Purification of Phosphatidylethanolamine N-Methyltransferase from Rat Liver*

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Phosphatidylethanolamine (PE) N-methyltransferase catalyzes the synthesis of phosphatidylcholine by the stepwise transfer of methyl groups from S-adenosylmethionine to the amino head group of PE. PE N-methyltransferase was solubilized from a microsomal membrane fraction of rat liver using the nonionic detergent Triton X-100 and purified to apparent homogeneity. Specific activities of PE N-methyltransferase with PE, phosphatidyl-N-monomethylthanolamine (PMME), and phosphatidyl-N,N-dimethylthanolamine (PDME) as substrates were 0.63, 8.59, and 3.75 μmol/min/mg protein, respectively. The purified enzyme was composed of a single subunit with a molecular mass of 18.3 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Methylation activities dependent on the presence of PE, PMME, and PDME and the 18.3-kDa protein co-eluted when purified PE N-methyltransferase was subjected to gel filtration on Sephacryl S-300 in the presence of 0.1% Triton X-100. All three methylation activities eluted with a Stokes radius 2.1 Å greater than that determined for pure Triton micelles (molecular mass difference of 27.4 kDa). Two-dimensional analysis of PE N-methyltransferase employing nonequilibrium pH gradient gel electrophoresis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicates that the enzyme is composed of a single isoform.

Analysis of enzyme activity using PE, PMME, and PDME at various Triton X-100 concentrations indicated the enzyme follows the "surface dilution" model proposed for other enzymes that act at the surface of membrane. Initial velocity data for all three lipid substrates (at fixed concentrations of Triton X-100) were highly cooperative in nature. Hill numbers for PMME and PDME ranged from 3 at 0.5 mM Triton to 6 at 2.0 mM Triton. All three methylation activities had a pH optimum of 10. These results provide evidence that a single membrane-bound enzyme catalyzes all three methylation steps for the conversion of PE to phosphatidylcholine.

Rat hepatic PC† can be synthesized by either the CDP-choline pathway (1) or by methylation of pre-existing PE (2).

Successive transfer of methyl groups from AdoMet to PE results in the synthesis of the mono- and dimethylated intermediates, PMME and PDME, and finally PC. PE N-methyltransferase (EC 2.1.1.17) accounts for 20–30% of PC synthesis in hepatocytes (3) and is localized almost exclusively in a hepatic microsomal fraction (4).

PE N-methyltransferase is an integral membrane protein catalyzing a multistep reaction and as such there are inherent problems in assaying and purifying the enzyme. These problems are exemplified by the many disagreements over the number of enzymes involved in the PE methylation pathway. It has been reported that rat liver microsomes contain a methyltransferase that methylates PE to PMME and a second enzyme that converts PMME to PC (5). Similar conclusions based on kinetic data, pH, and Mg²⁺ dependence have been made for PE N-methyltransferase activity in erythrocyte membranes (6), rat brain synaptosomes (7), and bovine adrenal medulla (8). The theory that two enzymes convert PE to PC, drawn from kinetic data that did not take into account the steady state nature of intermediates in the methylation pathway, may be erroneous (9). In contrast, it has been reported that all three methylation activities in rat liver microsomes (9) have pH optima between 10 and 10.5 and similar Kₘ values for AdoMet. Data on partially pure PE N-methyltransferase also suggested the methylation of PE was catalyzed by a single enzyme (10, 11).

A lack of consensus on the nature of the enzyme(s) for methylation of PE can be reconciled on the basis of tissue differences and interpretation of kinetic data. True characterization, however, awaited purification of the enzyme. Previous attempts to purify Triton X-100-solubilized PE N-methyltransferase from mouse (12) and rat (10) liver were partially successful but met with a problem common to membrane enzyme purification: enzyme instability in detergents. PE N-methyltransferase has been purified from rat liver to a final specific activity of 0.27 μmol/min/mg protein (13) and reported to consist of a 25-kDa monomer and 50-kDa dimer. The catalytic subunit of CAMP-dependent protein kinase (14) and protein kinase C (15) phosphorylate the 50-kDa protein with a resultant increase in PE N-methyltransferase activity. The 24-kDa protein was photolabeled with 8-azido AdoMet (11). We reported (in abstract form) the purification of PE N-methyltransferase to a similar specific activity reported by Paijares et al. (13) and the presence in that preparation of a 50-kDa protein (16). However, we were concerned that the final specific activity was too low for a homogenous prepara-

