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Phospholipids Chiral at Phosphorus. Steric Course of the Reactions Catalyzed by Phosphatidylserine Synthase from *Escherichia coli* and Yeast[†]

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ABSTRACT: The steric courses of the reactions catalyzed by phosphatidylserine (PS) synthase from *Escherichia coli* and yeast were elucidated by the following procedure. *R_P* and *S_P* isomers of 1,2-dipalmitoyl-*sn*-glycero-3-[¹⁷O,¹⁸O]phosphoethanolamine ([¹⁷O,¹⁸O]DPPE) were synthesized with slight modification of the previous procedure [Bruzik, K., & Tsai, M.-D. (1984) *J. Am. Chem. Soc.* 106, 747–754] and converted to (*R_P*)- and (*S_P*)-1,2-dipalmitoyl-*sn*-glycero-3-[¹⁶O,¹⁷O,¹⁸O]phosphoric acid ([¹⁶O,¹⁷O,¹⁸O]DPPA), respectively, by incubating with phospholipase D. Condensation of [¹⁶O,¹⁷O,¹⁸O]DPPA with cytidine 5'-monophosphomorpholidate in pyridine gave the desired substrate for PS synthase, [¹⁷O,¹⁸O]cytidine 5'-diphospho-1,2-dipalmitoyl-*sn*-glycerol ([¹⁷O,¹⁸O]CDP-DPG), as a mixture of several isotopic and configurational isomers. Incubation of [¹⁷O,¹⁸O]CDP-DPG with a mixture of L-serine, PS synthase (which converted [¹⁷O,¹⁸O]CDP-DPG to phosphatidylserine), and PS decarboxylase (which catalyzes decarboxylation of phosphatidylserine) gave [¹⁷O,¹⁸O]DPPE. The configuration and isotopic enrichments of the starting [¹⁷O,¹⁸O]DPPE and the product were analyzed by ³¹P NMR following trimethylsilylation of the DPPE. The results indicate that the reaction of *E. coli* PS synthase proceeds with *retention* of configuration at phosphorus, which suggests a two-step mechanism involving a phosphatidyl-enzyme intermediate, while the yeast PS synthase catalyzes the reaction with *inversion* of configuration, which suggests a single-displacement mechanism. Such results lend strong support to the ping-pong mechanism proposed for the *E. coli* enzyme and the sequential Bi-Bi mechanism proposed for the yeast enzyme, both based on previous isotopic exchange experiments.

Phosphatidylserine (PS)¹ synthase (CDP-diglyceride:L-serine O-phosphatidyltransferase, EC 2.7.8.8) is a key enzyme in the biosynthesis of major phospholipids (phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine) in both bacteria (Raetz, 1978) and in yeast (Steiner & Lester, 1972). There appears to be two subclasses of PS synthase. The enzyme from *Escherichia coli* (Raetz & Kennedy, 1972) as well as other Gram-negative bacteria (Dutt & Dowhan, 1977) is associated with ribosomes in cell-free extracts. However, the PS synthase was found to be associated with membranes in yeast (Carson et al., 1982; Carman & Matas, 1981; Letts et al., 1983), in *Bacillus licheniformis* (Dutt & Dowhan, 1985), and in *Clostridium perfringens* (Cousminer et al., 1982). Table I summarizes the properties of PS synthases from three different sources, which have been purified to homogeneity and extensively characterized.

The problem this paper addresses is the comparison of the mechanism between the two subclasses of PS synthase. Since

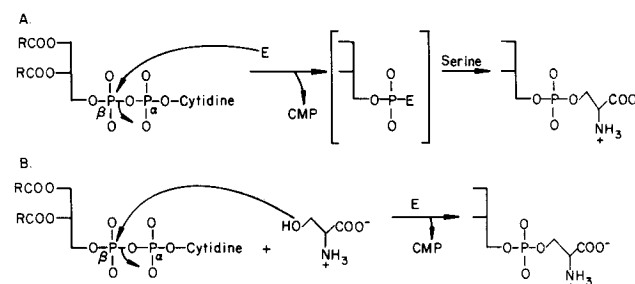
Table I: Properties of PS Synthases from Different Sources

property	<i>E. coli</i> ^a	<i>B. licheniformis</i> ^b	yeast ^c
location	ribosomes	membrane	membrane
mol wt (subunit)	54 000	53 000	23 000
M ²⁺ requirement	none	Mn ²⁺	Mn ²⁺ or Mg ²⁺
turnover no. (min ⁻¹)	3000	4	90
hydrolytic act.	+ ^d	—	—
proposed mechanism	ping-pong	sequential Bi-Bi	sequential Bi-Bi

^a From Raetz and Kennedy (1974) and Larson and Dowhan (1976).

^b From Dutt and Dowhan (1985). ^c From Bae-Lee and Carman (1984). ^d From Bulawa et al. (1983).

Scheme I: Possible Mechanisms of PS Synthase



detailed kinetic analysis is usually difficult for such enzymes requiring detergent in *in vitro* assays, the enzyme-catalyzed

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exchanges between isotopically labeled substrates and products (Cleland, 1970) have been investigated and used to suggest a ping-pong mechanism for the *E. coli* enzyme (Raetz & Kennedy, 1974; Larson & Dowhan, 1976) and a sequential Bi-Bi mechanism for the enzyme from *B. licheniformis* (Dutt & Dowhan, 1985) and yeast (Bae-Lee & Carman, 1984). Such proposals would predict a two-step mechanism involving a phosphatidyl-enzyme intermediate (Scheme IA) for the *E. coli* enzyme and a single-displacement reaction (Scheme IB) in the other two cases.

