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Biochemistry

Identification and properties of two methyltransferases in conversion of phosphatidylethanolamine to phosphatidylcholine

(adrenal medulla/*S*-adenosyl-L-methionine/membrane phospholipids)

FUSAO HIRATA, O. HUMBERTO VIVEROS*, EMANUEL J. DILIBERTO, JR.*, AND JULIUS AXELROD

Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, Maryland 20014

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ABSTRACT Two methyltransferases involved in the methylation of phosphatidylethanolamine to form phosphatidylcholine were demonstrated in a microsomal fraction of bovine adrenal medulla. The first methyltransferase catalyzes the methylation of phosphatidylethanolamine to form phosphatidyl-*N*-monomethylethanolamine. This enzyme has an optimum pH of 6.5, a low K_m for *S*-adenosyl-L-methionine (1.4 μM), and an absolute requirement for Mg^{2+}. The second methyltransferase catalyzes the two successive methylations of phosphatidyl-*N*-monomethylethanolamine to phosphatidyl-*N,N*-dimethylethanolamine and phosphatidylcholine. In contrast to the first methyltransferase, it has an optimum pH of 10 and a high K_m for *S*-adenosyl-L-methionine (0.1 mM) and does not require Mg^{2+}.

Several investigations have shown that enzymatic methylations can occur on the amino group of phospholipids to form phosphatidylcholine (1–4). The enzyme(s) catalyzing this sequence of methylation were shown to reside in the microsomes of rat liver and *Neurospora*. A preparation of rat liver microsomes has been described that catalyzed the stepwise methylation of phosphatidyl-*N*-monomethylethanolamine to phosphatidylcholine but not of phosphatidylethanolamine (1, 4). The enzyme catalyzing the first methylation step has been suggested to be rate-limiting (1), but its properties have not yet been described.

Recently, our laboratory reported on the ability of the enzyme, protein carboxymethylase, to transfer a methyl group from *S*-adenosyl-L-methionine to carboxy groups of membrane proteins of chromaffin granules in the adrenal medulla (5–7). In studies to examine the effects of cations on this enzyme activity with various membrane fractions, it was observed that methylation of lipids also occurred which depended upon the presence of Mg^{2+}. Since the methylation of phospholipids has not been shown to require Mg^{2+} (1–4), this led us to search for and characterize the Mg^{2+} dependent enzyme that methylates lipids. This communication presents evidence that this Mg^{2+}-dependent enzyme is involved in the conversion of phosphatidylethanolamine to phosphatidyl-*N*-monomethylethanolamine and that a second methyltransferase converts the latter compound to phosphatidylcholine.

METHODS AND MATERIALS

Assay of Phosphatide Methyltransferases. The methylation of phosphatidylethanolamine to phosphatidyl-*N*-monomethylethanolamine was assayed by measuring incorporation of the methyl group from *S*-adenosyl-L-[*methyl*-^{3}H]methionine into phospholipids. The assay medium, in a 6-ml stoppered polyethylene tube, contained 4 μM *S*-adenosyl-L-[*methyl*-^{3}H]-methionine (2 μCi), 10 mM $MgCl_2$, 0.1 mM sodium EDTA, 50 mM sodium acetate buffer (pH 6.5), and tissue extract (0.1 mg of protein) in a total volume of 50 μl. The reaction was started by the addition of radioactive *S*-adenosyl-L-methionine and the mixture was incubated at 37° for 30 min. Unless otherwise indicated, the reaction was stopped by the addition of 3 ml of chloroform/methanol/hydrochloric acid (2/1/0.02, vol/vol). After the addition of 2 ml of 0.1 M KCl in 50% methanol, the tube was vigorously shaken for 15 min and centrifuged at 2000 × *g* for 10 min. The aqueous phase was aspirated, the chloroform phase was again washed with 2 ml of 0.1 M KCl in 50% methanol, and 1 ml of the chloroform phase was transferred to a vial. After the solvent was evaporated to dryness at 80° in an oven, 10 ml of Aquasol was added and the radioactivity was measured. The radioactivity with a heated preparation of enzyme was approximately 1000 dpm (0.045 pmol).

The methylation of phosphatidyl-*N*-monomethylethanolamine to phosphatidylcholine was assayed with 50 mM sodium borate buffer (pH 10), 1 mM *S*-adenosyl-L-[*methyl*-^{3}H]methionine (1.6 μCi), and 100 μg of phosphatidyl-*N*-monomethylethanolamine by the procedure described above. The two methyltransferases can be assayed together at pH 10 with a high concentration of radioactive *S*-adenosyl-L-methionine in the presence of 10 mM $MgCl_2$.