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†The abbreviations used are: PC, phosphatidylcholine; PMME, phosphatidyl-N-monomethylthanolamine; PDME, phosphatidyl-

N,N-dimethylethanolamine; PE, phosphatidylethanolamine; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NEPHGE, nonequilibrium pH gradient gel electrophoresis; AdoMet, S-adenosyl-t-methionine.

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tion of PE N-methyltransferase. The relative abundance of the 50-kDa protein in crude soluble microsomes also suggested that this was not the methyltransferase.

In this paper we report the copurification of PE-, PMME-, and PDME-dependent methylation activities from Triton X-100-solubilized microsomes to final specific activities of 0.63, 8.59, and 3.75 pmol/min/mg protein, respectively. Purified PE N-methyltransferase was composed of a single protein of 18.3 kDa. PE N-methyltransferase activities were determined with each of the pure lipid substrates in a Triton X-100 mixed micelle assay. Kinetic analysis with regard to lipid substrates indicated an adherence to the "surface dilution" model proposed for enzymes that act on mixed micelle substrates (28, 29).

**EXPERIMENTAL PROCEDURES**

**Materials**

PMME and PDME were from Avanti Polar Lipids. AdoMet was purchased from Boehringer Mannheim, Canada. [methyl-3H]AdoMet was from Amersham Corp. Triton X-100, DT T, and bovine serum albumin were from Sigma. Molecular mass standards for gel filtration and SDS-gel electrophoresis, octyl Sepharose, Sephacyr S-300, and PBE 94 polybuffer exchanger for chromatofocusing were specific purchased from Pharmacia LKB Biotechnology Inc. Preparative (2.0 mm) and analytical (0.2 mm) silica Gel 60 thin layer plates were from Merck. All other materials were of reagent grade.

**Purification of PE N-Methyltransferase**

**Isolation of Microsomes**—Microsomes were isolated from the livers of female Wistar rats (175-225 g) in the following manner. Rats were put to death by cervical dislocation, and the livers were immediately removed and placed in ice-cold 10 mM Tris-HCl (pH 7.2) buffer containing 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT. The liver was cut into small pieces, suspended at a final concentration of 25% (w/v) in the Tris-saline buffer, and homogenized using a motor driven Potter-Elvehjem apparatus. The homogenate was centrifuged at 12,000 X g for 10 min. The supernatant fraction was then subjected to centrifugation at 120,000 X g for 1 h. The cytosol was immediately decanted and the microsomal pellet resuspended in 50 mM morpholine-ethanesulfonic acid buffer (pH 7.9) containing 1 mM EDTA, 1 mM DTT, and 250 mM sucrose using a hand-held glass Dounce homogenizer. Microsomes were also isolated from livers that had been perfused with 150 mM NaCl and 0.25 ml/min. When loading was complete the column was flushed in a stream of nitrogen and further dried under high vacuum for 30 min. Each of the pure lipid substrates in a Triton X-100 mixed micelle assay. Kinetic analysis with regard to lipid substrates indicated an adherence to the "surface dilution" model proposed for enzymes that act on mixed micelle substrates (28, 29).

**Chromatography on Whatman DE52 Cellulose**—Soluble PE N-methyltransferase was passed through a column of DE52 cellulose (30 X 2.5 cm) previously equilibrated in buffer A plus 0.7% Triton X-100 at a flow rate of 1.0 ml/min. PE N-methyltransferase activity was recovered in the unbound fractions.