Stereochemical studies may support or refute the above proposed mechanisms. It has been generally established that, except in special cases involving a 1,2-migration (Buchwald et al., 1984), a single-displacement catalyzed by enzymes will result in *inversion* of configuration at phosphorus, whereas a double-displacement reaction will result in overall *retention* [for reviews, see Eckstein (1985), Gerlt et al. (1983), Frey (1982), Tsai (1982), and Knowles (1980)]. Such studies, however, require relatively large quantities of enzymes. Since strains overproducing PS synthase have been constructed for *E. coli* (Raetz et al., 1977; Ohta et al., 1981; Sparrow & Raetz, 1983) and yeast (*Saccharomyces cerevisiae*) (Letts et al., 1983), we set forth to elucidate the steric course of the reaction catalyzed by PS synthase from these two sources, using substrates stereospecifically labeled with ^{17}O and ^{18}O .

MATERIALS AND METHODS

Materials. H_2^{17}O (51.0 atom % ^{17}O , 38.6 atom % ^{18}O) and H_2^{18}O (97.5 atom % ^{18}O) were purchased from Monsanto. BSA, CMP-morpholidate, and other biochemicals were obtained from Sigma. Bio-Sil A (200–400 mesh) was purchased from Bio-Rad. Pd/C (10%, type 1, 325 mesh) was obtained from Johnson Matthey, Inc. Silica gel (Kieselgel 60, 230–400 mesh) and precoated TLC plates (0.2-mm aluminum support, silica gel 60, F-254) were purchased from E. Merck. All other chemicals were of reagent grade or highest purity available commercially.

Enzymes. The international unit "U" ($\mu\text{mol}/\text{min}$) is used to describe the activity of all enzymes used in this paper. The yeast PS synthase was purified from an overproducing strain VAL2C(YEpCHO1) of *Saccharomyces cerevisiae* (Bae-Lee & Carman, 1984). The purified enzyme (3.7 U/mg of protein) contained some CDP-diacylglycerol derived from egg lecithin (Carman & Fischl, 1980), which was required to stabilize the enzyme. The *E. coli* PS synthase (35 U/mg) was purified from an overproducing strain AC5/pPS 4017 (Sparrow & Raetz, 1983). PS decarboxylase (49 U/mg) was purified from *E. coli* strain JA 200/pPSD2b (Li et al., 1986), as described in Dowhan et al. (1974). Phospholipase D (from cabbage, 0.3 U/mg of lyophilizate) was purchased from Boehringer.

Analytical Methods. Routine ^1H , ^{13}C , and ^{31}P NMR analysis were carried out on a Bruker WP-200 NMR spectrometer. ^{31}P NMR analysis of chiral [^{17}O , ^{18}O]DPPE was performed on a Bruker AM-500 NMR spectrometer. Broad-band ^1H decoupling was used in ^{31}P and ^{13}C NMR. ^1H and ^{13}C chemical shifts were referenced to internal Me_4Si standard, while ^{31}P chemical shifts were referenced to external 85% H_3PO_4 . When unlabeled pyridine was used as solvent,

an inner tube containing D_2O was used for deuterium lock. The positive sign represents a downfield shift in all cases. The justification of Gaussian multiplication in the quantitative analysis of the ^{31}P NMR spectra of [^{17}O , ^{18}O]DPPE was described in Bruzik and Tsai (1984a).

TLC plates were visualized by spraying with a 10% ethanolic solution of phosphomolybdic acid followed by heating. The solvent systems and R_f values are as follows. System A:² $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}/\text{HOAc}$ (25/15/4/2); DPPA (0), CMP-morpholidate (0.21), CDP-DPG (0.6), DPPE (0.8). System B: $\text{CHCl}_3/\text{pyridine}/\text{HOAc}$ (50/20/7); DPPA (0.7).

