Ribosomal-Associated Phosphatidylserine Synthetase from *Escherichia coli:* Purification by Substrate-Specific Elution from Phosphocellulose Using Cytidine 5′-Diphospho-1,2-diacyl-*sn*-glycerol†

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ABSTRACT: Cytidine 5′-diphospho-1,2-diacyl-*sn*-glycerol (CDPdiglyceride):L-serine *O*-phosphatidyltransferase (EC 2.7.8.8, phosphatidylserine synthetase) is bound tightly to the ribosomes in crude extracts of *Escherichia coli*. After separation of the enzyme from the ribosomes by the method of Raetz and Kennedy (Raetz, C. R. H., and Kennedy, E. P. (1974), *J. Biol. Chem. 249,* 5038), we have purified the enzyme to 97% of homogeneity. The major portion of the overall 5500-fold purification was attained by substrate-specific elution from phosphocellulose using CDP-diglyceride in the presence of detergent. The purified enzyme migrated as a single band with an apparent minimum molecular weight of 54 000 when subjected to electrophoresis on polyacrylamide disc gels containing sodium dodecyl sulfate. The purified enzyme catalyzed exchange reactions between cytidine 5′-monophosphate (CMP) and CDP-diglyceride and between serine and phosphatidylserine. The enzyme also catalyzed the hydrolysis of CDP-diglyceride to form CMP and phosphatidic acid. dCDP-diglyceride was equivalent to CDP-diglyceride in all reactions catalyzed by the enzyme. In addition, the purified enzyme catalyzed the formation of phosphatidylglycerol or phosphatidylglycerophosphate at a very slow rate when serine was replaced as substrate by glycerol or *sn*-glycero-3-phosphate, respectively. These results suggest catalysis occurs via a ping-pong mechanism through the formation of a phosphatidyl–enzyme intermediate.

In general, the enzymes utilizing lipid substrates in phospholipid biosynthesis in prokaryotic as well as eukaryotic organisms are tightly associated with a membrane component of the cell (Cronan and Vagelos, 1972; Van den Bosch et al., 1972). The sole exception to this general rule appears to be the CDP-diglyceride[1]:L-serine *O*-phosphatidyltransferase (EC 2.7.8.8, PS synthetase) of *Escherichia coli*. The majority of this enzymatic activity is found associated with the ribosomal fraction of whole-cell lysates with some activity also found in the cytoplasmic fraction (Raetz and Kennedy, 1972; Ishinaga and Kito, 1974). The enzyme can be dissociated from the ribosomal core only under conditions normally employed for dissociation of intrinsic ribosomal proteins (Raetz and Kennedy, 1974). Contrary to the above results, Machtiger and Fox (1973) were not able to assign any distinct subcellular localization to the enzyme, although they did report the majority of the enzyme was not associated with the membrane fraction. At present, the in vivo localization of this enzyme has not been established.

The PS synthetase is an important enzyme in the process of membrane biogenesis, since it catalyzes the first committed step in the synthesis of 3-*sn*-phosphatidylethanolamine, the major phospholipid of *E. coli*. The apparent non-membrane and possible ribosomal localization of this enzyme may play some role in the coordination of phospholipid metabolism with the synthesis of other macromolecules, such as proteins (Raetz and Kennedy, 1972). In order to facilitate studies related to the physical, chemical, and enzymological properties of the PS synthetase, we report, in this communication, the purification of the enzyme to near homogeneity by a method dependent on substrate affinity. Results from such a characterization of the enzyme should shed light on the in vivo localization of the enzyme, as well as the coordination of its control with other cellular processes. The minimum molecular weight of the enzyme is reported. Several catalytic properties of the enzyme were investigated, including exchange reactions, hydrolytic activities, dependence on detergent for activity, and alternate phosphatidyl acceptors. A preliminary report of some of these results has appeared elsewhere (Larson and Dowhan, 1976).

Materials and Methods

Reagents. All chemicals were reagent grade or better. Radiochemicals were purchased from Amersham/Searle. Ovalbumin, α-chymotrypsinogen A (bovine pancreas), dextran (average mol wt = 500 000), L-seryl-L-seryl-L-serine, L-serylglycine, and *N*-carbamyl-DL-serine, were purchased from Sigma. Bovine serum albumin was obtained from Miles Laboratories, phosphorylase *a* (rabbit muscle) from Worthington Biochemicals, L-serine from Calbiochem, and polyethylene glycol (Carbowax, PEG 6000) from Fisher Scientific. Phosphocellulose (P11) was purchased from Whatman and was washed with acid and base before use (Kurland et al., 1971). DEAE-Sephadex (A-50) was a product of Pharmacia. Sodium dodecyl sulfate (99% dodecyl) was purchased from Bio-Rad. Triton X-100 was obtained from Rohm and Haas; solutions containing Triton X-100 are expressed on a weight per volume basis. PS (bovine brain) was a product of Serdary. Phosphatidylglycerol and PGP standards were synthesized as described previously (Larson et al., 1976). *E. coli* B (¾ log), grown on rich medium, was purchased as the frozen cell paste from Grain Processing of 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; phosphatidylglycerol, 3-*sn*-phosphatidyl-1′-glycerol; PS, 3-*sn*-phosphatidyl-L-serine; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid.