Identification of Reaction Products. To avoid oxidation of phospholipids, the chloroform phase was dried under a stream of nitrogen gas and the residue was dissolved in 50 μl of chloroform/methanol (2/1, vol/vol). The samples were applied on a Silica gel G plate (Uniplate, Analtech Inc., Newark, DE) and chromatograms were developed in several solvent systems: (*a*) chloroform/methanol/7M ammonia (60/35/5, vol/vol), (*b*) chloroform/methanol/water (65/25/4, vol/vol), (*c*) chloroform/propionic acid/*n*-propyl alcohol/water (2/2/3/1, vol/vol), (*d*) *n*-butyl alcohol/acetic acid/water (6/2/2, vol/vol), and (*e*) chloroform/acetone/methanol/acetic acid/water (5/2/1/1/0.5, vol/vol). The authentic compounds of phosphatidylcholine and its intermediates were chromatographed and the spots were visualized by exposure to iodine vapor or by spraying with 0.06% Rhodamin 6 G solution.

The bases of phospholipids were analyzed with Dowex 50 W-X8 as described by Bremer (8), after hydrolysis with 6 M HCl in a sealed tube at 120° for 4 hr.

Subcellular Fractionation of Bovine Adrenal Medulla. Bovine adrenal glands were obtained from a local slaughterhouse and the medullas were removed and chilled. Subcellular fractions were prepared by differential centrifugation as described (7). A microsomal fraction was resuspended into 25 mM

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* Present address: Laboratory of Neurochemistry, Department of Medicinal Biochemistry, The Wellcome Research Laboratories, Burroughs Wellcome Company, Research Triangle Park, NC 27709.

Table 1. Requirement of magnesium ion for methylation of lipids in adrenal medulla

Addition (5 mM)	[^{3}H]Methyl group incorporated, pmol/25 min per mg protein
None	0
+ $MgCl_2$	96.7
+ $MnCl_2$	24.8
+ $CaCl_2$	0
+ $ZnCl_2$	0
+ $FeCl_2$	0
+ $NiCl_2$	0
+ $CuCl_2$	0

The microsomal fraction of bovine adrenal medulla was washed once with an equal volume of 5 mM EDTA (pH 7.0) and resuspended into 25 mM sodium acetate, pH 6.5 (15.78 mg of protein per ml). Ten microliters of the suspension was used for the assay. The reaction was carried out as described in the text, and the incubation was for 25 min.

sodium acetate buffer (pH 6.5) and used as a crude enzyme.

Materials. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine (10 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Phosphatidylethanolamine, phosphatidyl-*N*-monomethylethanolamine, phosphatidyl-*N,N*-dimethylethanolamine, and phosphatidylcholine were obtained from Grand Island Biological Company (Grand Island, NY). These phospholipids were derivatives of egg phosphatidylcholine by the exchange of bases in the presence of phospholipase D. Ethanolamine, monomethylaminoethanol, dimethylaminoethanol, and choline chloride were purchased from Eastman Organic Chemicals (Rochester, NY). Other reagents were of analytical grade.

RESULTS

Requirement of Mg^{2+} for Methylation of Lipids. When homogenates of bovine adrenal medulla were incubated with *S*-adenosyl-L-[*methyl*-^{3}H]methionine, a significant incorporation of [^{3}H]methyl group into the membranes was observed. Fractionation of the homogenate showed that the microsomal and mitochondrial fractions were more active than the nuclear fraction and the chromaffin vesicles. The methyl group incorporated into the membranes was extractable into organic solvents such as toluene/isoamyl alcohol (2/1, vol/vol) or chloroform/methanol (2/1, vol/vol) and was not volatile after heating at 80° for 10 hr, whereas methanol, formed from carboxymethylester by hydrolysis at alkaline pH, was volatile under these conditions (5–7). The rate of [^{3}H]methyl group incorporation into the organic solvent in the absence of Mg^{2+} was 30–50% of the maximal activity in the presence of Mg^{2+} when a crude preparation of microsomes was used. When this fraction was washed with 5 mM EDTA (pH 7.0), the incorporation of [^{3}H]methyl group was completely dependent upon the presence of Mg^{2+} in the medium (Table 1). Mg^{2+} was partially replaced by Mn^{2+}, but not by other divalent cations such as Ca^{2+}, Zn^{2+}, Fe^{2+}, Ni^{2+}, and Cu^{2+}.