Syntheses of [^{17}O , ^{18}O]DPPE (1a and 1b). This followed essentially the same procedure as described in Bruzik and Tsai (1984a), except the following modifications in the last two steps ($6 \rightarrow 7 \rightarrow 1$ in Scheme IV). To 1.0 g (1.28 mmol) of the dried **6** in 10 mL of freshly distilled (from lithium aluminum hydride) 1,2-dimethoxyethane (DME) was added 0.77 mL of H_2^{18}O , followed by dropwise addition of 1 N HCl in DME (dry HCl in dry DME) until the pH turned ca. 2 according to a pH paper. The hydrolysis was complete after stirring for ca. 1 h at room temperature as monitored by TLC (ether, R_f 0.43 and 0 for **6** and **7**, respectively) and by the precipitation of **7**. The above procedures were performed in a drybox. The thick suspension was then evaporated in vacuo, redissolved in CHCl_3 , and washed with aqueous NaHCO_3 followed with H_2O . After evaporation of the CHCl_3 phase the product **7** was obtained in 98% yield as a white powder, which gave R_f 0.55 in $\text{EtOH}/\text{CHCl}_3/\text{H}_2\text{O}$ (50/50/4). ^1H NMR (not reported previously) (CDCl_3 , 200 MHz): δ 0.86 (t, $J = 6.7$ Hz, 6 H, terminal CH_3), 1.23 (br s, 48 H, long-chain CH_2), 1.57 (m, 4 H, $\text{CH}_2\text{CH}_2\text{C}=\text{O}$), 1.71 (d, $J = 6.8$ Hz, 3 H, CH_3CHN), 2.26 (t, $J = 7.5$ Hz, 4 H, $\text{CH}_2\text{C}=\text{O}$), 2.9 (m, 2 H, $\text{OCH}_2\text{CH}_2\text{N}$), 3.97–4.21 (m, 3 H, $\text{OCH}_2\text{CH}_2\text{N}$ and CH_3CHN), 4.0 (dd, $^3J_{\text{CH}_A\text{CH}} = 5.4$ Hz, $^3J_{\text{CH}_A\text{OP}} = 7.2$ Hz, 1 H, $\text{CH}_A\text{H}_B\text{OP}$), 4.06 (dd, $^3J_{\text{CH}_B\text{CH}} = 5.4$ Hz, $^3J_{\text{CH}_B\text{OP}} = 7.2$ Hz, 1 H, $\text{CH}_A\text{H}_B\text{OP}$), 4.16 (dd, $^3J_{\text{CH}_A\text{CH}} = 6.4$ Hz, $^2J_{\text{H}_A\text{H}_B} = 12.0$ Hz, 1 H, $\text{CH}_A\text{H}_B\text{OC}=\text{O}$), 4.37 (dd, $^3J_{\text{CH}_B\text{CH}} = 3.5$ Hz, $^2J_{\text{H}_A\text{H}_B} = 12.0$ Hz, 1 H, $\text{CH}_A\text{H}_B\text{OC}=\text{O}$), 5.22 (m, 1 H, $\text{CHOC}=\text{O}$), 7.3–7.5 (m, 5 H, phenyl). The assignments were assisted by homonuclear ^1H decoupling.

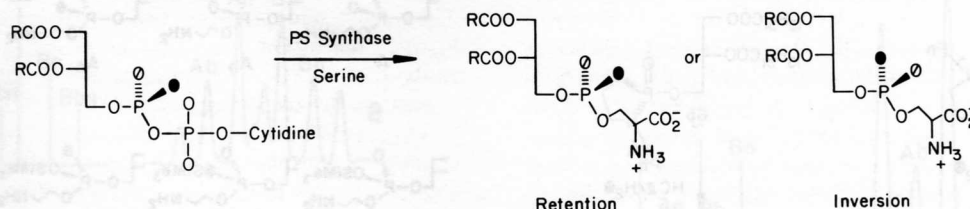
In the hydrogenolysis of **7** by H_2 over Pd/C, *n*-BuOH instead of acetic acid was used as solvent. The catalyst was removed by filtration through a short column of Celite 545 and washed with a 1/1 mixture of $\text{CHCl}_3/\text{CH}_3\text{OH}$. The product was then further purified by chromatography on silica gel.

[^{16}O , ^{17}O , ^{18}O]DPPA (**2a** and **2b**). The phospholipase D catalyzed hydrolysis of [^{17}O , ^{18}O]DPPE to [^{16}O , ^{17}O , ^{18}O]DPPA was carried out as described in Bruzik and Tsai (1984b). Alternatively, **7** was converted to **2** as follows. To a solution of thoroughly dried **7** (0.1 mmol) in 1 mL of freshly distilled CHCl_3 was added 70 μL (0.48 mmol) of iodotrimethylsilane. The reaction was complete after incubation at room temperature for ca. 3 h, as monitored by TLC, and by the formation of precipitates of byproducts. The precipitate was then filtered off and washed with cold acetone. The filtrate was evaporated to dryness and the solid residue redissolved in 1.5 mL of 95% MeOH and refluxed for 15 min. After cooling, crystalline

¹ Abbreviations: BSA, bovine serum albumin; CDP-DG, cytidine 5'-diphospho-1,2-diacyl-*sn*-glycerol; CDP-DPG, cytidine 5'-diphospho-1,2-dipalmitoyl-*sn*-glycerol; CMP-morpholidate, cytidine 5'-monophosphomorpholidate; DME, 1,2-dimethoxyethane; DPPA, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoric acid; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DPPS, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine; KPi , potassium phosphate; PS, phosphatidylserine; Me_4Si , iodotrimethylsilane.

² Although solvent system A is adequate for identification of CDP-DPG on TLC plates, an attempt to purify CDP-DPG on a silica gel column using solvent system A resulted in decomposition of the compound. The decomposed product, though uncharacterized, gave the same R_f on the TLC system A. Thus, it is possible that the R_f value reported for CDP-DPG is that of the decomposed product. Indeed the spot at R_f 0.6 for CDP-DPG was always accompanied by other spots at the origin and the solvent front.

Scheme II



DPPA formed, in 68% yield after filtration and drying, and was characterized by TLC and ^1H NMR.