Analytical Methods. Protein was determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard.

Preparation of Substrates. CDP-diglyceride and dCDP-diglyceride were prepared as described earlier (Larson et al., 1976). CDP-diglyceride and dCDP-diglyceride with tritium in the cytosine moiety were prepared enzymatically as described by Raetz and Kennedy (1974). *sn*-[2-^{3}H]Glycero-3-phosphate was prepared as described by Chang and Kennedy (1967). 3-*sn*-Phosphatidyl-L-[1-^{14}C]serine was prepared enzymatically by the procedure described by Kanfer and Kennedy (1964).

Enzyme Assays. All enzymatic assays were carried out at 30 °C. PS synthetase activity was determined in a 12-ml polypropylene tube by a modification of the method of Raetz and Kennedy (1972). The assay mixture (0.06 ml) contained 0.67 mM CDP-diglyceride, 0.5 mM L-[3-^{3}H]serine (200 cpm/nmol), 1 mg/ml of bovine serum albumin, and enzyme in the standard assay buffer (0.1 M potassium phosphate, pH 7.4, and 0.1% Triton X-100). The reaction was stopped after 10 min by the addition of 0.5 ml of methanol (0.1 N in HCl), followed by the addition of $CHCl_3$ (1.5 ml) and 1 M $MgCl_2$ (3 ml). After centrifugation, 1.0 ml of the chloroform phase was removed, evaporated to dryness, and counted for radioactivity. One unit of PS synthetase activity is defined as the amount of enzyme required to convert 1 nmol of L-serine to chloroform-soluble product in 1 min under the above conditions. CDP-diglyceride hydrolase activity was determined as described earlier (Raetz et al., 1972) using 0.4 mM [5-^{3}H]CDP-diglyceride (445 cpm/nmol) as substrate. Radioactivity released into the aqueous phase was measured. dCDP-diglyceride hydrolase activity was determined in an analogous manner using [5-^{3}H]dCDP-diglyceride. Serine–PS exchange was determined in the standard assay buffer, described above, using 0.15 mM 3-*sn*-phosphatidyl-L-[1-^{14}C]-serine (380 cpm/nmol) and 0.5 mM L-serine as substrates (Raetz and Kennedy, 1974). The release of radioactive serine into the aqueous phase was measured. CMP–CDP-diglyceride exchange was determined in the standard assay buffer using 0.33 mM of both [5-^{3}H]CMP (310 cpm/nmol) and CDP-diglyceride (Raetz and Kennedy, 1974). The incorporation of tritium into the chloroform phase was measured.

Polyacrylamide Disc Gel Electrophoresis. Electrophoresis in the presence of sodium dodecyl sulfate was carried out as described in the preceding paper of this issue (Hirabayashi et al., 1976), except the gel system described by Laemmli (1970) was used; the ratio of acrylamide to *N,N'*-methylenebisacrylamide was 40:1 in all gels.

Results

Purification of PS Synthetase. Steps 1 through 5 of Table I are, essentially, a scaled-up version of the method described by Raetz and Kennedy (1974) for the separation of the enzyme from nucleic acid. All procedures were carried out between 0 and 4 °C, except for the two phosphocellulose chromatography steps (steps 6 and 7), which were done at room temperature.

Frozen *E. coli* B cell paste (450 g) was suspended with the aid of a Waring blender in 1500 ml of 10 mM potassium phosphate buffer (pH 7.4), containing 10 mM 2-mercaptoethanol and 2 mM Na_2EDTA. The cells were broken by passing the suspension through a French pressure cell. The volume was adjusted to 3500 ml using the above buffer, and the cell supernatant fraction (step 2) was obtained by centrifugation at 13 500*g* for 2 h. The pellet was discarded and

TABLE I: Purification of PS Synthetase.[a]

Step	Total Vol (ml)	Total Protein (mg)	Sp Act. (Units/mg)	Yield (%)
(1) Broken cells	3 500	73 500	6.0	100
(2) Cell supernatant	3 200	51 200	7.5	88
(3) Streptomycin sulfate precipitation	1 220	15 900	18	64
(4) Polymer partitioning	1 980	12 500	20	58
(5) Ammonium sulfate	465	3 900	39	35
(6) Phosphocellulose I	125			26
(7) Phosphocellulose II	60			17
(8) DEAE-Sephadex	1.3	1.9	33 000	14

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

1 l. of 5% streptomycin sulfate–25% Triton X-100 (both w/v) was added to the above cell supernatant with rapid stirring. After holding the mixture on ice for 1 h, the precipitate was collected by a 20-min centrifugation at 10 000*g*. The pellet was dissolved by homogenization in a final volume of 1220 ml (step 3) of 20 mM potassium phosphate (pH 7.4), 10 mM 2-mercaptoethanol, 2 mM Na_2EDTA, and 5 M NaCl.

