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PURIFICATION AND PROPERTIES OF PHOSPHATIDYL-N-MONOMETHYLETHANOLAMINE N-METHYLTRANSFERASE, THE ENZYME CATALYZING THE SECOND AND THE THIRD STEPS IN THE PHOSPHATIDYLETHANOLAMINE N-METHYLTRANSFERASE SYSTEM, FROM MOUSE LIVER MICROSOMES

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SUMMARY: The phosphatidylethanolamine (PE) N-methyltransferase (MT) system is known to convert PE to phosphatidylcholine by three successive N-methylations. Phosphatidyl-N-monomethylethanolamine (PME) MT was purified 1,400-fold from mouse liver microsomes and separated from the PE-MT activity for the first time. This enzyme catalyzes N-methylations of PME and phosphatidyl-N,N-dimethylethanolamine, the intermediates of PE-MT system, but not PE, the initial substrate of the PE-MT system. In addition, a preparation with a different affinity to S-adenosyl-L-homocysteine catalyzing all the three methylations was obtained. These results suggest that at least two enzymes are involved in the PE-MT system.

INTRODUCTION

The phosphatidylethanolamine (PE) N-methyltransferase system [E.C.2.1.1.17, S-adenosyl-L-methionine (SAM): PE N-methyltransferase]

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converts PE to phosphatidylcholine (PC) by means of three successive N-methylations through two intermediate phospholipids, phosphatidyl-N-monomethyl-ethanolamine (PME) and phosphatidyl-N,N-dimethylethanolamine (PDME), with SAM as a methyl donor (1,2). The enzyme system was first found in microsomes of rat liver and other organs (3). We have also detected this enzyme system in various cultured mammalian cell lines (4-6). This pathway for PC synthesis can be expected to be important in expression of various membrane functions, since conversion of PE to PC within a biological membrane alters the physical character of the membrane near the reaction site and consequently the microenvironment of the membrane. Hirata and Axelrod (7,8) suggested coupling of these reactions with several membrane functions related to the receptor-mediated transfer of information across membranes, while other groups had opposed questions against their results and models (9). Thus the biological significance of these reactions is still obscure.

Beside solubilization studies (10,11), genetical (12-14) and kinetical (15-21) studies on this enzyme system also suggested that it consists of at least two enzymes. In yeast, two distinct methyl transferase genes were cloned and the existence of at least two methyltransferases, one catalyzing PE N-methylation and the other all the three N-methylations, were suggested (22). In mouse thymocytes, the presence of at least two enzymes comprising a PE N-methyltransferase system was also suggested by the partial purification of the enzymes (23). The enzymes partially purified from rat and mouse liver microsomes, however, showed all the three successive N-methylation activities (24-27). From the results of re-examination of kinetical studies on this enzyme system in rat liver microsomes, re-evaluation of the number of enzymes involved in the conversion of PE to PC was proposed (28), and a methyltransferase catalyzing all the three methylations was purified from rat liver (29), suggesting that one enzyme converts PE to PC by N-methylations.

In this paper we report purification of PME N-methyltransferase from mouse liver microsomes for the first time, that specifically catalyzes the conversion of PME to PC through PDME as an intermediate, and separation from PE N-methyltransferase activity that catalyzes the first step of the PE N-methyltransferase system, by S-adenosyl-L-homocysteine (SAH)-Sepharose 4B affinity column chromatography. In addition to this enzyme, we obtained another enzyme fraction with a different affinity to SAH catalyzing PE, PME and PDME methylations. From these results, we suggest that the PE N-

methyltransferase system in mouse liver microsomes consists of at least two enzymes.

MATERIALS AND METHODS

PME N-methyltransferase assay: PME N-methyltransferase activity was assayed by measuring the incorporation of methyl groups from [methyl- ^{14}C]SAM into PDME and PC as reported previously (25) with modifications. The assay mixture contained 0.5 mM PME, 42 μM [methyl- ^{14}C]SAM (3n Ci/nmol), 5 mM EDTA, 10 mM 2-mercaptoethylamine hydrochloride, 10% (w/v) glycerol, 0.1 M Tris-HCl (pH 8.8) and the enzyme solution. To measure N-methylation of endogenous phospholipids or PE, 1 mM MgCl_2 was used instead of 5 mM EDTA, with or without 0.5 mM PE. The reaction was started by addition of radioactive SAM, and then the mixture (0.2 ml) was incubated at 37 C for 30 min. The reaction was terminated by addition of 3 ml of chloroform/methanol (2/1, v/v). After vigorous shaking for one minute, the chloroform layer was separated by centrifugation at $1,500 \times g$ for 5 min and washed twice with 2 ml of 0.85% (w/v) NaCl. Separation of the reaction products recovered in the chloroform layer was performed by two-dimensional thin-layer chromatography as reported previously (25). During this experiment no radioactive spot other than PME, PDME, and PC was detected by autoradiography conducted as described (25). The radioactive spots corresponding to PME, PDME and PC on a thin-layer chromatogram were scraped off and counted by liquid scintillation spectrometry.