$[^{17}\text{O}, ^{18}\text{O}]\text{CDP-DPG}$ (3). The $[^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}]\text{DPPA}$ obtained from the phospholipase D reaction, possibly as a calcium salt, was converted to the acid form as follows: 122 mg of DPPA was dissolved in 2 mL of CHCl_3 and added with 3 mL of 6/4 $\text{CH}_3\text{OH}/\text{H}_2\text{O}$. The upper (aqueous) layer was adjusted to pH 2.5 with 0.1 N HCl and the mixture shaken. The CHCl_3 layer was then separated, washed with 3×3 mL of 25/75 $\text{CH}_3\text{OH}/\text{H}_2\text{O}$, evaporated, and dried in vacuo (0.01 mmHg) over P_2O_5 to give 109 mg of white powder (one spot on TLC, system B).

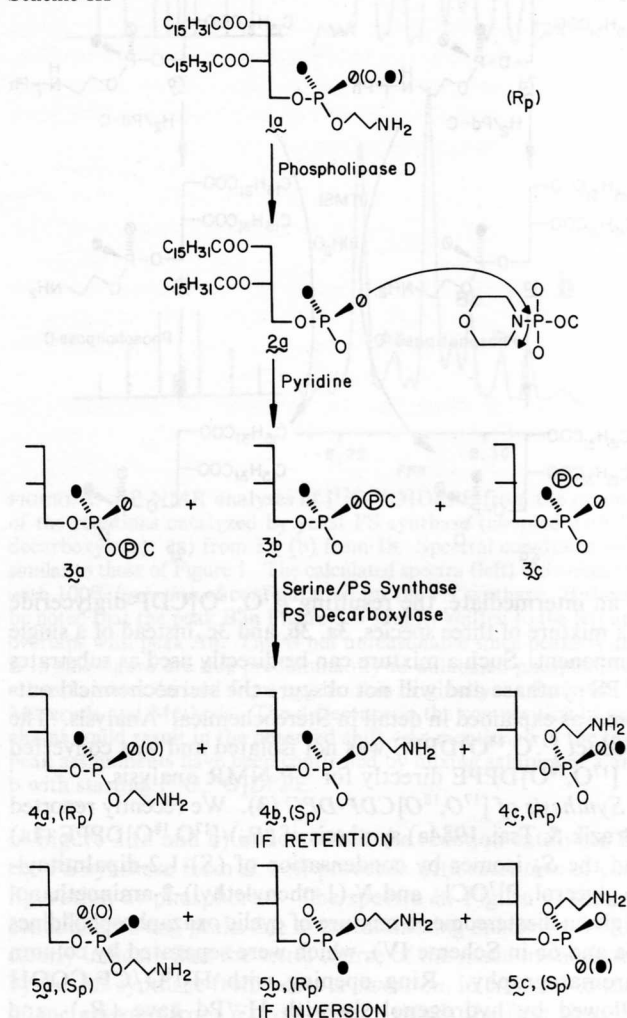
The CMP-morpholidate from Sigma gave two spots on TLC and several ^{31}P NMR signals. It was purified as follows [modified from Turcotte et al. (1980)]: 500 mg was dissolved in 3 mL of CH_3OH and mixed with 30 mL of anhydrous ether. The precipitate was centrifuged down and the solvent discarded. The procedure was repeated several times until the precipitate (white powder after drying in vacuo over P_2O_5) gave a single spot on TLC (system A).

The purified reactants (0.17 mmol each) were placed in a 50-mL flask, further dried for 20 h under 0.01 mmHg, and added with 15 mL of pyridine (distilled over CaH_2 in the vacuum line). The flask was then transferred under dry nitrogen to a drybox and stirred at room temperature. The reaction was monitored by TLC and by ^{31}P NMR (directly in pyridine, δ 2.2 for DPPA, 6.8 for CMP-morpholidate, and -9.8 and -10.1 for CDP-DPG). The conversion was 36% at 24 h, 75% at 54 h, and complete after 5–10 days. In some runs ca. 5–10% excess of CMP-morpholidate was used to ensure full conversion of 2 to 3. After evaporation of pyridine under vacuum, the product was used directly as a substrate of PS synthase without further purification.

Coupled Reactions of PS Synthase (*E. coli*) and PS Decarboxylase. The reaction mixture was prepared by mixing the following solutions: 37 mg of 3 in 15 mL of Triton X-100, 15 mL of 0.5 M potassium phosphate (KP_i) buffer (pH 7.0), 15 mL of 0.5% BSA (5 mg/mL), 15 mL of 10 mM L-serine (neutral form), 15 mL of H_2O , 200 μL *E. coli* PS synthase (40 $\mu\text{g}/\text{mL}$, in 0.1 M KP_i buffer, pH 7.0, containing 1 mg/mL of BSA), and 200 μL of PS decarboxylase (200 $\mu\text{g}/\text{mL}$, in the same buffer as PS synthase). The progress of the reaction was monitored by TLC. After incubation at 25 $^\circ\text{C}$ for ca. 40 h, the reaction was largely complete, and the reaction mixture was mixed with 85 mL of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1/1). The upper layer (water/methanol) was then adjusted to pH 1.5 with concentrated HCl and shaken again. The organic layer was collected and the aqueous layer further washed with 2×30 mL of CHCl_3 . The CHCl_3 fractions were combined, evaporated in vacuo, and loaded onto a Bio-Sil A column (10-mm diameter, 250-mm height). After Triton X-100 was washed off with ca. 150 mL of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (95/5), DPPE was eluted off by $\text{CHCl}_3/\text{CH}_3\text{OH}$ (70/30) in 60% yield and characterized by TLC and ^1H NMR.