In order to separate the enzyme from the bulk of nucleic acid present after step 3, phase partition between aqueous phases of polyethylene glycol and dextran was carried out. Dextran (200 ml of 20%, w/w) and polyethylene glycol (400 ml of 30%, w/w) were added, with stirring, to the above solution. After stirring for 30 min, the phases were separated by a 10-min centrifugation at 10 000*g*. The upper polyethylene glycol rich phase was decanted and the remaining dextran-rich phase was washed with 400 ml of fresh polyethylene glycol phase, previously equilibrated with dextran and the above buffer containing 5 M NaCl. After centrifugation, this upper polyethylene glycol phase was combined with the first upper phase (step 4).

The PS synthetase was precipitated from the polyethylene glycol phase by slowly adding solid ammonium sulfate to saturation (19 g/100 ml). The resulting precipitate was collected at the interface between the polyethylene glycol phase and the aqueous-salt phase by a 20-min centrifugation at 10 000*g*. The ammonium sulfate precipitate was dissolved in 400 ml (final volume) of 0.2 M potassium phosphate (pH 7.4), 10 mM 2-mercaptoethanol, and 10% glycerol. A clear supernatant was obtained after a 20-min centrifugation at 10 000*g*. The resulting pellet was reextracted with 70 ml of the above 0.2 M potassium phosphate buffer, and the resulting supernatant combined with the first supernatant (step 5).

The supernatant from step 5 was diluted threefold with 0.1 M NaCl, 0.5% Triton X-100, and 10% glycerol and then applied at 500 ml/h to a column (5 × 5 cm) of phosphocellulose, previously equilibrated with 50 mM potassium phosphate, pH 7.4, 0.1% Triton X-100, and 10% glycerol. The column was washed (500 ml/h) first with 600 ml of buffer A (0.1 M potassium phosphate, pH 7.4, 1% Triton X-100, 0.5 mM dithiothreitol, and 10% glycerol) containing 0.65 M NaCl, and then with 200 ml of buffer B (identical with buffer A, but with only 0.1% Triton X-100 and containing 0.5 M NaCl). The enzyme was finally eluted at 200 ml/h with 180 ml of buffer B, containing 0.4 mM CDP-diglyceride.

In order to purify the PS synthetase further, the phosphocellulose chromatography, described above, was repeated on a smaller scale. The fractions containing enzymatic activity,

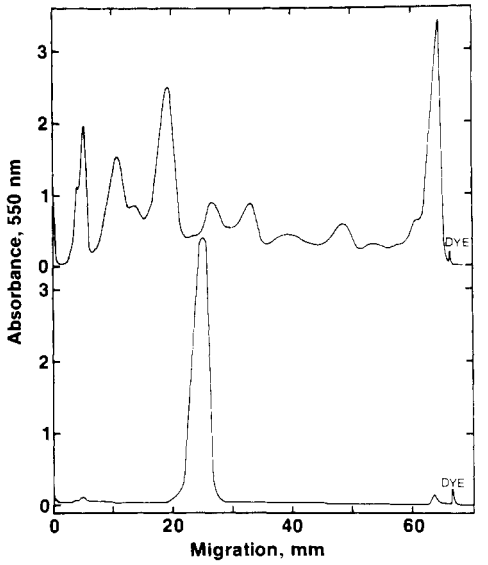

FIGURE 1: Sodium dodecyl sulfate polyacrylamide disc gel electrophoresis of PS synthetase. Upper scan: PS synthetase (140 μg of protein) after step 5, Table I. Lower scan: PS synthetase (24 μg of protein) after step 8, Table I. Samples were prepared for sodium dodecyl sulfate disc gel electrophoresis and electrophoresed in 10% acrylamide disc gels. After staining with Coomassie blue, the protein profile was determined by scanning the gels at 550 nm using a Gilford recording spectrophotometer equipped with a linear transporter and a No. 2412 cuvette.

which had been eluted from the first phosphocellulose column, were pooled (step 6), diluted threefold with 0.1% Triton X-100 and 10% glycerol, and then applied to a second phosphocellulose column (2.7 × 7 cm); the dilution allowed absorption by lowering the ionic strength and diluting any remaining unhydrolyzed CDP-diglyceride (see below). Less than 10% of the enzymatic activity applied to either the first or second phosphocellulose column was recovered in the flow-through and wash fractions. This column was washed (130 ml/h) first with 50 ml of buffer A, then with 150 ml of buffer A, containing 0.65 M NaCl, and, finally, with 150 ml of buffer B. After the above washes, the enzyme was eluted (60 ml/h) with 100 ml of buffer B, containing 0.4 mM CDP-diglyceride. The fractions containing enzymatic activity were pooled (step 7) and dialyzed overnight against 240 ml of buffer C (20 mM sodium phosphate, pH 7.0, 0.5 mM dithiothreitol), containing 0.2% Triton X-100. The dialyzed preparation was applied at 25 ml/h to a column of DEAE-Sephadex (1.5 × 6 cm), which had been previously equilibrated with buffer C containing 0.1% Triton X-100. The column was washed with 40 ml of buffer C, containing 0.2% Triton X-100 and 0.1 M NaCl, after which the enzyme was eluted (20 ml/h) with buffer C, containing 0.1% Triton X-100 and 1 M NaCl. The DEAE-Sephadex column not only concentrated the PS synthetase, but also removed the bulk of the phospholipid which was present after elution of the enzyme from phosphocellulose.