Product Identification of Mg^{2+}-Dependent Methylation. To identify the products, a microsomal fraction, equivalent to 2.0 mg of protein, was incubated with *S*-adenosyl-L-[^{3}H]methionine as described above for 15 min and the reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid. After centrifugation at 20,000 × *g* for 10 min, the precipitate was extracted with 3 ml of chloroform/methanol (2/1, vol/vol). The mixture was washed three times with 1 ml of 0.1 M KCl. More than 95% of the radioactivity remained in the organic phase. The chloroform phase was evaporated to dryness under a stream of N_2 and the residue was dissolved in 1.0 ml of chloroform. The extract was applied to a silica gel G column (1 × 10 cm) that had been equilibrated with chloroform. Only one major peak containing about 90% of the radioactivity was detected in the phosphatidylethanolamine fraction by a stepwise elution with increasing methanol concentration (9, 10). Although this fraction was resistant to the treatment with 0.1 M HCl containing 10 mM ($HgCl_2$) at 37°, it was easily converted to water-soluble products after the treatment with 0.1 M NaOH in methanol for 10 min at 37° (9). After the complete hydrolysis of the phosphatidylethanolamine fraction in 6 M HCl at 120° for 4 hr, the resulting hydrolysate was poured onto a column of Dowex 50 W-X8 (H^+ form) and was analyzed by the method described by Bremer and Greenberg (1). Most of the radioactivity was found in the fraction in which authentic monomethylethanolamine was eluted. Less than 5% of the radioactivity was detected in the dimethylethanolamine and choline fractions. These results, taken together, indicated that the amino group of phosphatidylethanolamine was methylated to form phosphatidyl-*N*-monomethylethanolamine by the Mg^{2+}-dependent methyltransferase in the microsomal fraction.

Properties of Phosphatidylethanolamine Methyltransferase. The properties of phosphatidylethanolamine methyltransferase were examined with a microsomal preparation of bovine adrenal medulla. The reaction rate was linear for at least 60 min and with an amount of enzyme up to 2 mg of protein. The optimum pH was 6.5 (Fig. 1*a*) and the K_m value for *S*-adenosyl-L-methionine was approximately 1.4 μM. *S*-Adenosyl-L-homocysteine was a potent competitive inhibitor with respect to *S*-adenosyl-L-methionine (Fig. 1*b*). The K_i value was approximately 1.6 μM. The concentration of Mg^{2+} required for half-maximal activation was about 0.4 mM (Fig. 1*c*). Since the microsomal fraction contained phospholipids (approximately 40% of the total weight), the K_m value for phosphatidylethanolamine could not be determined. However, the addition of phosphatidylethanolamine (100 μg) to the microsomes (100 μg of protein) caused 50–100% stimulation of the enzyme activity, whereas either monomethyl or dimethyl derivative at the same concentration was not effective.

Properties of Methyltransferase that Forms Phosphatidylcholine from *N*-Methylaminoethanol Phosphatides. Previous studies reported that the methylation of phospholipids to phosphatidylcholine has optimum pH of 10 and did not require Mg^{2+} (1, 2, 4). The K_m value for *S*-adenosyl-L-methionine has been reported to be about 0.1 mM. However, this enzyme has been reported to methylate only the exogenously added mono- and dimethyl derivatives of phosphatidylethanolamine, but not phosphatidylethanolamine (1, 4). To establish whether the second methyltransferase was present in the adrenal medulla, the microsomal fraction was incubated at pH 10 with 1 mM *S*-adenosyl-L-[*methyl*-^{3}H]methionine and at pH 6.5 with 4 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine. The methylated products were examined by thin-layer chromatography. The product of the reaction at pH 6.5 had only the one radioactive peak corresponding to the same R_f value as phosphatidyl-*N*-monomethylethanolamine (Fig. 2*a*). On the other hand, when the reaction was carried out at pH 10, three radioactive peaks were present (Fig. 2*b*). These had the same R_f values of the monomethyl, dimethyl, and trimethyl derivatives of phosphatidylethanolamine. Similar results were obtained with five different solvent systems (see *Methods and Materials*). Three radioactive peaks were also found when the reaction product was hydrolyzed with 6 M HCl and applied on a Dowex 50 W-X8 (H^+ form) column. The peaks of the radioactivity eluted

