Conversion of Phosphatidylethanolamine to Phosphatidylcholine in Rat Liver

PARTIAL PURIFICATION AND CHARACTERIZATION OF THE ENZYMATIC ACTIVITIES

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The enzymatic activities for the synthesis of phosphatidylcholine from phosphatidylethanolamine and S-adenosyl-l-methionine have been solubilized from rat liver microsomes by ultrasonic disruption in the presence of 0.2% Triton X-100. Significant co-purification of the activities was achieved by chromatography of the Triton extract on octyl-Sepharose CL-4B and, subsequently, on Sepharose 6B. Throughout purification, the ratio of the activities for the N-methylation of phosphatidylethanolamine, phosphatidyl-N-monomethylethanolamine, and phosphatidyl-N,N-dimethylethanolamine remained unchanged. Additional properties of the purified enzyme(s) were examined. The three activities had a similar pH profile with an optimum at pH 9.5 and were inhibited in an identical fashion by Triton X-100. It was not possible to estimate kinetic parameters with phosphatidylethanolamine as a substrate because of the rapid methylation of the product (phosphatidyl-N-monomethylethanolamine) to phosphatidyl-N,N-dimethylethanolamine and phosphatidylcholine. However, the initial reaction rate for the formation of phosphatidyl-N-monomethylethanolamine from phosphatidylethanolamine and S-adenosyl-l-methionine was estimated to be 0.08 nmol·min⁻¹·mg⁻¹. The apparent kinetic constants for the methylation of phosphatidyl-N-monomethylethanolamine and phosphatidyl-N,N-dimethylethanolamine were determined. S-Adenosyl-l-homocysteine was a competitive inhibitor with respect to S-adenosyl-l-methionine for the methylation of phosphatidyl-N-monomethylethanolamine and phosphatidyl-N,N-dimethylethanolamine. The results suggest that the conversion of phosphatidylethanolamine to phosphatidyl-N,N-dimethylethanolamine is the rate-limiting step.

The conversion of phosphatidylethanolamine to phosphatidylcholine by transfer of methyl groups from S-adenosyl-l-methionine (AdoMet) is catalyzed by enzymic activities localized on the cytoplasmic side of the microsomes from rat liver (1, 2). Synthesis of phosphatidylcholine by N-methylation has also been demonstrated with microsomes from Neurospora crassa (3, 4), Agrobacterium tumefaciens (5), dog lung (6), and bovine adrenal medulla (7). Partially methylated intermediates (phosphatidyl-N-monomethylethanolamine and phosphatidyl-N,N-dimethylethanolamine) have been identified in the conversion of phosphatidylethanolamine to phosphatidylcholine and the concentration of these lipids has been determined in rat liver (8). The importance of the methylation pathway in the biosynthesis of phosphatidylcholine in rat liver hepatocytes has been assessed and it may account for 20% of the phosphatidylcholine made in rat liver (9). The contribution of this pathway to phosphatidylcholine biosynthesis may be increased during choline deficiency since the activity of the phosphatidylethanolamine (phosphatidyl-N-monomethylthanolamine, phosphatidyl-N,N-dimethylethanolamine)-methyltransferase(s) is elevated nearly 2-fold (10). Despite the apparent importance of the methylation pathway for phosphatidylcholine biosynthesis in liver, detailed knowledge on the structure, properties, and regulation of the enzyme(s) is not available. As a first step in the characterization of this system, we report the first solubilization of the enzyme(s) from rat liver and a partial purification. In addition, some physical and kinetic properties of the N-methyltransferase(s) are reported.

EXPERIMENTAL PROCEDURES

The materials and methods used, some of the results, and the references are in the miniprint supplement that follows this paper.1

RESULTS

Phosphatidylethanolamine (Phosphatidyl-N-Monomethylethanolamine, Phosphatidyl-N,N-Dimethylethanolamine)-Methyltransferase(s) in Intact Microsomal Vesicles

Incubation of microsomal vesicles (Step 3, Table I) with labeled AdoMet2 in the presence or absence of exogenous substrate resulted in a rapid labeling of phosphatidylcholine. Regardless of incubation time or total concentration of phosphatidylethanolamine, phosphatidyl-N-monomethylethanolamine, or phosphatidyl-N,N-dimethylethanolamine, 97% of the radioactivity found in methylated phospholipids was localized in phosphatidylcholine. Addition of phosphatidyl-

1 Portions of this paper (including Figs. 2 to 9 and the references) are presented in miniprint at the end of this paper. The abbreviations used in the miniprint include PE, phosphatidylethanolamine; PM, phosphatidyl-N-monomethylethanolamine; PD, phosphatidyl-N,N-dimethylethanolamine; and PC, phosphatidylcholine. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9550 Rockville Pike, Bethesda, Md. 20014. Request Document No. 78M-1118, cite author(s), and include a check or money order for $2.55 per set of photocopies.