The procedure outlined above yielded a preparation of PS synthetase which had been purified 5500-fold over crude extracts of *E. coli* with a 14% yield. The results of the scaled-up procedure through step 5 are comparable to those reported by Raetz and Kennedy (1974). The major purification was attained in step 6. Step 7 removed only the remaining minor contaminants.

Enzyme Purity. Due to the tendency of the enzyme to aggregate in buffers of low ionic strength (see below), it was not possible to use native disc gel electrophoresis to assess the purity of the preparation. Electrophoresis, in the presence of sodium dodecyl sulfate, indicated a highly purified preparation with the major protein peak accounting for 97% of the total absorption profile (Figure 1); the small peak near the dye marker was variable from preparation to preparation and may be due to small amounts of phospholipids, which are known to stain positively with Coomassie blue (Carraway et al., 1972). Electrophoretic analysis of the ammonium sulfate fraction showed no significant band corresponding to the major band found in the purified preparation (Figure 1).

Further evidence indicating the major band in Figure 1 to be the PS synthetase comes from subjecting a portion of the sample from step 6 (Table I) to DEAE-Sephadex chromatography, using a linear gradient (0.1–1.0 M NaCl) for elution rather than step elution. Sodium dodecyl sulfate disc gel electrophoresis of samples taken across the peak of eluted enzymatic activity showed a proportional relationship between the number of enzyme units loaded on each gel and the total Coomassie blue absorbance of the major band. Several minor bands, which were subsequently removed by step 7 (Table I), appeared at various positions in the elution profile but exhibited no correlation with the amount of enzymatic activity applied to the gels. Finally, the method of elution of the enzyme from phosphocellulose should be selective for enzymes specific for CDP-diglyceride. No contaminating activities were found (see below) in the purified enzyme which could be attributed to other known CDP-diglyceride dependent enzymes of *E. coli*.

Enzyme Stability. After separation of the enzyme from nucleic acid, high ionic strength buffers were necessary to prevent irreversible precipitation and inactivation of the enzyme, as noted by Raetz and Kennedy (1974). The enzyme was also unstable in dilute protein solutions and may have a tendency to adsorb to glass; assays were generally carried out in polypropylene tubes in the presence of added bovine serum albumin. The purified enzyme only lost 25% of its activity upon storage for 1 month at 4 °C in the 1 M NaCl buffer used in step 8 of Table I. When glycerol (10%) was added to the purified enzyme, it could be stored at −20 °C for at least 1 month with no loss in activity.

Molecular Weight and Number of Molecules per Cell. Several difficulties were encountered in the determination of the "native" molecular weight of the enzyme. Polyacrylamide disc gel electrophoresis under nondenaturing conditions (Davis, 1964) was unsuccessful because of the tendency of the enzyme to precipitate from concentrated solutions at low ionic strength. Estimates of the molecular weight using Sephadex or Sepharose gel filtration were hampered by very low recovery of both enzymatic activity and protein. An additional complication, which may be encountered in determining the molecular weight of the enzyme, is the possible affinity of the enzyme for detergent micelles (see section on kinetic properties). Such binding will have to be quantitated before an accurate molecular weight can be determined for the enzyme (Tanford et al., 1974).

The minimum subunit molecular weight in sodium dodecyl sulfate was determined using polyacrylamide disc gel electrophoresis. The fully reduced and denatured enzyme showed a linear relationship between polyacrylamide concentration (7.5–12.5%) and relative mobility (Ferguson, 1964), which extrapolated to the same relative free mobility at 0% gel concentration as for the standard marker proteins (chymotrypsinogen, egg ovalbumin, bovine serum albumin, and phosphorylase *a*). Hence, this method should be reliable for determining the minimum molecular weight of the PS synthetase (see preceding paper in this issue for discussion, Hirabayashi

TABLE II: Levels of Phospholipid Biosynthetic Enzymes in *E. coli*.[a]

Enzyme	Turnover No. (37 °C)[b]	Molecules/ Cell	Molecules of Lipid Synthesized/ 30 min
PS synthetase	3000[e]	800	7.2×10^7
PS decarboxylase[c]	2800	1800	15×10^7
PGP synthetase[d]	910[e]	1400	3.8×10^7

[a] Based on 3.6 genomes/cell, and 60×10^7 amino acids in protein/genome (Kjeldgaard and Gausing, 1974). [b] Moles of product formed per min per mole of enzyme. [c] Data taken from Dowhan et al. (1974) and corrected for 95% purity. [d] Data for PGP synthetase taken from Hirabayashi et al. (1976), and corrected for 85% purity. [e] Turnover number at 30 °C was multiplied by 1.7.