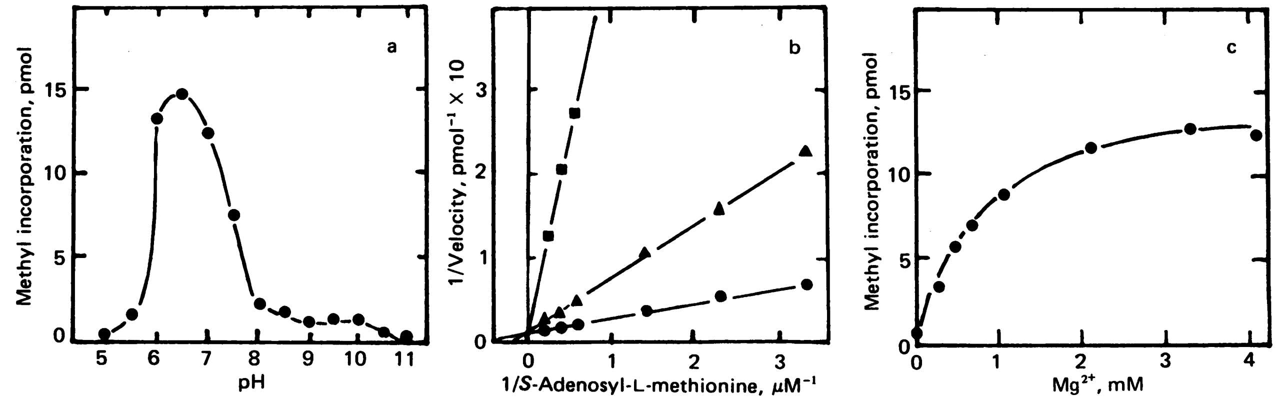

FIG. 1. Properties of the enzymatic N-methylation of phosphatidylethanolamine. (*a*) pH optimum. The reaction was performed with a microsomal fraction as described in *Methods and Materials* except that buffers at various pH were used. The buffers used were 0.1 M sodium acetate between pH 4.0 and 6.5, 0.1 M Tris-acetate between pH 7.0 and 9.5, and 0.1 M sodium borate between pH 10 and 11.5. (*b*) Effects of S-adenosyl-L-methionine concentrations on enzyme activity and inhibition by S-adenosyl-L-homocysteine. The reaction was at pH 6.5 in the absence (●) and presence of 10 μM (▲) and 100 μM (■) S-adenosyl-L-homocysteine and various concentrations of S-adenosyl-L-methionine. (*c*) Requirement for Mg^{2+}. The reaction was at pH 6.5 as described in the text except that the concentration of $MgCl_2$ was varied in the absence of EDTA.

corresponded to those of the added authentic monomethylaminoethanol, dimethylaminoethanol, and choline. Choline was further identified by repeated recrystallization of the Reinick's salt, which had a constant specific activity. These observations indicated the presence of the second methyltransferase that methylates phosphatidyl-N-monomethylethanolamine to di- and trimethylated phosphatidylethanolamine. The properties of this enzyme were similar to those described by Greenberg and his coworkers (1, 4). In contrast to the first methyltransferase, it had an optimum pH of 10 and a K_m value of 0.1 mM for S-adenosyl-L-methionine. The addition of mono- and dimethyl derivatives of phosphatidylethanolamine (100 μg) to the microsomes of bovine adrenal medulla (100 μg of protein) increased the incorporation of [^{3}H]methyl group by 70–80%, and this process did not require Mg^{2+}.

DISCUSSION

Phosphatidylcholine can be synthesized by two alternative pathways, the incorpation of CDP-choline to α,β-diacylglycerate or the stepwise methylation of phosphatidylethanolamine (1, 11). The possibility that two methyltransferases were involved in the methylating pathway has been postulated by the use of genetic variants of *Neurospora crassa* (3). One mutant has little methyltransferase activity for phosphatidylethanolamine, while the other requires N-methylated derivatives