2 The abbreviation used is: AdoMet, S-adenosyl-l-methionine.
ethanolamine, phosphatidyl-N-monomethylethanolamine, or phosphatidyl N,N-dimethylethanolamine to the incubation mixture only slightly stimulated methyl transfer (Table I).

The best condition for solubilization of the N-methyltransferase(s) was sonication of the microsomes (Step 3, Table I) for 15 min in Buffer A with 0.2% Triton X-100. After centrifugation of this suspension at 100,000 × g for 2 h, 44% of the enzyme(s) remained in the supernatant (Table I). After solubilization, the enzyme(s) was not stable to freezing at −70°C and was irreversibly inactivated by concentrations of Triton X-100 greater than 0.6%. In addition, the preparation was contaminated by significant amounts of thiol methyltransferase. This preparation was used immediately for subsequent studies since it lost 30% of its activity within 12 h at 2°C.

Chromatography of the Triton X-100 extract on Sepharose 6B (Fig. 1). The most active fractions (18 to 21) were combined and concentrated to 4 ml by ultrafiltration. It was necessary to add Buffer A during the filtration step so that Triton X-100 concentrations of greater than 0.6% were avoided. This fraction was chromatographed on Sepharose 6B or DEAE-Sepharose CL-6B did not result in an improved purification. However, the specific activity of the enzyme(s) was enhanced by hydrophobic chromatography on octyl-Sepharose CL-4B (Fig. 1). The most active fractions (18 to 21) were combined and concentrated to 4 ml by ultrafiltration. It was necessary to add Buffer A during the filtration step so that Triton X-100 concentrations of greater than 0.6% were avoided. This fraction was chromatographed on Sepharose 6B and was irreversibly inactivated by concentrations of Triton X-100 greater than 0.6%. In addition, the preparation was contaminated by significant amounts of thiol methyltransferase. This preparation was used immediately for subsequent studies since it lost 30% of its activity within 12 h at 2°C.

Characterization of Partially Purified Phosphatidylethanolamine (Phosphatidyl-N-Monomethylethanolamine, Phosphatidyl-N,N-Dimethylethanolamine)-Methyltransferase(s)

The rates of the conversion of all three substrates to labeled products increased in identical patterns as the pH was raised from 7 to 9.5 (Fig. 2). At a pH higher than 9.5, reproducibility of the results was poor, most likely due to the lability of the substrate AdoMet in the alkaline medium. None of the methyltransferase activities required Mg²⁺, nor could the requirement for any other cofactor be established. The partially purified preparation was dependent on a sulfhydryl reducing agent (5 mM cysteine) for stability. Inhibition characteristics with respect to Triton X-100 were identical for all three methyltransferase activities. In addition, the inhibition patterns for the partially purified phosphatidylethanolamine (phosphatidyl-N-N-monomethylethanolamine, phosphatidyl-N,N-dimethylethanolamine)-methyltransferases and for the crude extract were superimposable (Fig. 3).

Kinetic Properties Phosphatidylethanolamine as Substrate—At 68 µM AdoMet, maximum phosphatidylethanolamine-methyltrans-
ferase activity was obtained with 0.6 mg of phosphatidylethanolamine/ml (0.8 mM) assay mixture. When the formation of products was followed over a 20-min incubation, newly synthesized phosphatidyl-N-monomethylethanolamine and phosphatidylcholine (Fig. 4). Because of this complication and the low initial rate of formation of phosphatidyl-N-monomethylethanolamine, initial velocity studies were not possible for the evaluation of kinetic parameters. However, under optimal conditions, we estimated an initial reaction rate of 0.08 nmol-min⁻¹-mg⁻¹ for the synthesis of phosphatidyl-N-monomethylethanolamine.