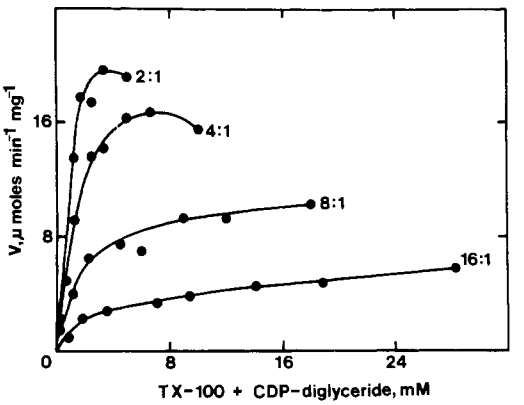

FIGURE 2: Dependence of PS synthetase on the sum of the CDP-diglyceride and Triton X-100 concentrations. Points represent averages of duplicate assays carried out at 30 °C in 0.1 M potassium phosphate buffer (pH 7.4) at the indicated ratios of Triton X-100 to CDP-diglyceride in the presence of 0.67 mM L-[3-^{3}H]serine (200 cpm/nmol) and 0.05 μg of purified enzyme. (Specific activity was 50% of freshly purified enzyme due to loss of activity during storage.)

et al., 1976). The average value for the minimum subunit molecular weight determined at three different gel concentrations was 54 000 ± 2000.

The number of subunits of the PS synthetase per cell, as well as the turnover number of the enzyme, can be calculated using the minimum molecular weight, the specific activity of the purified enzyme, and the overall purification factor. Assuming no change in the turnover number of the enzyme during purification, these two numbers can be used to estimate the number of PS molecules which could be synthesized per cell per generation time (about 30 min for *E. coli* B). This data is summarized in Table II and compared to similar data available for two other enzymes of phospholipid metabolism. Although strong arguments about in vivo activity based on in vitro assay conditions are difficult to make, it is significant that these three enzymes appear to be present in cells at about the same functional level.

Kinetic Properties. Figure 2 shows the dependence of the enzyme on detergent and its phospholipid substrate, CDP-diglyceride, for activity. Substrate saturation curves were determined at increasing ratios of detergent (Triton X-100) to CDP-diglyceride, where the substrate concentration was expressed as the sum of the CDP-diglyceride and Triton X-100 concentrations. Velocity at saturating substrate concentration was maximal at a Triton X-100 to liponucleotide ratio of 2:1; at higher ratios the maximum velocity was lower. Below a molar ratio of 2:1, the activity of the enzyme fell off rapidly to about 5% of maximal activity (standard assay conditions) at 0.017% Triton X-100. This data would indicate Triton X-100 must be in at least a twofold molar excess over liponucleotide in order for a uniform mixed micellar substrate population to be present. Higher ratios have little effect on the type of substrate saturation curve exhibited but do affect the maximum velocity reached. This observation is consistent with a mechanism in which free enzyme and enzyme bound to the mixed micelle are at equilibrium in the steady state; once binding of enzyme to the micelle occurs, the maximum velocity of the reaction would be dependent on the concentration (mole fraction) of the liponucleotide in the mixed micelle with Triton X-100.

Such a model has been proposed for the action of phospholipase A_2 from *Naja naja naja* (Dennis, 1973; Deems et al., 1975) and phosphatidylserine decarboxylase from *E. coli* (Warner and Dennis, 1975) toward their respective phospholipid substrates in mixed micelles with Triton X-100. Depending on the phospholipid substrate, ratios of Triton X-100 to lipid from 2:1 to 6:1 were required for maximum activity. In the case of the phospholipase, the ratio required for maximum activity correlated well with physical studies, which demonstrated the ratio necessary to form uniform mixed micelles (Dennis and Owens, 1973). Higher ratios of Triton X-100 to substrate caused an apparent dilution of lipid substrate in the mixed micelle with a parallel reduction in maximum velocity. In the case of the above two enzymes, sufficient data is available concerning the physical state of the mixed micellar substrates to allow quantitative calculation of the interaction constants for the enzyme with the micellar surface and with its substrate. Since such physical data is unavailable for mixed micelles of CDP-diglyceride and Triton X-100, we were only able to qualitatively fit our data to such a model. The PS synthetase also has the additional complication of catalyzing a two substrate reaction with one substrate being water soluble and, therefore, not present in the mixed micelle.

When the activity of the purified PS synthetase was studied as a function of CDP-diglyceride or dCDP-diglyceride concentration, the results shown in Figure 3 were obtained. The dependence of enzymatic activity on substrate concentration was indistinguishable for the two liponucleotides within the accuracy limitations of the assays. This is in contrast to the findings of Raetz and Kennedy (1973) in which the dependence of activity on concentration differed for the two liponucleotides; however, their studies were done with crude preparations in which the enzyme was associated with the ribosomal fraction. This may account for the observed differences.

