye front (f).

could be accounted for in these fractions. The column was then washed with 200 mL of the loading buffer (excluding hydroxylamine) and then allowed to stand in the same buffer containing 1 mM CDP-diacylglycerol overnight. Further development of the column with the CDP-diacylglycerol-containing buffer resulted in elution of phosphatidylserine synthase from the blue dextran–Sepharose (step 5). None of the eluted fractions contained any detectable phosphatidylglycerophosphate synthase activity.

The overall recovery of purified enzyme by using this procedure was low, but it is unlikely that this was due to multiple forms of the enzyme or enzyme inactivation. The major losses occurred in steps 4 and 5 during the extensive salt washes where the activity was slowly released with the majority of the contaminants.

Enzyme Purity. Polyacrylamide gel electrophoresis in the presence of SDS indicated a highly purified preparation with only one major band when either stained with Coomassie blue or silver stained. When samples taken across the peak of enzymatic activity eluted from the blue dextran–Sepharose column (Figure 1) were subjected to SDS–polyacrylamide gel electrophoresis, there was a qualitative correlation between the amount of phosphatidylserine synthase units applied and the intensity of the stained band. The band near the dye front was due to CDP-diacylglycerol which was present in the preparation. The apparent relative molecular mass of phosphatidylserine synthase determined by SDS gel electrophoresis was 53 000 daltons; the enzyme displayed a distinctly faster mobility than the phosphatidylserine synthase from *E. coli* (lane 8, band b).

While carrying out experiments to further establish that the 53 000-dalton band observed in SDS gels was indeed phosphatidylserine synthase, we found enzymatic activity associated with two different sizes of proteins; the smaller protein was apparently derived from the larger protein by proteolytic cleavage. The purified sample was subjected to gel electrophoresis under nondenaturing conditions in the presence of 1 mM CDP-diacylglycerol and 0.2% Triton X-100 as described under Materials and Methods. A lane into which phosphatidylserine synthase had been electrophoresed was cut out from the gel, divided into 0.25-cm slices, and assayed for phosphatidylserine synthase activity; Figure 2 shows the distribution of enzymatic activity along the entire length of the lane as measured after electrophoresis. Two peaks of phosphatidylserine synthase activity were evident, one at the very top of

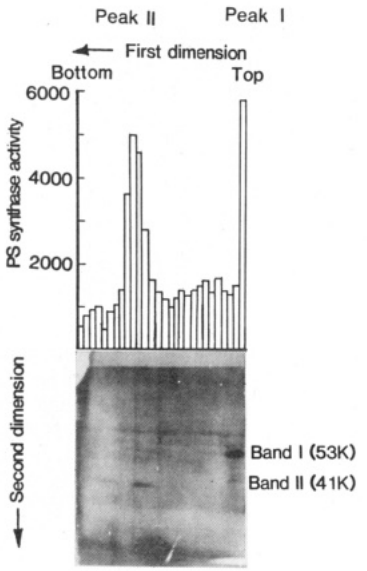

FIGURE 2: Polyacrylamide gel electrophoresis in two dimensions of purified phosphatidylserine synthase. First dimension: gel (5% acrylamide) run under nondenaturing conditions. Enzyme activity is expressed as total chloroform-soluble radioactivity after partitioning as described under Materials and Methods. Second dimension: gel (10% polyacrylamide) run in the presence of SDS.

the gel (peak I) and one two-thirds of the way into the gel (peak II). A parallel lane from the same gel loaded with phosphatidylserine synthase was then denatured and run in the second dimension into an SDS gel as described under Materials and Methods. Figure 2 also shows this SDS gel after silver staining. A dark-staining band was seen corresponding to the top of the nondenaturing gel, i.e., to peak I of enzymatic activity which migrated to a position (band I) identical with that of the major band (53 000 daltons) seen when phosphatidylserine synthase was subjected directly to SDS–polyacrylamide gel electrophoresis as seen in Figure 1. On the other hand, a faster moving band (band II) was found at the position corresponding to peak II (Figure 2). The molecular weight of the band II protein was 41 000. The other staining material in this gel corresponded to material which stained when a buffer sample lacking enzyme was treated in parallel. Band II (41 000 daltons) associated with peak II activity was apparently formed by proteolytic cleavage of band I by traces of endogenous protease present in the preparation of phosphatidylserine synthase. When a sample of the purified phosphatidylserine synthase was allowed to incubate in the elution buffer of the blue dextran–Sepharose column at 30 °C for 1 h, a lower molecular weight band (lane 1) in addition to the major band (lane 2) was seen after electrophoresis (Figure 3). This band was also observed when a phosphatidylserine synthase sample which had been stored at 4 °C for a week was similarly electrophoresed. The proteolytic degradation could be arrested if the enzyme was stored in the presence of 1 mM CDP-diacylglycerol and 20% glycerol at −80 °C in 20 mM Tris–maleate (pH 7.0) containing 1 mM $MnCl_2$, 0.25% Triton X-100, 1 mM dithiothreitol, and 1 mM *p*-aminobenzamidine hydrochloride.