Preparation of S-adenosyl-L-homocystein(SAH)-sepharose 4B: SAH-Sepharose 4B was prepared as described previously (30). Activated CH-Sepharose 4B (15g), washed with 3 l of 1 mM HCl on a sintered glass filter, was mixed with 300 mg of SAH in 75 ml of 0.1 M NaHCO_3 (pH 8.0), followed by incubation for an hour at 24 C. The excess SAH was washed away with 3 l of 0.1 M NaHCO_3 (pH 8.0), and then the remaining reactive groups in the activated CH-Sepharose 4B were blocked with 75 ml of a 1 M ethanolamine (pH 9.0) solution by incubation for an hour at 24 C. After three cycles of washing with 1 l each of 50 mM Tris-HCl (pH 8.0) containing 1 M NaCl, 50 mM formic acid (pH 4.0) containing 1 M NaCl, and distilled water, the resulting SAH-Sepharose 4B was equilibrated with buffer B [20 mM Tris-HCl (pH 8.8) containing 0.1 M NaCl, 0.2%(w/v) Triton X-100, 10%(w/v) glycerol and 5 mM 2-mercaptoethylamine hydrochloride]. The amount of SAH bound to Sepharose 4B was 12 $\mu\text{mol/ml}$.

Other methods: Protein concentrations were determined by the method of Lowry et al. (31), except for the fractions obtained by column chromatography (Fig. 1), in which the Bradford method (32) was used. Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (33) and the subsequent silver staining (34) were performed as described previously.

Fig. 1. SAH-Sepharose 4B column chromatography. The column was eluted as described in the text. At each point indicated by an arrow the buffer was changed. The PME N-methyltransferase activity (○) was measured as described under MATERIALS AND METHODS except for further separation of the chloroform layer by thin-layer chromatography and the expression of the enzyme activity as pmol of [^{14}C]methyl group incorporated into the chloroform layer. Protein concentrations (●) were determined by the method of Bradford (32).

Materials: The buffers used were buffer A [20 mM Tris-HCl (pH 8.8) containing 0.1 M NaCl, 10% (w/v) glycerol and 5 mM 2-mercaptoethylamine hydrochloride], buffer B [buffer A containing 0.2% (w/v) Triton X-100], buffer C (buffer B containing 0.5 M NaCl), and buffer D [20 mM Tris-HCl (pH 7.0) containing 1 M NaCl, 0.2% (w/v) Triton X-100, 10% (w/v) glycerol and 5 mM 2-mercaptoethanolamine hydrochloride]. PE, PME, PDME, PC and sodium deoxycholate (DOC) were prepared as described previously (25). Water-dispersions of phospholipids were prepared as described (35). Male ddY mice (5 weeks of age) were obtained from Shizuoka Laboratory Animal Cooperation, Hamamatsu, Japan. SAM and SAH were from Sigma Chemical Co. (St. Louis, MO). [Methyl- ^{14}C]SAM obtained from Amersham (Buckinghamshire, England) was sufficiently pure to use without further purification when analyzed by thin-layer chromatography. Triton X-100 was obtained from Nacalai Tesque

Inc. (Kyoto, Japan). Octyl- β -D-glucopyranoside was from Calbiochem (La Jolla, CA) and EDTA from Dojin Kagaku Inc., Kumamoto, Japan. DEAE cellulose (DE-52) was purchased from Whatman Ltd. (Maidstone, England) and activated CH-Sepharose 4B from Pharmacia Fine Chemicals, Uppsala, Sweden. Bio-Gel P-300 was obtained from Bio-Rad Laboratories (Richmond, VA) and Immersible CX-10 from Millipore Corporation (Bedford, MA). Other chemicals were of reagent grade.