**Chromatography on Whatman P-11 Phosphocellulose**—A column of Whatman P-11 phosphocellulose (16 X 1.6 cm) was equilibrated in buffer A containing 0.7% (w/v) Triton X-100 and 100 ml containing 0.25% (w/v) Triton X-100. PE N-methyltransferase activity was eluted from the column with a linear gradient of NaCl from 0 to 0.5 M in 0.25% (w/v) Triton X-100. PE N-methyltransferase activity eluted in a broad peak from 0.2 to 0.6 M. We found it necessary to use P-11 phosphocelulose that had been used for several purification attempts (and not otherwise recycled) in order to achieve good recoveries from this as well as from the following steps. The phosphocelulose could also be pretreated with 0.2% (w/v) bovine serum albumin in buffer A containing 0.7% Triton X-100 followed by elution with 2.0 M NaCl in the same buffer. This procedure seemed to block high affinity sites on the resin that would otherwise cause recovery of PE N-methyltransferase and change its chromatographic characteristics on octyl Sepharose.

**Chromatography on Octyl Sepharose CL-4B**—Active fractions from the phosphocellulose column were pooled and diluted with buffer A (no Triton X-100) to a final Triton X-100 concentration of 0.05% (w/v). The enzyme solution was pumped (0.5 ml/min) onto a column of octyl Sepharose (17 X 1.6 cm) equilibrated in buffer A containing 0.05% (w/v) Triton X-100. The column was flushed with 100 ml of buffer A containing 0.05% Triton X-100. It is critical to the final purity of the enzyme that this column be eluted so that the main PE N-methyltransferase peak is well separated from the major protein peak that precedes it (Fig. 1). The best results were obtained by eluting the column with 100 ml of buffer A containing 0.15% (w/v) Triton X-100 followed by 250 ml of a linear gradient of Triton X-100 from 0.15 to 0.5% (w/v) in the same buffer.

**Chromatography on PBE 94**—The final step in the purification takes advantage of the very basic nature of PE N-methyltransferase. The pooled fractions from the previous step, adjusted to pH 9.4 with 250 mM ethanolamine, were applied to a column of PBE 94 (15 X 1.6 cm) equilibrated in 25 mM ethanolamine (pH 9.4), 5 mM DTT, 10% (v/v) glycerol, and 0.1% (w/v) Triton X-100. The column was loaded at a flow rate of 0.2 ml/min. Similar to the DE52 cellulose step, the PE N-methyltransferase activity was not bound to this anion exchange resin. Concentration of the diluted purified enzyme was achieved by reapplying the enzyme solution to a 3-ml column of hydroxyapatite and eluting the activity with 0.5 M K2HPO4 (pH 7.9) in 0.1% Triton X-100.

**PE N-Methyltransferase Assay**

The presence of microsomal phospholipids in partially purified fractions (steps 1-4) necessitated the use of higher concentrations of Triton X-100 and exogenous phospholipid substrates to achieve maximal expression of PE N-methyltransferase activity. The complete removal of endogenous phospholipids after phosphocellulose chromatography (Table I) allowed the use of lower Triton X-100 and exogenous lipid substrate concentrations. All assays were in 125 mM Tris-HCl (pH 9.2) and 5 mM DTT with a final assay volume of 150 pl. Samples from steps 1-4 contained 1.0 mM Triton X-100 and either no addition, 2.0 mM PE, 0.4 mM PMME, or 0.4 mM PDME. No more than 25 mg of protein was assayed in these first 4 steps. Purification steps 5-7 were assayed in the presence of 0.5 mM Triton X-100 and 2.0 mM PE, 0.25 mM PMME, or 0.46 mM PDME. Dilution of the enzyme source to 0.5 mM Triton X-100 was the major factor in deciding the volume of enzyme to be assayed in the final three purification steps. PE, PMME, and PDME were added to the assay as vesicles prepared in the following manner. Lipids were dried under a stream of nitrogen and further dried under high vacuum for 30 min. The dry lipids were resuspended in 20 mM Tris-HCl (pH 9.2), 0.01% (w/v) EDTA, and 0.02% (w/v) Triton X-100 by vortexing for 1 min and immediately sonicated at 37 °C for 3 min. Following the addition of these assay components the mixture was placed on ice and the enzyme assay was added and allowed to equilibrate for 10 min. [methyl-3H]AdoMet (21 μCi/μmol) was added to a final concentration of 200 μM, and the mixture was incubated at 37 °C for 10 min to assay PMME- and PDME-dependent activity or 30 min to assay PE-dependent activity unless otherwise specified. PE, PMME, and PDME methylolation was linear for 40 min.