Although the purified enzyme from *B. licheniformis*, as has been observed for the enzyme from *E. coli* (Larson & Dowhan, 1976), does not move appreciably under nondenaturing conditions during gel electrophoresis, the coincidence of enzymatic activity (peak I) with a protein of 53 000 daltons (band I) supports the identification of the major protein species as the phosphatidylserine synthase. This conclusion is further strengthened by the apparent proteolytic conversion of the

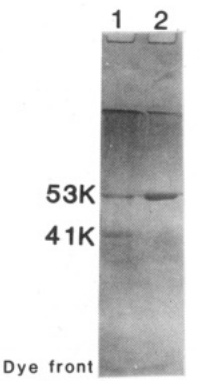

FIGURE 3: SDS–polyacrylamide gel of phosphatidylserine synthase. The gel was 10% in acrylamide and was stained with Coomassie blue. Lane 1, phosphatidylserine synthase after 1-h incubation at 30 °C; lane 2, phosphatidylserine synthase stored at −80 °C.

53 000-dalton species to an enzymatically active 41 000-dalton species. The higher mobility of the 41 000-dalton species in the absence of denaturants could be due to a reduced tendency to aggregate or a reduced affinity for detergent micelles. Therefore, the native enzyme may have, in addition to a catalytic domain, a domain involved in membrane association or self-aggregation.

Stoichiometry of the Phosphatidylserine Synthase Reaction. When phosphatidylserine synthase was assayed separately either by the CDP-diacylglycerol-dependent incorporation of radiolabeled L-serine into phosphatidylserine (in the absence of additional phosphatidylserine) or by the L-serine-dependent release of radiolabeled CMP from CDP-diacylglycerol, there was a 1:1 relationship between the amount of radiolabeled serine incorporated and radiolabeled CMP released; in an average of six determinations, 22.1 ± 2.1 nmol of phosphatidylserine was formed as compared to 24.4 ± 2.8 nmol of CMP.

Doubly labeled phosphatidylserine formation was measured in order to determine the ratio of incorporation of both the phosphatidyl and serine moieties from [^{32}P]CDP-diacylglycerol and L-[U-^{14}C]serine, respectively; this was done under standard assay conditions except for the absence of additional unlabeled phosphatidylserine in the system. The ratio of cpm per nanomole of ^{32}P to cpm per nanomole of ^{14}C in phosphatidylserine (isolated by two-dimensional chromatography) was 1.06 ± 0.16 (average of six determinations). This indicates that both the phosphatidyl and serine moieties were incorporated into phosphatidylserine in a proportion of 1:1. These data establish that the purified phosphatidylserine synthase catalyzes a reaction in which 1 mol each of CDP-diacylglycerol and L-serine combine to yield 1 mol of phosphatidylserine and 1 mol of CMP; no radiolabeled phosphatidic acid was detected, indicating the absence of hydrolytic activities. Measurement of doubly labeled phosphatidylserine formation was difficult to make accurately using crude preparations of the enzyme (Dutt & Dowhan, 1981) due to contaminating activities.

Enzymological Properties of Phosphatidylserine Synthase. In general, the properties of the purified enzyme were very similar to those reported earlier for the enzyme in crude extracts. The reduction of the level of CDP-diacylglycerol hydrolase, phosphatidylserine hydrolase, phosphatidylserine decarboxylase, and phosphatidylglycerophosphate synthase to less than 0.001% the level of phosphatidylserine synthase rules out the involvement of the above enzymes in this reaction; the levels of the above activities in crude extracts are on the same order as the synthase. The purified enzyme was still dependent on divalent metal ion concentration (Figure 4) for activity

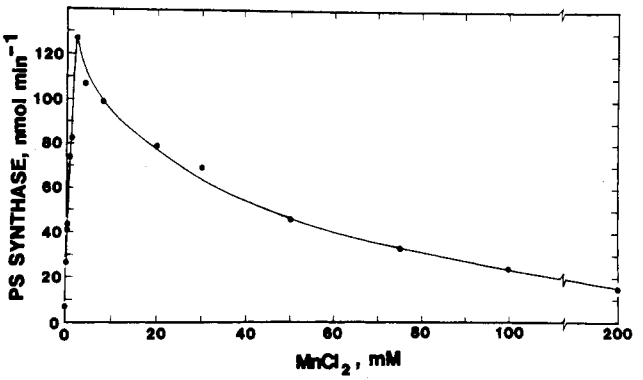

FIGURE 4: Dependence of purified phosphatidylserine synthase activity on $MnCl_2$ concentration. Standard assay conditions were used, except the $MnCl_2$ concentration was varied as indicated.