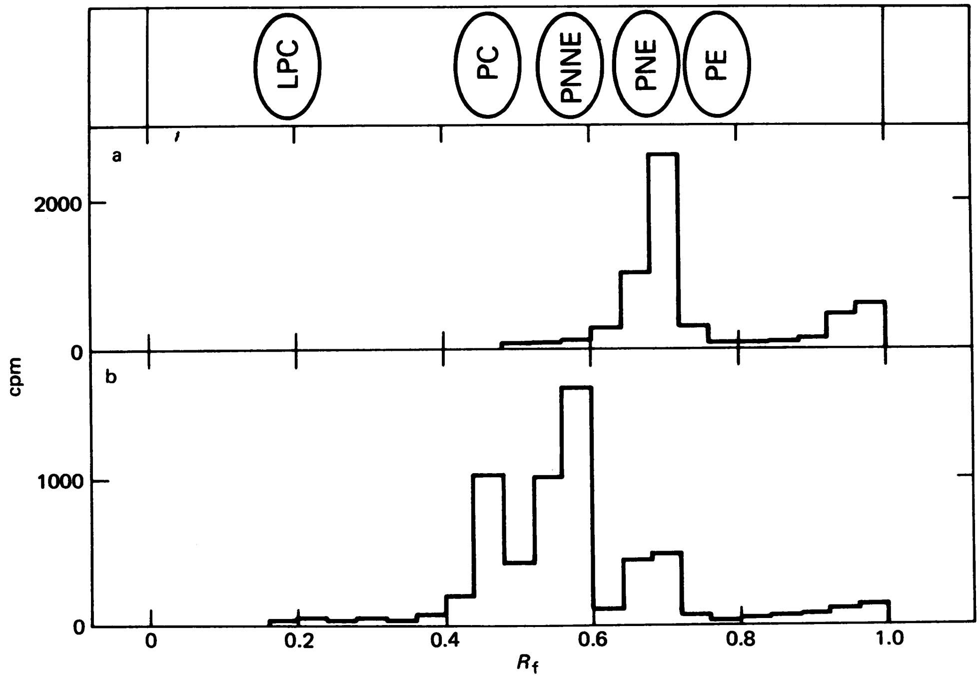

FIG. 2. Chromatographic pattern of the [^{3}H]methylated phospholipids of reaction products at pH 6.5 (*a*) and pH 10 (*b*) on the silica gel G plate. The reaction was with 4 μM (*a*) or 1 mM (*b*) S-adenosyl-L-methionine in the presence of 10 mM $MgCl_2$. The reaction products were isolated as described in the text. Chromatography was performed with a solvent system of chloroform/propionic acid/n-propyl alcohol/water (2/2/3/1, vol/vol). The solvent front migrated about 16 cm. PE, phosphatidylethanolamine; PNE, phosphatidylmonomethylethanolamine; PNNE, phosphatidyldimethylethanolamine; PC, phosphatidylcholine; LPC, lysophosphatidylcholine.

of ethanolamine for growth. When the microsomal fractions from the two variants were mixed, all three methylations of phosphatidylethanolamine could be demonstrated (3). The properties of these enzymes, however, had not been described. This communication demonstrates the two methyltransferases in the synthesis of phosphatidylcholine from phosphatidylethanolamine in mammals and also described the properties of these enzymes.

The two methyltransferases that synthesize phosphatidylcholine have markedly different properties. The enzyme that catalyzes the first methylation step requires Mg^{2+}, has an optimum pH of 6.5, and a low K_m value for *S*-adenosyl-L-methionine, and utilizes phosphatidylethanolamine in the substrate. The second methyltransferase that catalyzes the stepwise methylation of phosphatidyl-*N*-methylethanolamine to form phosphatidylcholine has an optimum pH of 10 and a high K_m value for *S*-adenosyl-L-methionine and does not require Mg^{2+}. The first methyltransferase has a higher affinity for *S*-adenosyl-L-methionine and is inhibited by low concentrations of *S*-adenosyl-L-homocysteine, indicating that this might be a regulatory enzyme. This is supported by the observation that negligible amounts of a monomethyl derivative of phosphatidylethanolamine were found *in vitro* and *in vivo* in mammalian tissues (1, 10).

Both methyltransferases are highly localized in the mitochondrial and microsomal fractions. The second methyltransferase could easily be removed by washing these fractions with 5 mM EDTA, pH 7.0, or by sonication, whereas the first methyltransferase could not be solubilized unless nonionic detergents such as Triton X-100 or Nonidet P-40 were used (unpublished data). These properties might be attributed to their different localization in the membranes and suggest a possible role of these enzymes in the asymmetrical arrangement of phosphatidylethanolamine and phosphatidylcholine in biomembranes (12). Preliminary experiments in our laboratory have shown that both methyltransferases are also present in red blood cell membranes. Using red blood cell ghosts and *S*-adenosyl-L-[*methyl*-^{3}H]methionine, we found that the methylation of phosphatidylethanolamine takes place on the interior side of the membranes and that newly synthesized phosphatidylcholine was rapidly (under 2 min) transferred (flip-flopped) to the outside of the membrane (unpublished data).

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