The assay was stopped by the addition of 2 ml of chloroform:methanol (2:1, v/v), and methylated phospholipids were extracted as previously described (9). Total chloroform:methanol (2:1, v/v) soluble counts were determined directly and expressed as mass of CH4 groups transferred to phospholipid/min/mg protein or products of the assay were separated by thin layer chromatography in a solvent system of chloroform:methanol:acetic acid (50:30:5, v/v/v). Carrier PE, PC, PMME, and PDME were added to the extracts prior to separation. Phospholipids were visualized by exposing the plates to iodine vapor. Scortonizing into scintillation vials containing 250 ml of water, 5 ml of ACS scintillation fluid was added, and radioactivity was measured after 24 h.
RESULTS

PE N-Methyltransferase Purification—Table I shows a typical purification of PE N-methyltransferase from a crude microsomal fraction of rat liver. Activities for methylation of PE, PMME, and PDME copurify but do not show the same degree of purification. The activities for the methylation of PMME and PDME purify to the same degree to the phospho-
cellulose step, after which the PMME-dependent activity shows a substantially higher fold purification. Microsomal PE-dependent methylation activity was found to purify 429-fold. These results can be rationalized by considering that the individual lipid substrates are also providing the enzyme with an unique phospholipid environment in each case. Differences in phospholipid head group and acyl composition would affect the properties of the mixed micelle substrate thereby influencing PE N-methyltransferase activity. Unlike previous purification schemes that relied on endogenous PE in order to assay PE N-methyltransferase (11), it was found that PE N-methyltransferase activity was completely dependent on exogenous substrate following the phosphocellulose step. This is consistent with the observation that all measurable lipid phosphorous was removed following this step (Table I).

![Image](image-url)
The hydrophobic character of PE N-methyltransferase is illustrated by the high Triton X-100 concentration necessary to release it from microsomal membranes and by its high affinity for octyl Sepharose CL-4B (Fig. 1). This step offered the most substantial purification (100-fold) and was critical with regard to final enzyme purity. As illustrated in Fig. 1, PE N-methyltransferase elutes in a broad peak following single step elution with 0.15% Triton X-100. The gradient was necessary to keep the volume of the pooled fractions to a minimum.

The distribution of methylated products at each step of the purification and for all 3 lipid substrates is shown in Table II. As previously reported (9), PMME and PDME methylation resulted in the formation of predominately PDME and PC, respectively. Methylation of these two substrates using the Triton X-100 mixed micelle assay showed the same trend with 95–99% of the radioactivity found in these two products. Purification and for all 3 lipid substrates is shown in Table II and is critical. The most substantial purification (100-fold) and was critical with regard to final enzyme purity. This trend was observed throughout the entire purification. PC (the major product of PE methylation) showed a 10–15% increase in content with a concomitant decrease in PMME and PDME, as the enzyme was purified.

**Molecular Mass of PE N-Methyltransferase**—Analysis of the purified enzyme by SDS-PAGE in 10% acrylamide gels indicated that PE N-methyltransferase was composed of a single 18.3 ± 0.7 (n = 3) kDa subunit (Fig. 2, lane 1). It is apparent that during the purification of PE N-methyltransferase from microsomes there is an increase in the amount of an 18.3-kDa protein. The 50-kDa protein previously thought to be PE N-methyltransferase (11, 13–15) steadily decreased in content (lanes 7–2) and was absent from the pure enzyme.