RESULTS

Purification of Phosphatidyl-N-monomethylethanolamine N-methyltransferase

A summary of purification is given in Table I. All the procedures were performed at 4 C.

Step 1.

Mouse liver microsomes were obtained as described previously (25) and stored at -70 C until use.

Step 2.

The microsomes (10 mg protein/ml) were mixed with 0.25% (w/v) DOC in buffer A, followed by stirring with a magnetic stirrer for 30 min on ice, and then centrifuged at $105,000 \times g$ for 60 min. The precipitates and fluffy materials near the bottom were collected (referred to as "DOC-enzyme"). On DOC treatment, the specific activities for phospholipid N-methylation assayed with PME and PE increased 1.5 and 2.3-fold, respectively (Table I).

Step 3.

DOC-enzyme (1 mg/ml) was solubilized on ice for 30 min with 0.2% (w/v) Triton X-100 (buffer B). The mixture was then centrifuged at $105,000 \times g$ for 60 min and the supernatant (referred to as "Triton-enzyme") was collected. As shown in Table I, through this solubilization the specific activity for phospholipid N-methylation assayed with PME as a substrate increased three-fold over that of DOC-enzyme, while that assayed with PE did not change. Furthermore, Triton-enzyme showed an activity only one-third that of DOC-enzyme when incubated without any exogenous substrate.

Table I. Purification of PME N-methyltransferase from mouse liver microsomes

The enzyme activity was assayed as described under MATERIALS AND METHODS without lipids(+ None), with 0.5 mM PE(+ PE) or with 0.5 mM PME(+ PME) added exogenously to the assay mixture. In this assay, radioactivities recovered in the chloroform layer were measured directly without further separation. The total activity was expressed as μmol of $[^{14}\text{C}]$ methyl group incorporated into the chloroform layer during 30 min, and the specific activity as nmol of $[^{14}\text{C}]$ methyl group incorporated during 30 min per mg protein.

Purification step	Volume (ml)	Total protein (mg)	Total activity			Specific activity		
			+None	+PE	+PME	+None	+PE	+PME
1 Microsomes	0.958	52.5	0.32	0.60	2.27	6.14	11.5	43.2
2 DOC-treatment (DOC-enzyme)	0.189	4.9	0.10	0.13	0.30	21.4	26.8	62.0
3 Solubilization (Triton-enzyme)	4.5	4.0	0.02	0.11	0.76	5.8	27.8	191
4 DEAE cellulose (DEAE-enzyme)	6.7	0.8	0.001	0.004	0.22	1.6	5.0	272
5 SAH-Sepharose								
peak I	16	0.05	— ¹⁾	0.002	0.053	—	40	1,100
peak II	2.0	0.00002 ²⁾	—	—	0.0013	—	—	63,000

1): Not detectable.
2): This amount of protein is the detection limit for a silver stained SDS-polyacrylamide gel electrophoregram of the enzyme preparation concentrated by ultrafiltration with Immersible CX-10.

Step 4.

Four portions of Triton-enzyme, each containing 1 mg of protein, were separately applied to four DEAE-cellulose columns (1.5 cm × 3.5 cm) equilibrated with buffer B. All columns were eluted with buffer B simultaneously. As the enzyme activity is very labile after purification (the half life on ice was less than 12 hr), we saved time by this procedure. The PME N-methyltransferase activity was recovered in the “flow through” fraction from each column (referred to as “DEAE-enzyme”). In DEAE-enzyme, the phospholipid N-methyltransferase activity with PE and without exogenous substrates decreased to less than one-fifth and one-third of that of Triton-enzyme, respectively, while the PME N-methyltransferase activity increased 1.5-fold.

Step 5.

For the last step of purification, we used affinity column chromatography. SAH is known to be a competitive inhibitor of methyltransferases (36-40), and SAH-Sepharose 4B affinity chromatography was reported to be useful for purifying RNA methyltransferase (30). This method was also reported to be useful for the partial purification of phospholipid N-methyltransferases from murine thymocyte microsomes (23). We applied this method to purification of PME N-methyltransferase. DEAE-enzyme (0.8 mg) was applied to an SAH-Sepharose 4B (12 μ mol/ml of SAH) column (0.9 cm $\times$ 16 cm) equilibrated with the buffer containing 0.5 M NaCl (buffer C), and finally peak II was eluted with the buffer containing 1 M NaCl at a lower pH (buffer D) (Fig. 1).