Phosphatidyl-N-Monomethylethanolamine as Substrate—There was a 10 to 20% methylation of newly formed phosphatidyl-N,N-dimethylethanolamine to phosphatidylethanolamine 5 min after the start of the reaction (Fig. 5). Thus, initial velocity studies for the determination of true kinetic parameters were unreliable. However, at a fixed concentration of 68 μM AdoMet, the apparent Kₘ for phosphatidyl-N-monomethylethanolamine was estimated by a double reciprocal plot to be 0.06 mg/ml (0.08 mM) in 5-min incubations. Maximum activity was observed with 0.28 mg of phosphatidyl-N-nanomethylethanolamine/ml (0.37 mM). Inhibition studies with the product S-adenosyl-L-homocysteine were performed in 5-min incubations. S-Adenosyl-L-homocysteine was demonstrated to be a competitive inhibitor of AdoMet (Fig. 6). The apparent Kₛ for the conversion of phosphatidyl-N-monomethylethanolamine to phosphatidyl-N,N-dimethylethanolamine was estimated to be 0.24 mg/ml (0.37 mM) at a concentration of 0.37 mM phosphatidyl-N-monomethylethanolamine and 68 μM AdoMet is 0.69 nmol-min⁻¹·mg⁻¹. The apparent Kₛ for AdoMet was 22 μM at a concentration of 0.37 mM phosphatidyl-N-monomethylethanolamine.

Phosphatidyl-N,N-Dimethylethanolamine as Substrate—Conversion of phosphatidyl-N,N-dimethylethanolamine to phosphatidylethanolamine was linear for up to 40 min (Fig. 7) and 100 μg of protein. When the concentration of each substrate was varied at fixed concentrations of the other, two sets of straight lines were obtained in double reciprocal plots (Fig. 8). The lines intersected in each case at one common point above the ordinate against the reciprocal of the nonvaried substrate, providing some support for a sequential type of reaction mechanism (12). From replots of the intercepts on the ordinate against the reciprocal of the nonvaried substrate, the true Kₛ of 16.0 μM and 0.34 mg/ml (0.45 mM) for AdoMet and phosphatidyl-N,N-dimethylethanolamine, respectively, were obtained. The Vₘₐₓ is 0.88 nmol of phosphatidylethanolamine-min⁻¹·mg⁻¹. The last step in the synthesis of phosphatidylethanolamine from phosphatidylethanolamine was also inhibited competitively by S-adenosyl-L-homocysteine with an apparent Kₛ of 6.7 μM at a concentration of phosphatidyl-N,N-dimethylethanolamine of 0.85 mg/ml (1.1 mM) (Fig. 9).

Phosphatidylethanolamine inhibited the incorporation of [3H]-methyl groups from AdoMet into phosphatidyl-N,N-dimethylethanolamine and phosphatidyl-N,N-dimethylethanolamine. The rate of [3H]methyl transfer with phosphatidylethanolamine as substrate was too low to demonstrate end product inhibition unambiguously. However, the addition of 0.24 mg of phosphatidylethanolamine/ml (0.3 mM) inhibited the incorporation of radioactivity into phosphatidyl-N,N-dimethyllethanolamine by 56%, and into phosphatidyl-N,N-dimethyllethanolamine by 38%. In the assay mixtures, the maximum possible concentration of endogenous phosphatidylethanolamine was only 7.5 μg/ml. Thus, endogenous phosphatidylethanolamine would exert, if at all, only negligible end product inhibition.

DISCUSSION

This is the first report of a solubilization and partial purification of the phosphatidylethanolamine (phosphatidyl-N-monomethylethanolamine, phosphatidyl-N,N-dimethylethanolamine)-N-methyltransferase(s) from any mammalian source. Although the phosphatidylethanolamine methyl transferase was partially purified from dog lung, it seems that it was still associated with membrane fragments (6). We were unable to achieve purification to homogeneity even though many different approaches were tried. Nevertheless, our preparation after chromatography on octyl Sepharose CL 4B and Sepharose 6B was somewhat purified relative to the Triton X-100 extract and allowed us to characterize the N-methyltransferase activities for the conversion of phosphatidylethanolamine to phosphatidylethanolamine.

One question we tried to answer is whether or not the same enzyme from rat liver catalyzes all three methylations in the conversion of phosphatidylethanolamine to phosphatidylethanolamine. The three N-methyltransferase activities co-purified during all attempts at resolution and the ratio of specific activities for the methylation of phosphatidylethanolamine, phosphatidyl-N,N-monomethylethanolamine, and phosphatidyl-N,N-dimethylethanolamine remained constant within experimental error (Table I). The optimal pH was similar for the three transferase activities (Fig. 2) as was the inhibition by Triton X-100 (Fig. 3). Although there is some indication that a single enzyme is involved, it is unlikely that this question can be answered satisfactorily until the activities have been separated or a homogeneous enzyme preparation obtained that catalyzes all three methylations.