Molecular mass analysis of the native enzyme in Triton X-100 by gel filtration on Sephacryl S-300 showed that PE-, PMME-, and PDME-dependent activities co-chromatographed with a Stokes radius of 55.2 Å (n = 2, Fig. 3A). Pure Triton micelles were found to have a Stokes radius of 53.1 Å when chromatographed on the same column. The apparent molecular mass difference between PE N-methyltransferase and pure micelles was determined to be 24.7 kDa. These results indicated that there was a single subunit/Triton micelle. Indeed, attempts to cross-link the enzyme subunits with dimethylsuberimidate were negative. Analysis of the elution profile of the 18.3-kDa protein on Sephacryl S-300 is shown in Fig. 3B. SDS-PAGE and silver staining of concentrated column fractions revealed that the putative methyltransferase protein (inset) co-chromatographed with all three methylation activities. This strong evidence to suggest that the 18.3-kDa protein is indeed PE N-methyltransferase and that a single enzyme performs all three methylation steps.

During purification it became apparent that PE N-methyltransferase possessed an extremely basic pI. This observation was corroborated by the finding that PE N-methyltransferase (solubilized in urea and Triton X-100) would not enter conventional isoelectric focusing gels (pH 3–10). A two-dimensional electrophoresis system employing NEPHGE (the method of choice for resolving basic proteins) in the first dimension and SDS-PAGE in the second revealed that PE N-methyltransferase is composed of a single isoprotein (Fig. 4).

**Kinetic Analysis of PE N-Methyltransferase**—We have developed a simple Triton X-100 mixed micelle assay to individually determine PE-, PMME-, and PDME-dependent PE N-methyltransferase activity. The purified enzyme was found to

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**Table II**

| Fraction       | Substrate     | PC Recovery | PDME Recovery | PMME Recovery |
|----------------|---------------|-------------|---------------|---------------|
| Microsomes     | NA*           | 77.1 ± 17.7 | 10.5 ± 3.4    | 12.5 ± 4.9    |
| Membranes      | NA            | 75.1 ± 25.2 | 12.3 ± 8.3    | 12.5 ± 17.2   |
| Soluble mem-   | NA            | 46.6 (2)    | 32.4 (2)      | 21.1 (2)      |
| branes         | PE            | 82.0 ± 9.7  | 91.1 ± 4.1    | 8.8 ± 6.7     |
| DE52 cellulose | NA            | 39.6 (2)    | 32.4 (2)      | 21.1 (2)      |
| P-11 cellulose | PE            | 71.8 ± 15.9 | 20.6 ± 16.3   | 7.6 ± 4.8     |
| Octyl Sepharose| PE            | 81.2 ± 12.9 | 14.7 ± 8.3    | 3.8 ± 4.8     |

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*NA, no addition.*
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**Fig. 3.** Sephacryl S-300 chromatography of purified PE N-methyltransferase. A 96 × 1.6-cm column of Sephacryl S-300 was equilibrated in buffer A containing 0.1% Triton X-100. Panel A, 3.68 µg of PE N-methyltransferase (1.5 ml) was chromatographed at a flow rate of 16 ml/h. Fractions were assayed for PE- (+), PMM- (□) and PDME- (•) dependent activities as described under "Experimental Procedures" except that incubation times were 20 min. Triton X-100 micelle size was determined by equilibrating the column in 0.35 mM Triton X-100 and chromatographing 1 ml of 8 mM Triton X-100 at a flow rate of 16 ml/h. The elution position of the Triton X-100 micelles was determined by absorbance at 275 nm. The column was calibrated using aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa). The void volume was determined using dextran blue 2000. A plot of Stokes radii versus √-log Kav is shown in the inset. Arrows 1 and 2 show the positions of PE N-methyltransferase and pure Triton X-100 micelles, respectively. Panel B, 3.68 µg of PE N-methyltransferase was chromatographed at a flow rate of 16 ml/h, and fractions were assayed for PE- (+) PMM- (□), and PDME- (•) dependent methyltransferase activity. Fractions around the activity peak were precipitated with 10% trichloroacetic acid and analyzed by SDS-PAGE in a 10% acrylamide gel (inset).