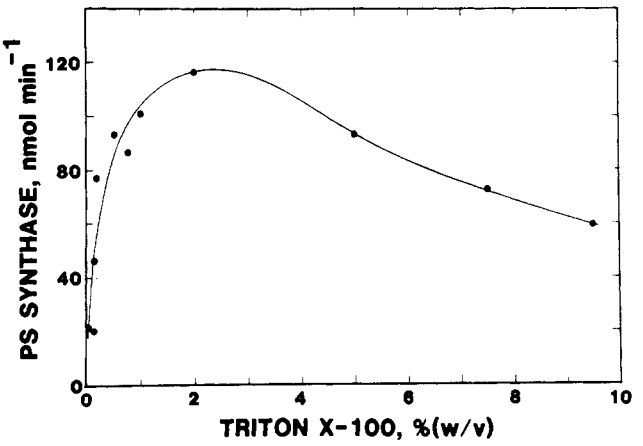

FIGURE 5: Triton X-100 concentration dependence of phosphatidylserine synthase activity. Standard assay conditions were employed, except the Triton X-100 concentration was varied as indicated.

although maximum activity occurred at 2 mM $MnCl_2$ rather than 10 mM as reported for the enzyme in crude extracts (Dutt & Dowhan, 1981). The dependence on Triton X-100 concentration was somewhat different in that maximum activity occurred at 2% (1% in crude extracts) and higher concentrations showed inhibition of activity (see Figure 5) which did not occur with crude extracts. This difference in detergent dependence may be due to the presence of phospholipids in crude extracts. The apparent inhibition at higher detergent concentrations has been attributed to substrate dilution for several detergent-dependent enzymes (Eaton & Dennis, 1976) including the enzyme from *E. coli* (Carman & Dowhan, 1979).

An apparent K_m of 0.23 mM for L-serine was obtained when a double-reciprocal plot was made of reaction velocity vs. increasing L-serine concentrations at a fixed concentration (0.125 mM) for CDP-diacylglycerol and Triton X-100 (2%). It was not possible to determine a K_m value for CDP-diacylglycerol as the CDP-diacylglycerol concentration present in the phosphatidylserine synthase preparation was apparently saturating even when diluted 1:40 into the assay system, i.e., at the level of 0.025 mM; concentrations of CDP-diacylglycerol higher than 0.15 mM caused inhibition of phosphatidylserine synthase activity.

Reaction Mechanism. The properties of phosphatidylserine synthase in cell-free extracts of *B. licheniformis* previously reported (Dutt & Dowhan, 1981) indicate the reaction catalyzed proceeds via a sequential Bi-Bi mechanism in the following order:

CDP-diacylglycerol + L-serine $\rightleftharpoons$
phosphatidylserine + CMP

Isotope exchange reactions catalyzed by an enzyme in the presence of defined combinations of substrates and products (one of the components being radiolabeled) are diagnostic of the reaction mechanism (Cleland, 1970). In sequential Bi-Bi reaction mechanisms involving two substrates and two products, both substrates or both products have to bind to the enzyme before the first product or first substrate can be released. We have previously reported that the incorporation of radiolabeled L-serine into phosphatidylserine is dependent on CMP in the presence of phosphatidylserine when CDP-diacylglycerol is absent. A feature of a Bi-Bi mechanism is that the presence of one of the products can enhance the rate of exchange between that product and one of the substrates, but only in the presence of the other substrate. Phosphatidylserine stimulates the initial rate of CDP-diacylglycerol-dependent incorporation of radiolabeled serine into lipid 5–6-fold (Dutt & Dowhan, 1981).