Coupled Reactions of PS Synthase (Yeast) and PS Decarboxylase (3 \rightarrow 5). A 25-mg portion of 3 was dissolved in 7 mL of 1% Triton X-100 and mixed with 60 mL of 50 mM

Scheme III

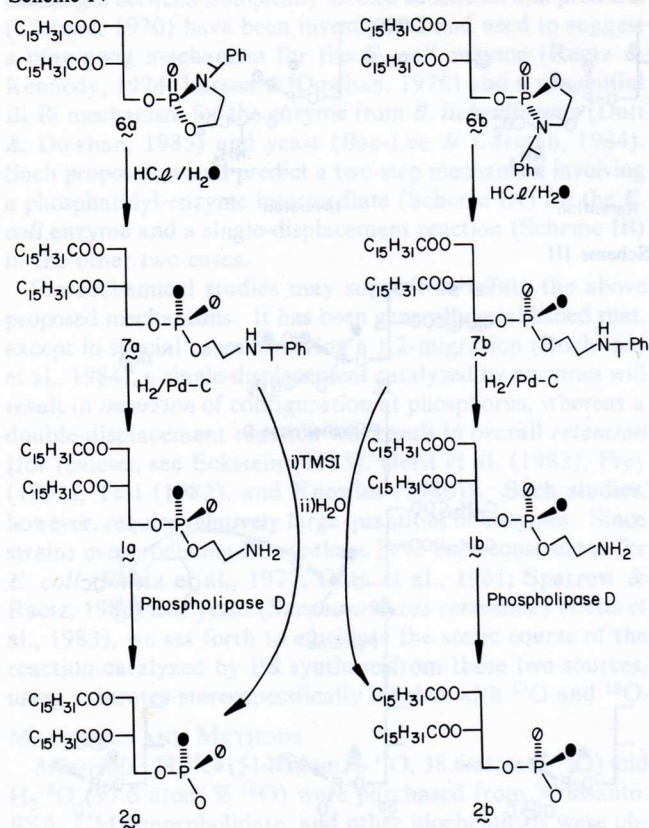


Tris-HCl buffer (pH 8.0) containing 0.6 mM MnCl_2 and 12.5 mg of serine. The reaction was started by addition of 12 mL (in the case of Figure 3a) or 20 mL (in the case of Figure 3b) of PS synthase from yeast (3 $\mu\text{g}/\text{mL}$) in 20 mM Tris-HCl buffer, pH 8.0 (containing 10 mM MgCl_2 , 0.3 mM MnCl_2 , 20% glycerol, 1.5 mM CDP-DG, 10 mM 2-mercaptoethanol, and 0.5% Triton X-100), and 100 μL of PS decarboxylase from *E. coli*. The reaction mixture was incubated at room temperature for 24 h and monitored and worked up as described above. The yield was ca. 60%.

RESULTS AND DISCUSSION

General Approach. In principle, we would synthesize $[\beta\text{-}^{17}\text{O}, \beta\text{-}^{18}\text{O}]\text{CDP-DG}$ of known configuration and determine the configuration of the product, as shown in Scheme II. Our strategy is outlined in Scheme III. Since we were able to synthesize $[^{17}\text{O}, ^{18}\text{O}]\text{DPPE}$ and analyze its configuration (Bruzik & Tsai, 1982, 1984a), our procedure started with $[^{17}\text{O}, ^{18}\text{O}]\text{DPPE}$ (1) as a precursor for $[^{17}\text{O}, ^{18}\text{O}]\text{CDP-DPG}$ (3) (detailed synthetic procedures are described in the next section). Since the procedure involved $[^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}]\text{DPPA}$ (2)

Scheme IV



as an intermediate, the resulting [^{17}O , ^{18}O]CDP-diglyceride is a mixture of three species, **3a**, **3b**, and **3c**, instead of a single component. Such a mixture can be directly used as substrates of PS synthase and will not obscure the stereochemical outcome, as explained in detail in Stereochemical Analysis. The product [^{17}O , ^{18}O]DPPE was not isolated and was converted to [^{17}O , ^{18}O]DPPE directly for ^{31}P NMR analysis.

Synthesis of [^{17}O , ^{18}O]CDP-DPG (3). We recently reported (Bruzik & Tsai, 1984a) synthesis of (R_P)-[^{17}O , ^{18}O]DPPE (**1a**) and the S_P isomer by condensation of (S)-1,2-dipalmitoyl-*sn*-glycerol, $\text{P}^{17}\text{OCl}_3$, and N -(1-phenylethyl)-2-aminoethanol to give a diastereomeric mixture of cyclic oxazaphospholidines (**6a** and **6b** in Scheme IV), which were separated by column chromatography. Ring opening with $\text{H}_2^{18}\text{O}/\text{CF}_3\text{COOH}$ followed by hydrogenolysis with H_2/Pd gave (R_P)- and (S_P)-[^{17}O , ^{18}O]DPPE with $\leq 70\%$ enantiomeric purity.