**Fig. 4.** Two-dimensional gel electrophoresis of PE N-methyltransferase. Two µg of purified PE N-methyltransferase was resolved in a NEPHGE system containing pH 3-10 ampholytes followed by SDS-PAGE in the second dimension. The gel was fixed in 30% (v/v) methanol, 10% (w/v) trichloroacetic acid, 3.5% (w/v) sulfosalicylic acid for 2 h prior to silver staining.

be maximally active when assayed in the presence of 0.5 mM Triton X-100 and 2.0 mM (88 mol %) PE, 0.25 mM (49 mol %) PMM, or 0.4 mM (61 mol %) PDME. The mol % of Triton X-100 at which maximum PMME and PDME methylation occurred is in the region where a homogenous population of micelles exists (27). The 88 mol % of PE required for maximal activity is well above 68 mol % (Triton X-100 mol fraction of 0.32) sphingomyelin, which is the effective limit for a monodisperse population of mixed micelles (27). Also, freeze fracture analysis of 91 mol % PE revealed the presence of large multilamellar vesicles (43).

The role of Triton X-100 in this mixed micelle assay was investigated to determine if PE N-methyltransferase had kinetic properties similar to those described for other membrane-bound enzymes. Fig. 5 illustrates that all three methylation activities are subject to "surface dilution" inhibition, a result often seen for enzymes that act on mixed micelle substrates (28, 29). All three methylation activities showed a definite peak of activity between 0.5 and 2.5 mM Triton X-100. However, PE and PDME methylation activities were not inhibited as much as PMME methylation by low surfactant concentrations. The inhibition of PE, PMME, and PDME methylation was not the result of increasing micelle concen-
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Fig. 5. Surface dilution of PE, PMME, and PDME methylatation activities. Assay conditions were as described under "Experimental Procedures" except 2 mM PE (■), 0.5 mM PMME (●), and 0.5 mM PDME (□) were assayed at increasing Triton X-100 concentrations. The inset shows PE- (■), PMME- (●), and PDME- (□) dependent activities assayed at fixed values of 88, 49, and 61 mol %, respectively, over a range of Triton X-100 concentrations.

Methylation (inset). Optimal enzyme activity was essentially linear over a range of Triton X-100 concentrations and at fixed PE, PMME, and PDME of 88, 49, and 61 mol %, respectively.

Double reciprocal plots of initial velocity versus lipid substrate concentration (at fixed Triton X-100) were found to be nonlinear and highly cooperative. Plots of PE-dependent activity at 0.5 mM Triton X-100 (Fig. 6) gave a Hill number of 3.7. Similar behavior was noted for PMME and PDME. In the case of PMME, there was a marked increase in the Hill number with higher concentrations of detergent (Fig. 7). Hill numbers for 0.5, 1.0, and 2.0 mM Triton X-100 were 3.1, 4.7, and 6.4, respectively. Similarly, Hill numbers for 2.5, 2.8, 6.1, and 12.8 were determined for PDME methylation at 0.5, 1.0, 2.0 and 3.0 mM Triton, respectively (Fig. 8). This type of cooperative kinetic behavior has been described by Deems et al. (31) using phospholipase A, and has been attributed to changes in the size and shape of the mixed micelle as the concentration of phospholipid in the mixed micelle increases.

As illustrated in Fig. 9, all three methylation activities of the purified enzyme have a pH optimum at 10. The alkaline pH optima of the three methylation activities probably reflects the pK₅ values of the substrate's amino group. It is feasible that protonation of the substrate's amino group results in poor binding to the active site of PE N-methyltransferase. PMME- and PDME-dependent activities were found to have K₅ values for AdoMet of 40.8 and 23.7 μM, respectively. The K₅ for AdoMet for the complete methylation of PE to PC was 36.7 μmol. These pH optima and K₅ values are similar to that reported for microsomes (9).

Methylation of PE, PMME, and PDME was found to be linear for up to 30 min. As previously reported (9), the major
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FIG. 8. Cooperativity of PDME-dependent methylation. PE N-methyltransferase activity was determined in the presence of various concentrations of PDME at 0.5 (□), 1.0 (●), 2.0 (●), and 3.0 mM (●) Triton X-100 essentially as described under "Experimental Procedures." The inset shows the corresponding Hill plot for the four Triton X-100 concentrations.