The above observations and conclusions concerning the reaction mechanism were further verified by the following results obtained by using the purified phosphatidylserine synthase. The apparent 5–6-fold stimulation of the forward reaction by unlabeled phosphatidylserine was still observed; the specific activity of the peak fraction from the final purification step was 3500 nmol min^{-1} (mg of protein)$^{-1}$ under standard assay conditions and 700 nmol min^{-1} (mg of protein)$^{-1}$ when phosphatidylserine was left out of the assay mixture. The serine-dependent release of labeled CMP from cytidine-labeled CDP-diacylglycerol was not enhanced by the presence of either phosphatidylserine (0.5 mM) or CMP (1.0 mM) in the system; this observation is consistent with the above proposed order for the reaction and also establishes that the apparent stimulation by phosphatidylserine of incorporation of serine is due to an increase in exchange rate, and not net synthesis of lipid. When phosphatidylserine labeled in the serine moiety was included in the standard phosphatidylserine synthase reaction, the release of labeled serine was absolutely dependent on the presence of L-serine in the system; in a typical experiment, 23% of the serine label in phosphatidylserine was released in 1 h in the presence of L-serine (1 mM) vs. less than 0.05% in the absence of L-serine. The dependence of such a release of labeled serine on CDP-diacylglycerol as well was demonstrated earlier in crude extracts (Dutt & Dowhan, 1981); the presence of the liponucleotide in the final purification step prevented such a determination with the purified enzyme. An important diagnostic test of a Bi-Bi-ordered mechanism is the inhibition of exchange between the first substrate and last product by increasing levels of the second substrate and first product added at a constant ratio. Although the expected result was observed when such an experiment was carried out, the interpretation of the result was not straightforward because the ratio of phospholipid to detergent in the assay system was also changed, which altered the physical properties of the mixed micelle substrate. As demonstrated in Figure 5, the initial velocity of this enzyme shows a dependence on the ratio of phospholipid substrate to detergent.

Physical Properties of Phosphatidylserine Synthase. Results of studies on the behavior of the enzyme from *B. licheniformis* during density gradient centrifugation were consistent with the membrane location of this enzyme. The properties of the enzyme were also in marked contrast to those of the enzyme from *E. coli*. After centrifugation of the purified enzyme from *B. licheniformis* through a 10–30% glycerol gradient containing no Triton X-100, all recoverable activity was found at the bottom of the gradient presumably in an aggregated form. When 1% Triton X-100 was included in the

Table II: Properties of Phosphatidylserine Synthases from *B. licheniformis* and *E. coli*

property	*B. licheniformis*	*E. coli*
location	membrane	ribosomes
mol wt	53 000	54 000
ion requirements	Mn^{2+}	none
detergent affinity	high	substrate induced
detergent requirement	++	+
mechanism	sequential Bi-Bi	ping-pong
stimulation by product	+	–
turnover no.	4[a]	3000[b]
hydrolytic act.	–	+
antigenic similarity	–	–

[a] The turnover number (moles of product per mole of enzyme per minute) was determined by using the molecular weight and specific activity in the absence of phosphatidylserine reported here; the latter value is a true measure of net phosphatidylserine formation since added phosphatidylserine only stimulates exchange. [b] Larson & Dowhan (1976).

gradient, the activity was recovered near the top of the gradient as a well-defined peak. These results are in marked contrast to those for the enzyme from *E. coli* (Carman & Dowhan, 1979) where detergent caused a smearing of activity throughout the gradient; only the addition of liponucleotide substrate induced the formation of a sharply defined peak near the top of the gradient which was attributed to binding to the detergent–substrate mixed micelle. The two purified phosphatidylserine synthases were also dissimilar with regard to their affinity for ribosomes. The purified *B. licheniformis* enzyme when incubated with *E. coli* crude extract was found associated with both the membrane and supernatant fractions of *E. coli* and not the ribosomal fraction after glycerol gradient centrifugation. In this experiment, *E. coli* strain RA80 was used, and the enzyme from *B. licheniformis* was distinguished from residual *E. coli* enzyme activity by the dependence of the former enzyme on divalent metal ion concentrations. The purified *E. coli* enzyme after incubation with a *B. licheniformis* crude extract was found associated with the ribosomal and supernatant fractions of *B. licheniformis*. These results are consistent with the differences in the affinity of these two enzymes for both membranes and ribosomes when they are present in their native extracts, respectively.

The *B. licheniformis* phosphatidylserine synthase was also antigenically dissimilar to the *E. coli* enzyme. Immune blotting with the *E. coli* synthase antibody after SDS–polyacrylamide electrophoresis indicated no cross-reactivity with purified phosphatidylserine synthase from *B. licheniformis*.

Discussion

The detailed studies in *B. licheniformis* and *E. coli* when compared to studies on crude preparations from other organisms suggest at least two distinct forms of CDP-diacylglycerol utilizing phosphatidylserine synthase in nature. One form, primarily found in Gram-negative bacteria (Dutt & Dowhan, 1977) and most extensively studied from *E. coli* (Larson & Dowhan, 1976), is found tightly associated with the ribosomal fraction of crude cell extracts. In several Gram-positive bacteria (Dutt & Dowhan, 1981; Cousminer et al., 1982; Langley et al., 1979) and in yeast (Carson et al., 1982; Carman & Matas, 1981; Letts et al., 1983), this same enzymatic activity is found tightly associated with the membrane fraction of crude cell extracts. This report has been the first detailed study of a homogeneous preparation of the latter form of this enzyme and has detailed many of the differences and similarities between these two forms of the enzyme.