Data have been published which indicate that two N-methyltransferases for the synthesis of phosphatidylethanolamine from phosphatidylethanolamine are present in N. crassa (4) and in bovine adrenal medulla (7). One of the activities from the bovine adrenal had a pH optimum of 6.5 and required Mg²⁺. Our preparations had low activity at pH 6.5 (Fig. 2) and were not stimulated by Mg²⁺.

The formation of products from the substrate phosphatidylethanolamine was consistent with the first methylation being rate-limiting for the synthesis of phosphatidylethanolamine (Fig. 4). In addition, the initial rate of phosphatidyl-N-nanomethylethanolamine formation from phosphatidylethanolamine was 7 to 9 times smaller than the N-methylation of phosphatidyl-N,N-monomethylethanolamine and phosphatidyl-N,N-dimethylethanolamine (Figs. 4, 6, and 8). These findings, together with the low levels of phosphatidyl-N,N-monomethylethanolamine and phosphatidyl-N,N-dimethylethanolamine in rat liver (8), support previous suggestions that in rat liver (1) and in bovine adrenal (7) the first step in the methylation pathway is rate-limiting.

Acknowledgment—We thank Pat Choy for a critical reading of the manuscript.

REFERENCES

References are found on p. 3890.
Phosphatidylcholine Biosynthesis by N-Methylation

SUPPLEMENT

Conversion of Phosphatidylethanolamine to
Phosphatidylcholine in This Line

Partial Purification and Characterization of the Enzymatic Activities
With the technical assistance of Lorraine Schneider

EXPERIMENTAL PROCEDURES

Materials - S-Adenosyl-L-[methyl-3H]choline (free of S-Adenosyl-L-[methyl-3H]choline and S-Adenosylmethionine) were ob-
tained from New England Nuclear Corp. (Boston, Mass.). S-
Adenosylmethionine and S-Adenosylmethionine were ob-
tained from Sigma Chemical Co. (St. Louis, Mo.). S-Adenosyl-
choline and phosphatidylcholine (plus lipids) were purchased from Bio-
Research Products (Cedar Crest, N. J.). S-Adenosyl-L-[methyl-
choline] was purchased from the National Institute of Ar-
thritis, Diabetes, and Digestive and Kidney Diseases (Beth-
esda, Md.). S-Adenosyl-L-[methyl-3H]choline was purchased from New
England Nuclear Corp. (Boston, Mass.).

Enzymatic Assays - Water was sterilized, 150-200 μl were main-
tained in the anaerobic chamber and tap water was sterilized in a
heating- and temperature-controlled room.

Preparation of Lipid Substrates - Lipid (4.3-μg) was
subtracted to each sample of the reaction mixture (0.3 ml) and
methylation was achieved for 0.5 hour at 35°C.

Decomposition of Phosphatidylethanolamine Activity - The
method is a modification of the procedure described by Topor and
Gomerov (1960). The reaction mixture was added to a micro-
scope slide, and the mixture was taken on the microscope slide
and examined under a microscope. The mixture was then
washed with 5 ml of chilled water.

Identification of Methylated Choline Activity - The
method is a modification of the procedure described by Topor and
Gomerov (1960). The reaction mixture was added to a micro-
scope slide, and the mixture was taken on the microscope slide
and examined under a microscope. The mixture was then
washed with 5 ml of chilled water.

Results

Solubilization of PM (N-Methyl)transferase (a) - Prelim-
inary experiments on the solubilization of PM (N-Methyl)trans-
ferase showed optimal activity for predition, soluble enzyme. (b) -
All attempts to solubilize the phosphatidylethanolamine methyl-
ating enzyme from the supernatant after centrifugation at 100,000 x g for 1
hour, but the activity eluted in the void volume was not identified as
being subsequently eluted at 100,000 x g for 1 hour.

If sufficient enzymes were mixed with the enzyme mixture in the
supernatant after centrifugation at 100,000 x g for 1 hour, the
activity eluted in the void volume was not identified as
being subsequently eluted at 100,000 x g for 1 hour.