FIG. 9. pH curves for the methylation of PE, PMME, and PDME by purified PE N-methyltransferase. PE N-methyltransferase was assayed as described under "Experimental Procedures." Assays contained 125 mM potassium phosphate (□), Tris-HCl (●), or glycine KOH (▲) at the indicated pH values.

product of PDME methylation is PC. When PMME methylation was examined the major product formed was PDME (Fig. 10); however, with increasing time there was a proportional increase in counts in PC and a concomitant decrease in counts in PDME. Total methylation remained constant for 30 min (inset). This result would indicate that as the proportion of PDME in the mixed micelle increases it can compete effectively for methylation with PMME. The linearity of the methylation reactions is a good indication that the rate of exchange of phospholipid substrate between micelles is not rate-limiting.

DISCUSSION

PE methylation is considered to be a minor route for the synthesis of PC in liver but may be more important in situations where PC synthesis via the CDP-choline pathway is compromised (32, 33). Alternatively, it has been demonstrated that factors that regulate hepatic PC synthesis, we have purified PE N-methyltransferase to apparent homogeneity from rat liver microsomes. The 7-step purification scheme we employed illustrates some properties of this enzyme. Contrary to previous reports from this laboratory (10), we were successful in using the nonionic detergent Triton X-100 as a solubilizing agent after substituting 20 mM potassium phosphate with 10% glycerol for the standard Tris-HCl buffers used in other purification attempts. Purified PE N-methyltransferase showed no perceivable loss in activity for at least 2 months when stored in buffer A plus 0.1% Triton X-100 at 4 °C. This is in sharp contrast to the 80% loss of activity reported for the partially pure enzyme after 16 h at 2 °C (10). A second property that became apparent during our purification efforts was that PE N-methyltransferase is a very basic protein. PE N-methyltransferase passed unretained through an anion exchange resin (PBE 94, step 7) at pH 9.4 and can only be resolved into a single isoprotein when electrophoresed toward the anode in a NEPHGE system. What relation this alkaline pI has to enzyme function is yet unknown.

The subunit molecular mass (18.3 kDa) of PE N-methyltransferase is unusually small considering the fairly complex series of methylation reactions it catalyzes. Analysis of the pure enzyme in Triton X-100 micelles by gel filtration indicated that a single subunit is present per micelle. No information is yet available concerning the subunit structure in phospholipid membranes. Low molecular masses have been reported for several other phospholipid biosynthetic enzymes: the 34-kDa phosphatidylinositol synthase (37) and 23-kDa phosphatidylserine synthase (29) purified from Saccharomyces cerevisiae and the 13.2-kDa diacylglycerol kinase (38) and 27-kDa CDP-diacylglycerol synthetase (30) from Escherichia coli. PE N-methyltransferase, purified to homogeneity from Zymomonas mobilis, has a reported molecular mass of 42 kDa (39).

Two reports by Pajares et al. (11, 13) have claimed purification of PE N-methyltransferase from rat liver. These researchers have co-purified PE N-methyltransferase with a 50-kDa protein. The specific activity of this preparation (assayed in the presence of a mixture of PE, PMME, and PDME) was 0.27 μmol/min/mg protein (13). Based on the data we have presented, two lines of argument would indicate that the 50-kDa protein bears no relation to PE N-methyltransferase. First, we have achieved final specific activities that are 2.3-, 32-, and 14-fold higher than that reported by Pajares et al. (13), using PE, PMME, and PDME as sub-
be independent of the concentration of mixed micelles when phospholipid on the micelle surface. This inhibition was found to occur reconstituted with those and Triton X-100 mixed micelles becomes more ellipsoidal as the mol % of phospholipid increases (31). It is more likely, however, that PE, PMME, and PDME are activators as well as substrates for PE N-methyltransferase. PE N-methyltransferase may require a structural feature of the phospholipid-Triton X-100 micellar substrate for full reconstitution of activity since, unlike phospholipase A₂ for which the kinetic scheme was formulated (31), PE N-methyltransferase is an integral membrane protein with hydrophobic domains contiguous with phospholipid or detergent. Also, because of its integral nature, PE N-methyltransferase has no true dissociation constant for binding to micelles. Kinetic analyses of E. coli diacylglycerol kinase, a hydrophobic 13.2-kDa enzyme, have shown that 1,2-diacylglycerol-dependent activity is nonlinear and cooperative (40). Addition of nonsubstrate lipid activates the kinase and linearizes the diacylglycerol-dependent activity. An analogous situation may occur for PE N-methyltransferase if nonsubstrate lipids were added to the mixed micelles.