The saturation curves for these two liponucleotides appear sigmoidal and reciprocal plots of the data are hyperbolic. These results are most easily explained by the inhibitory effect of high ratios of Triton X-100 to liponucleotide discussed above. In Figure 3, this ratio is highest at the lower liponucleotide concentrations.

Reaction Mechanism. The association of various exchange reactions and hydrolytic activities with the purified PS synthetase is consistent with a ping-pong reaction mechanism for the enzyme (Cleland, 1970) with the formation of a phosphatidyl-enzyme intermediate during the course of the enzymatic reaction (Raetz and Kennedy, 1974). Raetz and Kennedy (1974) found the partially purified enzyme catalyzed exchange reactions between CMP and CDP-diglyceride and between serine and PS. In addition, the enzyme catalyzed the

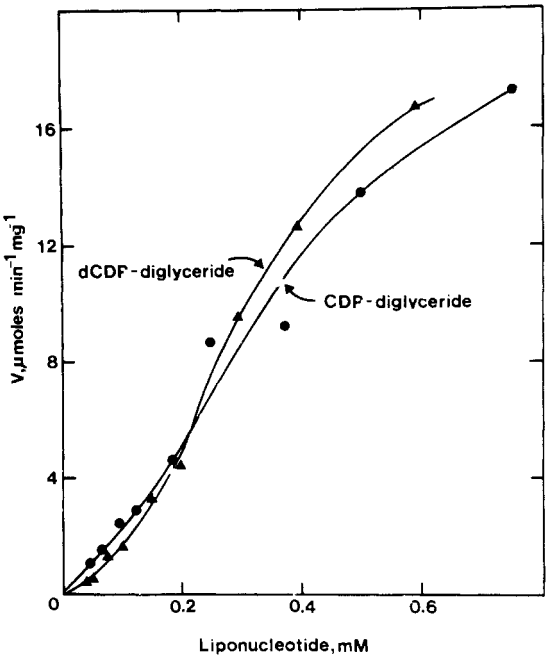

FIGURE 3: Dependence of PS synthetase on liponucleotide concentration at constant Triton X-100 concentration. Points represent averages of duplicate assays which were carried out in 0.1 M potassium phosphate buffer (pH 7.4), containing 0.67 mM L-[3-^{3}H]serine (990 cpm/nmole), 0.1% Triton X-100, and 0.02 μg of purified PS synthetase (same enzyme as in Figure 3) per assay. Liponucleotide concentrations were determined by enzymatic assay with purified PGP synthetase (Hirabayashi et al., 1976).

hydrolysis of both CDP-diglyceride and PS. After an additional 40-fold purification over that obtained by Raetz and Kennedy (1974), these activities were still present (Table III) at about the same levels originally reported; the reduced rate of PS hydrolysis reported here was most likely due to the threefold lower PS concentration used in our experiments. The purified enzyme was not only capable of hydrolyzing CDP-diglyceride to phosphatidic acid and CMP but was able to carry out an analogous reaction with dCDP-diglyceride at an equal rate. This fact, along with the observation that AMP had no inhibitory effect on either hydrolytic activity, demonstrated this activity was not due to the presence of the membrane-associated CDP-diglyceride specific hydrolase (Raetz et al., 1972,1976). The purified enzyme also catalyzed an exchange reaction between dCMP and dCDP-diglyceride (data not shown).

The purified enzyme was also capable of transferring the phosphatidyl moiety to both glycerol and glycero-3-phosphate. The phospholipid composition of a sample from step 7 (Table I) was determined after standing at 4 °C for 24 h by partitioning the lipid components into chloroform. The chloroform-soluble material was chromatographed in chloroform–methanol–glacial acetic acid–water (50:30:4:8) on silica gel thin-layer plates. Only small amounts of CDP-diglyceride (R_f 0.4) were found, since a large amount of the liponucleotide had been converted to phosphatidic acid by the inherent CDP-diglyceride hydrolase activity. In addition to phosphatidic acid (R_f 0.9), however, a significant amount of phosphate-positive material chromatographed as phosphatidylglycerol (R_f 0.55). Mild alkaline hydrolysis (Kates, 1972) of the chloroform-soluble material from the sample yielded mainly glycero-3-phosphate and glycerophosphorylglycerol, which are the expected deacylation products of phosphatidic acid and phosphatidylglycerol, respectively; the deacylation products were identified by comparison with authentic standards using ascending chromatography on Whatman no. 1 paper in the solvent system 1 M ammonium acetate, pH 7.5–ethanol (35:65). The PS synthetase apparently catalyzed the formation of phosphatidylglycerol from CDP-diglyceride and glycerol (1 M), which were present in the elution buffer. The effectiveness of glycerol as a phosphatidyl acceptor was found to be extremely poor. No phosphatidylglycerol was formed when [2-^{3}H]glycerol (1.7 mM, 1100 cpm/nmol) was substituted for serine using the standard assay buffer, 0.67 mM CDP-diglyceride, a 30-min incubation and 2.2 μg of purified enzyme. On the other hand, when *sn*-[2-^{3}H]glycero-3-phosphate (0.8 mM, 2400 cpm/nmol) was incubated for 30 min in the place of serine in the standard assay buffer, containing 4.2 μg of purified enzyme and 0.67 mM CDP-diglyceride, a small amount of tritium was incorporated into chloroform-soluble material, which cochromatographed with standard PGP on silica gel thin-layer plates in chloroform–methanol–glacial acetic acid–water (50:30:4:8). The observed PGP synthetase activity was not due to contaminating levels of the membrane-bound PGP synthetase, since no formation of PGP occurred when the assay conditions for this enzyme were used (Hirabayashi et al., 1976).