As shown in Table I, the PME N-methyltransferase activities recovered in peaks I and II corresponded to 24% and 0.6% of the applied enzyme (DEAE-enzyme) activity, respectively, and the protein in the two peaks to 6% and less than 0.003% of the applied amount, respectively. In the "flow through" fraction, 43% of the activity and more than 75% of the protein applied to the column were eluted (data not shown). This purification procedure was repeated seven times with reproducible results. After affinity column chromatography, the peak II fraction showed 1,400-fold higher specific activity for PME N-methylation and also no activity without exogenous lipid substrates.

This PME N-methyltransferase activity was lost on boiling for 10 min or just standing on ice for 12 hr. We used peak II fraction as the purified preparation of PME N-methyltransferase (referred to as "peak II fraction") for further characterization. As the enzyme was labile at this stage, we used this fraction immediately after purification. It is noteworthy, however, that the enzyme is rather stable when bound to SAH-Sepharose 4B, because about 25% of the PME N-methyltransferase activity was recovered in peaks I and II (Table I), even when chromatography took more than 40 hr. On SDS-polyacrylamide gel electrophoresis of peak II fraction after concentration by ultrafiltration with Immersible CX-10, no band was detected even on silver staining, suggesting that only a very small amount of protein remained in this fraction.

Peak I fraction also showed no phospholipid N-methyltransferase activity without addition of lipid substrates (Table I), although it exhibited all of PE, PME and PDME N-methyltransferase activities in the presence of exogenous substrates. This enzyme fraction was eluted faster than the peak II fraction from the SAH-Sepharose column (Fig. 1), suggesting a lower affinity to SAH than peak II fraction. Peak I fraction gave 10 bands in a silver stained SDS-polyacrylamide

gel electrophoregram (data not shown).

To improve this affinity chromatography, we changed the concentration of SAH bound to Sepharose 4B from 5 $\mu\text{mol/ml}$ to 12 $\mu\text{mol/ml}$, the amount of DEAE-enzyme applied to the column from 15 mg to 0.5 mg, and the concentration of Triton X-100 in the buffer solution for equilibration of the column from 0.05% (w/v) to 0.2% (w/v). We failed, however, to get a higher recovery of the PME N-methyltransferase activity in peak II fraction. When a smaller amount of DEAE-enzyme was loaded on the column, both the enzyme activities eluted in "flow through" and the peak II fractions were reduced. This could be explained by the lability of the enzyme and/or by the difficulty in eluting the enzyme activity bound tight to the affinity column. Triton X-100, sodium deoxycholate, SDS and octyl- β -D-glucopyranoside at higher concentrations did not improve the chromatography.

Substrate Specificity and the Reaction Products of PME N-methyltransferase

As shown in Table II, PME and PDME were N-methylated by the purified enzyme and no phospholipid N-methylation was observed without substrate phospholipids added exogenously or with PC or PE. The reaction products were PDME and PC, or only PC when PME or PDME was used as a substrate.

Effect of EDTA and MgCl_2 on PME N-methyltransferase and the pH Optimum of the Enzyme

The effect of Mg^{2+} and EDTA on purified PME N-methyltransferase was studied because the first step in the PE N-methyltransferase system, i.e. the conversion of PE to PME, was reported to be stimulated by Mg^{2+} in rat liver microsomes (10) and MOPC-31C cells (5). Although exogenously added MgCl_2 had no effect on the PME N-methyltransferase activity in peak II fraction, it was stimulated twofold by addition of 5 mM EDTA. This activation, however, was reversed and inhibited by 5 mM MgCl_2 (Table III). It is feasible that EDTA removed endogenous metal ions from the enzyme, which made the enzyme more susceptible to MgCl_2 . These results suggest that PME N-methyltransferase activity is suppressed by Mg^{2+} and activated by the removal of Mg^{2+} with EDTA.

Table II. Substrate specificity and reaction products of PME N-methyltransferase

The enzyme reaction and product analysis were carried out as described under MATERIALS AND METHODS in the presence of each substrate phospholipid (0.5 mM). The amounts of reaction products were expressed as pmol of [^{14}C]methyl group incorporated into each phospholipid during 30 min. Peak II fraction (20 μl) was used for the measurement. When peak II fraction (20 μl) was boiled for 10 min and then subjected to the assay in the presence of PME as a substrate, the [^{14}C]methyl group incorporated into each phospholipid was less than 0.1 pmol.