We have now improved the enantiomeric purity of [^{17}O , ^{18}O]DPPE by using dry HCl as a catalyst and DME as a solvent in the ring-opening reaction. The resulting (R_P)-[^{17}O , ^{18}O]DPPE (**1a**) and (S_P)-[^{17}O , ^{18}O]DPPE (**1b**) were analyzed by ^{31}P NMR following silylation, as shown in Figure 1. The quantitative analysis of isotopic enrichments and diastereomeric purity are described in Stereochemical Analysis, and the results are summarized in Table II.

Scheme IV shows two possible ways of obtaining (R_P)- and (S_P)-[^{16}O , ^{17}O , ^{18}O]DPPA (**2a** and **2b**, respectively). One of them is phospholipase D catalyzed hydrolysis of (R_P)- and (S_P)-[^{17}O , ^{18}O]DPPE, which has been shown to proceed with complete retention of configuration (Bruzik & Tsai, 1982, 1984b). Alternatively, **2a** and **2b** can be obtained directly from **7a** and **7b**, respectively, by treating with Me_3SiI followed with 95% MeOH . The yield (68%) was comparable to that of the phospholipase D catalyzed hydrolysis of DPPE, but the overall yield was better since the hydrogenolysis step was omitted. Since no P-O bond is cleaved in this reaction, the isotopes and configuration at phosphorus should be quantitatively retained.

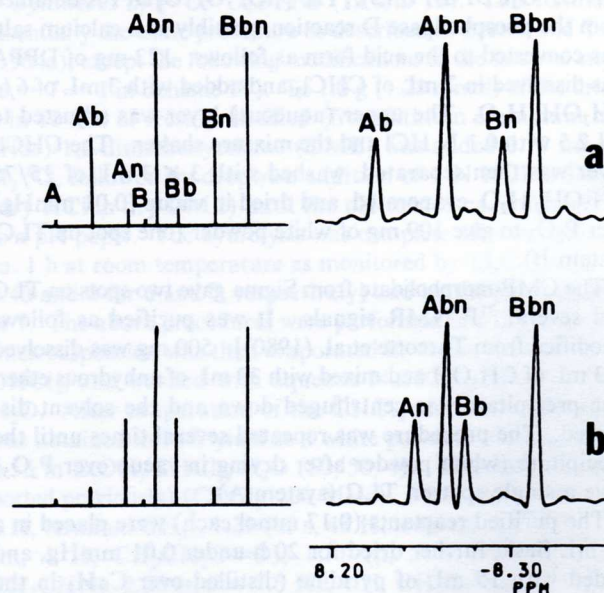
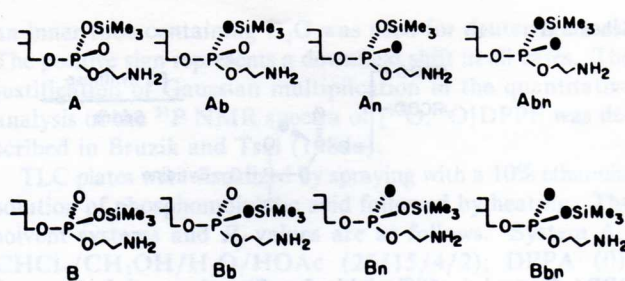


FIGURE 1: ^{31}P NMR analyses of (a) (R_P)-[^{17}O , ^{18}O]DPPE (**1a**) and (b) (S_P)-[^{17}O , ^{18}O]DPPE (**1b**). The calculated intensities are shown on the left. Spectral conditions: spectral frequency, 202.4 MHz; spectral width, 1000 Hz; acquisition time, 8.2 s; 16K data points; line broadening, -0.8; Gaussian broadening, 0.05.

Table II: Summary of Isotopic and Configurational Analyses of Chiral [^{17}O , ^{18}O]DPPE

[^{17}O , ^{18}O]DPPE	1a	1b	[^{17}O , ^{18}O]DPPE	1a	1b
^{17}O position			diastereomeric compn		
atom % ^{16}O	17	17	% R_P isomer	95	5
atom % ^{17}O	47	47	% S_P isomer	5	95
atom % ^{18}O	36	36	purity (%)	45	45
^{18}O position			chirality (%)	90	90
atom % ^{16}O	5	5			
atom % ^{18}O	95	95			

(R_P)- and (S_P)-[^{16}O , ^{17}O , ^{18}O]DPPA (**2a** and **2b**, respectively) obtained from the phospholipase D catalyzed hydrolysis of (R_P)- and (S_P)-[^{17}O , ^{18}O]DPPE, respectively, were then condensed with CMP-morpholidate to give [^{17}O , ^{18}O]CDP-DPG (Moffatt & Khorana, 1961). As illustrated in Scheme III for one of the isomers, **3** is indeed a mixture of three isomers, not assignable to a single configuration.

Enzymatic Conversion of [^{17}O , ^{18}O]CDP-DPG to [^{17}O , ^{18}O]DPPE. The reaction of PS synthase was coupled with that of PS decarboxylase from *E. coli*. The use of PS decarboxylase served two purposes: to convert [^{17}O , ^{18}O]DPPE to [^{17}O , ^{18}O]DPPE, which can be analyzed by ^{31}P NMR, and to drive the reaction of PS synthase to completion. The ^{31}P NMR analysis of [^{17}O , ^{18}O]DPPE obtained from the reaction catalyzed by *E. coli* PS synthase (coupled with PS decarboxylase) is shown in Figure 2.