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Supernatant Activity - Supernatant activity was eluted
from the void volume at 0.5 hour in the void volume. The
activity was eluted in the void volume after centrifugation at
100,000 x g for 1 hour. The activity in the void volume was
identified as being subsequently eluted at 100,000 x g for 1 hour.

Supernatant Activity - Supernatant activity was eluted
from the void volume at 0.5 hour in the void volume. The
activity was eluted in the void volume after centrifugation at
100,000 x g for 1 hour. The activity in the void volume was
identified as being subsequently eluted at 100,000 x g for 1 hour.

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Phosphatidylcholine Biosynthesis by N-Methylation

In a preliminary experiment, 5 ml of the void volume
was used and the activity in the void volume was not identified as
being subsequently eluted at 100,000 x g for 1 hour.

If sufficient enzymes were mixed with the enzyme mixture in the
supernatant after centrifugation at 100,000 x g for 1 hour, the
activity eluted in the void volume was not identified as
being subsequently eluted at 100,000 x g for 1 hour.

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Supernatant Activity - Supernatant activity was eluted
from the void volume at 0.5 hour in the void volume. The
activity was eluted in the void volume after centrifugation at
100,000 x g for 1 hour. The activity in the void volume was
identified as being subsequently eluted at 100,000 x g for 1 hour.
Phosphatidylcholine Biosynthesis by N-Methylation

FIG. 5. Effect of pH on the magnesium-dependent phosphorylation (MN-enzyme) of HMP (MN), and the incorporation of [1-14C]labeled NADPH into PC (CCP-enzyme). (A) Phosphorylation of HMP (MN) with the partially purified MN-enzyme. (B) Incorporation of [1-14C]labeled NADPH into PC (CCP-enzyme). The reaction mixtures contained HMP (MN), and [1-14C]labeled NADPH, respectively, and were incubated at 37°C for 30 min. The reaction was stopped by the addition of 5% trichloroacetic acid. The samples were then analyzed by chromatography on paper, and the radioactivity was determined by scintillation counting.

FIG. 6. Effect of NADPH on the phosphorylation of HMP (MN) by the MN-enzyme. The reaction mixtures contained HMP (MN), and various concentrations of NADPH, and were incubated at 37°C for 30 min. The reaction was stopped by the addition of 5% trichloroacetic acid. The samples were then analyzed by chromatography on paper, and the radioactivity was determined by scintillation counting.

FIG. 7. Effect of NADPH on the incorporation of [1-14C]labeled NADPH into PC (CCP-enzyme). The reaction mixtures contained [1-14C]labeled NADPH, and various concentrations of NADPH, and were incubated at 37°C for 30 min. The reaction was stopped by the addition of 5% trichloroacetic acid. The samples were then analyzed by chromatography on paper, and the radioactivity was determined by scintillation counting.

FIG. 8. Effect of NADPH on the incorporation of [1-14C]labeled NADPH into PC (CCP-enzyme). The reaction mixtures contained [1-14C]labeled NADPH, and various concentrations of NADPH, and were incubated at 37°C for 30 min. The reaction was stopped by the addition of 5% trichloroacetic acid. The samples were then analyzed by chromatography on paper, and the radioactivity was determined by scintillation counting.

FIG. 9. Effect of NADPH on the incorporation of [1-14C]labeled NADPH into PC (CCP-enzyme). The reaction mixtures contained [1-14C]labeled NADPH, and various concentrations of NADPH, and were incubated at 37°C for 30 min. The reaction was stopped by the addition of 5% trichloroacetic acid. The samples were then analyzed by chromatography on paper, and the radioactivity was determined by scintillation counting.
Phosphatidylcholine Biosynthesis by N-Methylation

**FIG. 3.** Formation of PC from PE. Incubations contained 45 μg phosphatidylethanolamine (Table 1) and 0.95 mg/ml PE. Experimental details are provided in the Methods section.

**FIG. 4.** Double reciprocal plots of initial velocity of PC synthesis as a function of PE substrate at different concentrations of the other substrate. In A, the fixed concentrations of PE were 0.02 (1), 0.0125 (2), 0.009 (3), 0.006 (4), and 0.009 (5). In B, the concentrations of PE were 0.006 (1), 0.009 (2), and 0.0125 (3). In C, the double reciprocal plot of concentrations of PE versus maximum reaction velocity is depicted. In D, the concentrations of PC from the above reaction are plotted. Other details are provided in the Methods section.