Optimal PE methylation, unlike PMME or PDME, occurs at pH 9.0, which is the range where nonmicellar structures do not exist. It is conceivable that pure PE, rather than PE in a micellar form, is more readily methylated by PE N-methyltransferase. Pure PE in solution is proposed to exist in a hexagonal II array (41). This is probably not the case in our assay where the pH is 9.0 and 12 mol % of Triton X-100 is present. Both pH >9.0 (42) and a Triton X-100/PE ratio of 0.1 or 9 mol % Triton X-100 (43) favor the formation of bilayer structure. Robinson and Waite (44) have reported a lysosomal phospholipase A₂ that preferentially hydrolyzes PE in what appears to be a hexagonal II phase. PC, phosphatidylinositol, and phosphatidylglycerol underwent maximal hydrolysis when in mixed micelles. The propensity of PE N-methyltransferase for PE bilayers versus micellar structures is quite intriguing and clearly requires more study in a reconstituted system free of detergent.

There is now strong evidence that PE methylation is catalyzed by a single enzyme. It is possible, however, that there are multiple active sites for the lipid substrates. We feel this is unlikely for several reasons. The major product of PMME methylation is PDME (9), which suggests that excess PMME is competing with and releasing PDME from the enzyme. Further evidence for competition between PMME and PDME can be seen in Fig. 10. As the concentration of newly formed PDME approaches 0.02 mM in the assay it competes efficiently with PMME for methylation. These results would seem to indicate that there is competition between PMME and PDME for methylation by PE N-methyltransferase. However, other explanations for Fig. 10 such as micelle modification and specific time-dependent inactivation of an active site cannot be ruled out. Analysis of the products formed by microsomal PE N-methyltransferase, using PMME as a substrate, indicated almost complete inhibition of PC formation (>95%) from endogenous PE (Table II). It seems likely that PMME is competitive with PE for an active site on the enzyme. It is interesting to note that like methylation of microsomal PE, methylation of pure microsomal PE by purified PE N-methyltransferase results in the formation of PC (92%). Clearly PE methylation is the rate-limiting step in the reaction sequence and PE does not compete with and release the two partially methylated intermediates.

Controversy concerning the molecular nature of the methylation system has also extended to possible modes of regulation. PE N-methyltransferase is regulated by the cellular levels of AdoMet and S-adenosyl-L-homocysteine (34, 45), fatty acids (36), and PE/PC ratios (46). There is a growing
body of evidence that suggests PE N-methyltransferase is regulated by phosphorylation. Glucagon and cAMP analogues caused a 2-fold activation of PE N-methyltransferase in hepatocytes in suspension and in primary culture (47, 48). Oddly, glucagon and cAMP analogues were found to inhibit PE to PC conversion in cultured hepatocytes (48, 49). Recent reports (14, 15) have suggested that purified PE N-methyltransferase is phosphorylated and activated by cAMP-dependent protein kinase and protein kinase C. The 50-kDa protein, claimed to be PE N-methyltransferase, is phosphorylated on serine in both cases. In light of the findings presented in this paper that indicate that the 50-kDa protein is a persistent contaminant in partially pure PE N-methyltransferase, the role of phosphorylation in PE N-methyltransferase regulation should be re-evaluated. Preliminary evidence has shown that the 18.3-kDa protein is phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. No information is yet available on the effects of enzyme activity. With the availability of pure PE N-methyltransferase we should presently be able to assess accurately what effect phosphorylation and other putative regulatory mechanisms have on PE N-methyltransferase in vivo and in vitro.

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