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

(adenal medulla/S-adenosyl-L-methionine/membrane phospholipids)

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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 nM), and an absolute requirement for Mg^2+. The second methyltransferase catalyzes the two successive methylations of phosphatidyl-N-monomethyl ethanolamine 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 catalyzes 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 Phosphatidyl Methyltransferases. The methylation of phosphatidylethanolamine to phosphatidyl-N-monomethylethanolamine was assayed by measuring incorporation of the methyl group from S-adenosyl-L-[methyl-^3H]methionine into phospholipids. The assay medium, in a 6-ml stoppered polyethylene tube, contained 4 µM S-adenosyl-L-[methyl-^3H]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°C 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 X g for 10 min. The aqueous phase was aspirated, the chloroform phase was then 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°C 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-^3H]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/proponic acid/1-n-propyl alcohol/water (2/2/5/1, 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°C 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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Table 1. Requirement of magnesium ion for methylation of lipids in adrenal medulla

<table>
<thead>
<tr>
<th>Addition (5 mM)</th>
<th>[3H]Methyl group incorporated, pmol/25 min per mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>+ MgCl₂</td>
<td>96.7</td>
</tr>
<tr>
<td>+ MnCl₂</td>
<td>24.8</td>
</tr>
<tr>
<td>+ CaCl₂</td>
<td>0</td>
</tr>
<tr>
<td>+ ZnCl₂</td>
<td>0</td>
</tr>
<tr>
<td>+ FeCl₂</td>
<td>0</td>
</tr>
<tr>
<td>+ NiCl₂</td>
<td>0</td>
</tr>
<tr>
<td>+ CuCl₂</td>
<td>0</td>
</tr>
</tbody>
</table>

The micromolar 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-3H]methionine (10 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Phosphatidylethanolamine, phosphatidyl-N-monomethyl ethanolamine, 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 phosphopipase D. Ethanolamine, monomethylaminoethanol, dimethylaminoethanol, and choline chloride were purchased from Eastman Organic Chemicals (Rochester, NY). Other reagents were of analytical grade.

RESULTS

Requirement of Mg²⁺ for Methylation of Lipids. When homogenates of bovine adrenal medulla were incubated with S-adenosyl-L-[methyl-3H]methionine, a significant incorporation of [3H]methyl group into the membranes was observed. Fractionation of the homogenate showed that the micromolar 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/isooamylic 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 carboxymethylster by hydrolysis at alkaline pH, was volatile under these conditions (5–7). The rate of [3H]methyl group incorporation into the organic solvent in the absence of Mg²⁺ was 30–50% of the maximal activity in the presence of Mg²⁺ when a crude preparation of microsomes was used. When this fraction was washed with 5 mM EDTA (pH 7.0), the incorporation of [3H]methyl group was completely dependent upon the presence of Mg²⁺ in the medium (Table 1). Mg²⁺ was partially replaced by Mn²⁺, but not by other divalent cations such as Ca²⁺, Zn²⁺, Fe²⁺, Ni²⁺, and Cu²⁺.

Product Identification of Mg²⁺-Dependent Methylation.

To identify the products, a microsomal fraction, equivalent to 2.0 mg of protein, was incubated with S-adenosyl-L-[methyl-3H]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 5 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 N₂ 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 (HgCl₂) 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 monomethyl ethanolamine was eluted. Less than 5% of the radioactivity was detected in the dimethyl ethanolamine and choline fractions. These results, taken together, indicated that the amino group of phosphatidylethanolamine was methylated to form phosphatidyl-N-monomethyl ethanolamine by the Mg²⁺-dependent methyltransferase in the micromolar 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. 1a) and the Kₘ 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. 1b). The Kᵢ value was approximately 1.6 µM. The concentration of Mg²⁺ required for half-maximal activation was about 0.4 mM (Fig. 1c). Since the microsomal fraction contained phospholipids (approximately 40% of the total weight), the Kₘ 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 Mg²⁺ (1, 2, 4). The Kₘ value for S-adenosyl-L-methionine has been reported to be 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-3H]methionine and at pH 6.5 with 4 µM S-adenosyl-L-[methyl-3H]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 R₁ value as phosphatidyl-N-nucleoethanolamine (Fig. 2a). On the other hand, when the reaction was carried out at pH 10, three radioactive peaks were present (Fig. 2b). These had the same R₁ 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
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-monomethyllethanolamine 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 [3H]methyl group by 70–80%, and this process did not require Mg$^{2+}$.

**DISCUSSION**

Phosphatidylcholine can be synthesized by two alternative pathways, the incorporation of CDP-choline to α,β-diacylglycerol 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
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-3H]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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