The PS synthase from yeast requires CDP-DG for stabilization (Bae-Lee & Carman, 1984). Thus, the enzyme solution contained ca. 1.5 mM of unlabeled CDP-DG derived

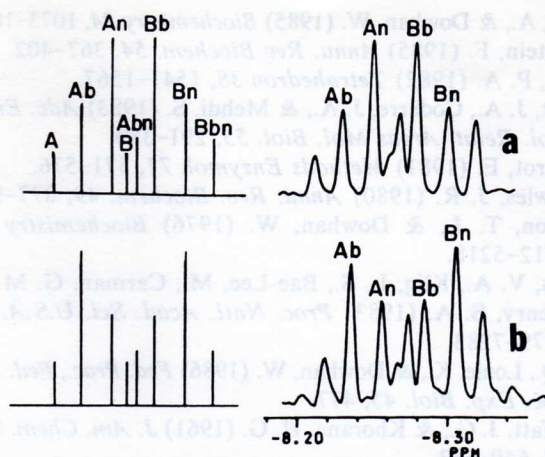


FIGURE 2: ^{31}P NMR analyses of $[\text{}^{17}\text{O}, \text{}^{18}\text{O}]$ DPPE from the product of the reactions catalyzed by *E. coli* PS synthase (coupled with PS decarboxylase): (a) from **1a**; (b) from **1b**. Spectral conditions were similar to those of Figure 1. The calculated spectra (left) were obtained with 100% retention of the configuration for PS synthase.

from egg lecithin (Carman & Fischl, 1980). Since the enzyme may be denatured upon removal of CDP-DG, no attempt was made to remove unlabeled CDP-DG. Thus, the product $[\text{}^{17}\text{O}, \text{}^{18}\text{O}]$ DPPE was contaminated with a substantial amount of unlabeled phosphatidylethanolamine derived from egg lecithin. The ^{31}P NMR analysis of $[\text{}^{17}\text{O}, \text{}^{18}\text{O}]$ DPPE obtained from the reaction catalyzed by PS synthase from yeast is shown in Figure 3.

Stereochemical Analysis. The quantitative analysis of the isotopic enrichments and configuration of **1a** and **1b** have been explained in detail previously (Bruzik & Tsai, 1984a). The method involves silylation of one of the two diastereotopic oxygen atoms of the phosphate group to give two sets of species, A and B, as shown in Figure 1. Since all species containing ^{17}O will not give rise to a detectable sharp ^{31}P NMR signal due to the quadrupolar effect of the ^{17}O nucleus (Tsai & Bruzik, 1983), the ^{31}P NMR spectrum consists of eight peaks due to unlabeled species, A and B, and ^{18}O -containing species, Ab, An, Abn, Bb, Bn, and Bbn (b and n designate bridging and nonbridging ^{18}O , respectively). The ^{18}O -induced isotope shifts in ^{31}P NMR has been reviewed by Cohn (1982). It is important to note that in the ^{31}P NMR analysis of (*R_p*)- $[\text{}^{17}\text{O}, \text{}^{18}\text{O}]$ DPPE (Figure 1a) we are observing predominantly (*R_p*)- $[\text{}^{16}\text{O}, \text{}^{18}\text{O}]$ DPPE (Ab and Bn) and $[\text{}^{18}\text{O}_2]$ DPPE (Abn and Bbn), while in that of (*S_p*)- $[\text{}^{17}\text{O}, \text{}^{18}\text{O}]$ DPPE (Figure 1b) the predominant species are (*S_p*)- $[\text{}^{16}\text{O}, \text{}^{18}\text{O}]$ DPPE (An and Bb) and $[\text{}^{18}\text{O}_2]$ DPPE (Abn and Bbn).

As illustrated in Scheme III, the $[\text{}^{17}\text{O}, \text{}^{18}\text{O}]$ DPPE obtained from (*R_p*)- $[\text{}^{17}\text{O}, \text{}^{18}\text{O}]$ DPPE (**1a**) should consist of ca. 33% each of (*R_p*)- $[\text{}^{17}\text{O}, \text{}^{18}\text{O}]$ DPPE (**4a**), (*S_p*)- $[\text{}^{18}\text{O}, \text{}^{16}\text{O}]$ DPPE (**4b**), and (*R_p*)- $[\text{}^{17}\text{O}, \text{}^{16}\text{O}]$ DPPE (**4c**), if the steric course of PS synthase is retention. In the ^{31}P NMR analysis, (*S_p*)- $[\text{}^{18}\text{O}, \text{}^{16}\text{O}]$ DPPE (**4b**) is fully observable, whereas species **4a** and **4c** will each give rise to $1/3 \times 33\%$ of (*R_p*)- $[\text{}^{18}\text{O}, \text{}^{16}\text{O}]$ DPPE (plus ^{17}O -labeled, double ^{18}O -labeled, and unlabeled species). Thus, the ^{31}P NMR analysis will show an apparent inversion if the reaction catalyzed by PS synthase proceeds with retention of configuration. In other words, (*R_p*)- $[\text{}^{17}\text{O}, \text{}^{18}\text{O}]$ DPPE (**1a**), which gives $\text{Ab} > \text{An}$ and $\text{Bn} > \text{Bb}$ in ^{31}P NMR analysis, will result in an opposite pattern in the product ($\text{An} > \text{Ab}$ and $\text{Bb} > \text{Bn}$) in the case of retention. The relative intensities of the peaks, however, will change substantially in the product DPPE, due to loss of 33% of both ^{17}O and ^{18}O isotopes during the reaction catalyzed by the PS synthase, as well as the formation of both *R_p* and *S_p* isomers in the product. The spectra shown