Phospholipid added to the assay mixture	Total	Phospholipid produced		
		PME	PDME	PC
None	— ¹⁾	—	—	—
PE	—	—	—	—
PME	12.6	—	3.7	8.9
PDME	4.0	—	—	4.0
PC	—	—	—	—

¹⁾: Less than 0.1 pmol.

Table III. Effect of EDTA and MgCl_2 on the purified PME N-methyltransferase activity

The enzyme activity was assayed as described under MATERIALS AND METHODS with various concentrations of MgCl_2 or/and EDTA. Peak II fraction (10 μl) was used for the measurement.

Additions	PME N-methyltransferase activity (pmol of [^{14}C]methyl group incorporated into PDME and PC in 30 min)
None	2.3
MgCl_2 (5 mM)	2.1
MgCl_2 (10 mM)	2.3
EDTA(5 mM)	4.5
EDTA(10 mM)	5.3
EDTA(5 mM)+ MgCl_2 (5 mM) ¹⁾	0.9
EDTA(5 mM)+ MgCl_2 (10 mM) ¹⁾	1.3

¹⁾: 10 μl of the peak II fraction was added to the assay mixture containing 5 mM EDTA, and then various concentrations of MgCl_2 were added to the mixture.

Fig. 2. Optimal pH for the final PME N-methyltransferase preparation. Peak II fraction in Fig. 1 (10 µl) was added to assay mixtures of different pHs, and then the enzyme activity was assayed as described under MATERIALS AND METHODS, and expressed as pmol of [^{14}C]methyl group incorporated into PDME and PC. 0.1 M sodium phosphate buffer (pH 6.4-6.9), 0.1 M Tris-HCl buffer (pH 7.4-8.7) and 0.1 M sodium carbonate buffer (pH 9.5-10.0) were used for the measurement. A similar pH dependency was observed for the PME N-methyltransferase activity of peak I fraction in Fig. 1 (step 5) and also for DEAE-enzyme (step 4).

Optimal pH of peak II fraction was 8.0 (Fig. 2). DEAE-enzyme (step 4) and peak I fraction (step 5) also showed their optimal pH values at 8.0. However, we found that optimal pH of Triton-enzyme (step 3) was 10.5 (data not shown), and this was in good agreement with that of the partially purified enzyme from mouse

(25) or rat (24) liver microsomes assayed with PE, PME or PDME, and that of the enzyme catalyzing PME N-methylation in rat liver microsomes (10). These differences in pH optima could be explained by the changes in the circumstances, such as phospholipids, detergents and other proteins around the enzyme(s).

DISCUSSION

PME N-methyltransferase was purified for the first time from mouse liver microsomes by affinity column chromatography on SAH-Sepharose 4B. The specific activity of the final enzyme preparation (peak II fraction) was more than 1,400-fold that of microsomes in terms of PME N-methylation (Table I). It also exhibited catalytic activity for PDME N-methylation but not for PE N-methylation (Table II). The reaction products of PME N-methyltransferase were PDME and PC when PME was used as a substrate, while those of peak I fraction N-methylated PE as well as PME and PDME. In this case, the reaction products were PME, PDME and PC when PE was used as a substrate (data not shown). It is suggested that peak II fraction catalyzes conversion of PME to PC through PDME as an intermediate by two successive N-methylations, while the enzyme(s) in peak I fraction catalyzes conversion of PE to PC through PME and PDME as the intermediates by three successive N-methylations.

As to the number as well as the characteristics of the methyltransferases involved in conversion of PE to PC in liver microsomes (24,28,29,41), the present situation is still controversial. In this study, we separated two enzyme fractions, one (peak II fraction) catalyzing PME and PDME N-methylation and the other (peak I fraction) PE, PME and PDME N-methylation. The former had a higher affinity to SAH-Sepharose than did the latter. As peak I fraction contained 10 protein bands, we could not exclude the possibility that this fraction still contains another enzyme involved in the PE N-methyltransferase system. From these results, we suggest that at least two enzymes are involved in the PE N-methyltransferase system in mouse liver microsomes. Recently, purification of a PE N-methyltransferase converting PE to PC from rat liver microsomes was reported (29). Peak I fraction in our experiment might contain such an enzyme.

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