TABLE III: Reactions Catalyzed by PS Synthetase.[a]

Reaction	Additions	Ratio of PS Synthetase: Indicated Reaction
CDP-diglyceride:CMP exchange		22
PS:Serine exchange[b]		24
CDP-diglyceride hydrolase[c]		103
CDP-diglyceride hydrolase	1.67 mM AMP	109
dCDP-diglyceride hydrolase[c]		98
dCDP-diglyceride hydrolase	1.67 mM AMP	112

[a] Conditions are described under Materials and Methods. [b] Rate of PS hydrolysis in the absence of serine was less than 10% of exchange rate. [c] Phosphatidic acid identified by silica gel thin-layer chromatography. Cytosine nucleotide monophosphate identified by paper chromatography.

Several other possible phosphatidyl acceptors were found not to be effective substrates (mM range) for the enzyme under standard assay conditions (30 min using 3.2 μg of purified enzyme). Among these were radiolabeled choline, ethanolamine, and inositol. These acceptors might be phosphatidyl acceptors if tested in the molar range, as was the case with glycerol. Finally, L-seryl-L-seryl-L-serine, L-serylglycine and *N*-carbamyl-DL-serine were not inhibitors of the enzyme at a 30-fold molar excess over serine under standard assay conditions; therefore, both the carboxyl and amino functions of serine are involved in recognition of the substrate.

Discussion

The utilization of substrate-specific elution from phosphocellulose for the purification of the PS synthetase complements the use of CDP-diglyceride Sepharose affinity chromatography for the purification of PGP synthetase from *E. coli* (Hirabayashi et al., 1976). In each case, large purifications were effected by the utilization of enzyme specificity. Both affinity procedures are compatible with and, in fact, require the presence of nonionic detergent. Substrate elution from an ion-exchange resin affords an alternative purification method for CDP-diglyceride dependent enzymes, such as the PS synthe-

tase, which have no affinity for CDP-diglyceride Sepharose (data not shown).

The in vivo localization of the PS synthetase has not, as yet, been settled. The isolation of point mutations, which are temperature sensitive for both growth and PS synthetase activity (Raetz, 1976; Ohta et al., 1975), strongly suggests the presence of only one biosynthetic PS synthetase in *E. coli*. These mutants also suggest the minor non-ribosomal, cytoplasmic activity (Raetz and Kennedy, 1972; Ishinaga and Kito, 1974) and the major ribosomal-associated activity are due to the same enzyme. Since the purified enzyme has a high affinity for polyphosphates, this cytoplasmic activity may be due to enzyme bound to RNA or fragmented ribosomes.

The PS synthetase is, however, not a major intrinsic ribosomal protein (Traub et al., 1971), even though it exhibits many of the properties of this group of proteins (Raetz and Kennedy, 1974). Assuming two enzyme binding sites per 70S ribosome (1/subunit), there are about 150 000 such sites per cell grown on rich medium (Kjeldgaard and Gausing, 1974); there are only about 800 PS synthetase subunits per cell (Table II). In addition, none of the major intrinsic ribosomal proteins have subunit molecular weights in sodium dodecyl sulfate near 54 000 (Wittmann, 1974). Until ribosome saturation studies and binding specificity studies are carried out, the possibility the enzyme is associated with some subclass of ribosomes involved, for instance, in membrane protein synthesis, cannot be ruled out; however, it is apparent the enzyme is neither a major intrinsic ribosomal protein nor an integral membrane protein.

Although the level of PS synthetase in cells is low, this level, along with that of PS decarboxylase and PGP synthetase, is in the right order of magnitude to account for the amount of phospholipid synthesis required for cell growth (Table II). Phospholipid makes up about 5% of the dry weight of *E. coli* (Ames, 1968, and Cronan, 1968), which represents about 2×10^7 molecules of phospholipid per cell; about 70–80% of this phospholipid is phosphatidylethanolamine with the remainder being phosphatidylglycerol and cardiolipin (Cronan and Vagelos, 1972). These enzymes (Table II), functioning near half-maximal activity, would be capable of making sufficient phospholipid to account for the doubling of the cell membrane during the generation time of *E. coli* B (30 min at 37 °C).