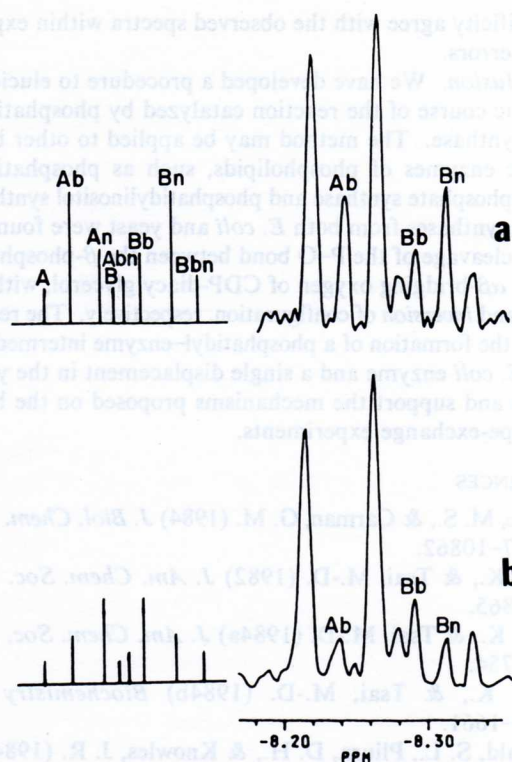


FIGURE 3: ^{31}P NMR analyses of $[\text{}^{17}\text{O}, \text{}^{18}\text{O}]$ DPPE from the product of the reactions catalyzed by yeast PS synthase (coupled with PS decarboxylase): (a) from **1a**; (b) from **1b**. Spectral conditions were similar to those of Figure 1. The calculated spectra (left) were obtained with 100% inversion of configuration for the PS synthase. It should be noted that the peak B in Figure 3 is slightly shifted to the left and overlaps with peak An. This is not unreasonable since peaks A and B in this figure are due predominantly to unlabeled phosphatidylethanolamines derived from egg lecithin originally, as described in Materials and Methods. The difference in the composition of acyl chains could result in the observed shift. As mentioned in the text, peak assignments have been confirmed by mixing samples of a and b with starting $[\text{}^{17}\text{O}, \text{}^{18}\text{O}]$ DPPE.

in Figure 2 (a and b) indicate that the reaction catalyzed by the PS synthase from *E. coli* proceeds with retention of configuration at phosphorus. The spectra in Figure 3, though containing strong peaks due to contaminating unlabeled DPPE, clearly indicate that the steric course of the reaction catalyzed by the PS synthase from yeast is inversion, in contrast to that of the enzyme from *E. coli*. Since this is the first time we analyzed the configuration of $[\text{}^{17}\text{O}, \text{}^{18}\text{O}]$ DPPE obtained after random (yet stereospecific) substitution of an oxygen atom of $[\text{}^{16}\text{O}, \text{}^{17}\text{O}, \text{}^{18}\text{O}]$ DPPA, we confirmed the peak assignments by adding starting $[\text{}^{17}\text{O}, \text{}^{18}\text{O}]$ DPPE to samples in Figures 2 and 3, to ensure all the peaks match (spectra not shown).

The quantitative determination of isotopic enrichments at the ^{17}O and the ^{18}O positions, as well as the diastereomeric purity, of **1a** and **1b** was achieved by varying the three parameters within the proximity of theoretical values to obtain the best fit simulation, as described in Bruzik and Tsai (1984). The calculated spectra are shown on the left side of the observed spectra in Figure 1, and the data are summarized in Table I. From these three parameters, we calculated the "purity", defined as the percentage of the chirally labeled species (i.e., the $M + 3$ species), and the "chirality", defined as the optical purity of the chirally labeled species (Tsai, 1980; Bruzik & Tsai, 1984a). The data for **1a** and **1b** were then used to calculate the spectra of the products, by varying the degree of retention or inversion. The best fit spectra thus obtained are shown on the left hand side of Figures 2 and 3. In all cases the spectra calculated on the basis of 100% ste-

reospecificity agree with the observed spectra within experimental errors.

Conclusion. We have developed a procedure to elucidate the steric course of the reaction catalyzed by phosphatidylserine synthase. The method may be applied to other biosynthetic enzymes of phospholipids, such as phosphatidylglycerolphosphate synthase and phosphatidylinositol synthase. The PS synthases from both *E. coli* and yeast were found to involve cleavage of the P-O bond between the β -phosphorus and the $\alpha\beta$ -bridging oxygen of CDP-diacylglycerol, with *retention* and *inversion* of configuration, respectively. The results suggest the formation of a phosphatidyl-enzyme intermediate in the *E. coli* enzyme and a single displacement in the yeast enzyme and support the mechanisms proposed on the basis of isotope-exchange experiments.

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