The properties of the phosphatidylserine synthase from *E. coli* and *B. licheniformis* are summarized in Table II. The major differences are in their respective affinities for subcellular components and in their reaction mechanisms and turnover numbers. The lower number for the enzyme from *B. licheniformis* is not due to enzyme inactivation during purification, since the specific activity of this enzyme in crude extracts of several Gram-positive bacteria has been noted to be 50–100 times lower than that in *E. coli* (Dutt & Dowhan, 1981; Langley et al., 1979). In addition, the total recoverable activity at each step of purification would argue against a significant reduction in catalytic efficiency through inactivation. Further conclusions about the significance of this wide difference in turnover numbers are complicated by the difference in optimum assay conditions for the two enzymes. Although the enzymes from other Gram-positive bacteria and yeast have not been as extensively studied, they appear to be similar in their kinetic and physical properties as well as their dependence on divalent metal ion to the enzyme from *B. licheniformis*. In addition, other membrane-associated CDP-diacylglycerol-dependent phosphatidyltransferases such as the phosphatidylglycerophosphate synthase from *E coli* (Hirabayashi et al., 1976), *B. licheniformis* (Larson et al., 1976), and yeast (Carman & Belunis, 1983) and the phosphatidylinositol synthase of yeast (Fischl & Carman, 1983) appear to be similar to the phosphatidylserine synthase from *B. licheniformis*; the *E. coli* phosphatidylglycerophosphate synthase is nearly identical in its kinetic properties with the enzyme from *B. licheniformis*. The physiological basis for the differences in reaction mechanism for the two forms of phosphatidyltransferases is not immediately apparent but may be related to their membrane affinity and mode of regulation. It has been speculated that in the course of the phosphatidylserine synthase reaction in *E. coli* a covalent phosphatidyl-enzyme intermediate is formed which enables the enzyme to be tightly membrane bound to facilitate catalysis (Raetz & Kennedy, 1974; Larson & Dowhan, 1976). The phosphatidylserine synthase from *B. licheniformis*, however, like the *E. coli* phosphatidylglycerophosphate synthase (Hirabayashi et al., 1976), catalyzes the formation of product by a sequential ordered mechanism which may not go through such an intermediate. Being membrane-embedded proteins, they have ready access to their lipid substrates and a requirement for binding of a second water-soluble substrate, viz., serine, or glycerophosphate before product formation may prevent unnecessary hydrolysis of CDP-diacylglycerol.

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Registry No. Phosphatidylserine synthase, 9068-48-8; L-serine, 56-45-1.

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Net Mass Transfer of Galactosylceramide Facilitated by Glycolipid Transfer Protein from Pig Brain: A Monolayer Study†

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ABSTRACT: A net mass transfer of galactosylceramide (GalCer) and galactosyldiacylglycerol (GalDG) is catalyzed by the glycolipid transfer protein from pig brain. GalCer and GalDG are transferred from a monolayer to phosphatidylcholine vesicles in the subphase or from a glycolipid monolayer to a phosphatidylcholine monolayer. No transfer of phosphatidylcholine is measured under these conditions. It is found that the glycolipid transfer protein functions as a carrier and that glycolipid is bound to less than 50% of the transfer protein. The presence of lipid-free proteins fits with the proposed mechanism of net mass transfer. The glycolipid transfer is influenced by the fluidity of the lipid interface and by the matrix lipid of the interface. GalCer transfer is stimulated in the presence of GalDG.

Proteins which facilitate the transfer of phospholipids between membranes in vitro have been widely found in various cells [for reviews, see Zilversmit et al. (1976), Kader (1977), and Akeroyd & Wirtz (1982)]. Phosphatidylcholine-specific phospholipid transfer protein, phosphatidylinositol transfer protein, and nonspecific lipid transfer protein have been purified. The mechanisms of lipid transfer facilitated by these proteins have been studied (Demel et al., 1984). The phosphatidylcholine transfer protein acts mainly as an exchange protein (Demel et al., 1973; Helmkamp et al., 1976) when both membranes contain phosphatidylcholine. Under certain conditions, a small percentage of net transfer can be found (Devaux et al., 1977; Wirtz et al., 1980).

For the phosphatidylinositol transfer protein, which binds primarily phosphatidylinositol but also phosphatidylcholine,

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