The PS synthetase and PGP synthetase (Hirabayashi et al., 1976) differ widely in physical properties, intracellular distribution, reaction mechanism, and dependence on Triton X-100 for activity, yet these two enzymes both catalyze an almost identical phosphatidyl transfer between CMP and an alcohol. The differences in physical properties are responsible for the difference in intracellular localization, which, in turn, may be important for the control of phospholipid metabolism and coordination of phospholipid metabolism with the synthesis of other macromolecules. The differences in reaction mechanism and dependence on Triton X-100 may be necessary, due to the apparent difference in intracellular distribution of these two enzymes. The liponucleotide pool of *E. coli* is less than 0.05% of the total phospholipid (Raetz and Kennedy, 1973); therefore, there are fewer than five liponucleotide molecules in the membrane per PS and PGP synthetase molecule in the cell. The apparent affinity of the PS synthetase for an interface coupled with the possible formation of a covalent phosphatidyl–enzyme intermediate may be a means of holding the enzyme on the membrane surface to facilitate catalysis; such a peripheral membrane association does not rule out interaction with ribosomes at a second site on the enzyme. The PGP synthetase, on the other hand, is embedded in the cytoplasmic membrane along with the liponucleotide where their motion is restricted to only two dimensions. This would result in a large enhancement in catalytic efficiency. The sequential ordered reaction mechanism would prevent unnecessary hydrolysis of the liponucleotide and prevent the formation of a covalent phosphatidyl–enzyme intermediate in the absence of glycerophosphate. The importance of these differences in properties to enzyme function and control of phospholipid metabolism will become clearer as more detailed studies are carried out on these two enzymes.

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Molecular Control of Membrane Properties During Temperature Acclimation. Fatty Acid Desaturase Regulation of Membrane Fluidity in Acclimating *Tetrahymena* Cells†

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ABSTRACT: This is a study of the molecular mechanisms employed by *Tetrahymena pyriformis* to change the lipid composition and thereby the fluidity of its various membranes during temperature acclimation. By quantitatively measuring the intramembrane particle aggregation using freeze-fracture electron microscopy, membrane physical properties in 39.5 °C grown cells shifted to 15 °C were found to be correlated with the degree of phospholipid fatty acid desaturation. Alteration of the phospholipid polar head group distribution from that of 39.5 °C-grown cells to the significantly different pattern of 15 °C grown cells appeared not to be of critical importance in the acclimation process. Changes in fatty acid desaturation during acclimation from high to low temperatures and vice versa were analyzed using normal cells and cells fed large amounts of polyunsaturated fatty acids. Fatty acid desaturase activity corresponded to the degree of membrane fluidity but not to the cell temperature. All evidence was compatible with the hypothesis that membrane fluidity is self-regulating, with the action of fatty acid desaturases being modulated by the physical state of their membrane environment.

The matrix of biological membranes appears to exist as a bilayer of mobile lipids, the relative motion of which determines the fluidity or viscosity of the membrane interior (Singer and Nicolson, 1972; also see review by Singer, 1974). The fluidity of the membrane, primarily determined by its lipid composition, is apparently of considerable importance to the organism in that it affects the activity of membrane-bound enzymes (Esfahani et al., 1971; Mavis and Vagelos, 1972; Kimelberg and Papahadjopoulos, 1972), membrane transport (Shechter et al., 1974; Thilo and Overath, 1976; Linden et al., 1973), and the mobility of membrane proteins (Horwitz et al., 1974). These observations indicate that optimal fluidities may exist to maintain the proper function of cell membranes and that cells may therefore have need to regulate their membrane lipid compositions to conform to a given temperature and to other environmental parameters. This would be especially true of poikilothermic organisms, which sometimes encounter wide ranges of growth temperature. The fact that these organisms do undergo changes in membrane lipids has been shown in a number of cases (Fukushima et al., 1976; Wunderlich et al., 1973; Marr and Ingraham, 1962; McElhaney, 1974). Furthermore, Nozawa et al. (1974) have demonstrated by the use of spin-label probes that the lipid changes induced by low temperature do have a fluidizing effect on the membranes of *Tetrahymena*.

In a previous publication, we have described the adaptive ability of *Tetrahymena pyriformis*, strain NT-1, which responds to varying temperatures by altering not only the degree of fatty acid unsaturation in its membrane phospholipids but also the relative proportions of the phospholipid species themselves (Fukushima et al., 1976). Another way to produce rapid and drastic lipid alterations in the membranes of this strain is by feeding large amounts of unsaturated fatty acids to exponentially growing cells. We found it instructive to compare the adaptive process in cells whose membranes had been perturbed by one or both of the above methods, since this would seem to provide an opportunity for discriminating between the roles of temperature per se and membrane fluidity in the regulation of the adaption process.

In this communication, we describe the effects of altered membrane lipid composition on acclimation by cells grown at high temperatures and shifted to low growth temperatures and vice versa. The following paper in this issue (Kasai et al., 1976) describes the adaptive changes which occur isothermally following even greater dietary modification of the membranes.

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