Purification and photoaffinity labelling of lipid methyltransferase from rat liver

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An enzyme that catalyses the three-step methylation of phosphatidylethanolamine to phosphatidylcholine as well as the methylation of fatty acids and that uses *S*-adenosylmethionine as the methyl donor has been purified about 200-fold from rat liver. Irradiation of the purified enzyme with a short-wavelength u.v. light in the presence of [*methyl*-³H]8-azido-*S*-adenosylmethionine followed by electrophoresis results in the incorporation of radioactivity into a single protein band of about 25 kDa. It is concluded that a single catalytic subunit catalyses the conversion of phosphatidylethanolamine into phosphatidylcholine and fatty acid methylation.

The transfer of labelled methyl groups from *S*-adenosylmethionine to lipids in liver microsomes were first described by Bremer & Greenberg (1961). The lipids that serve as substrate in this process are PtdEtn, PtdEtnMe, PtdEtnMe₂ and fatty acids (Alemany *et al.*, 1982). The synthesis of fatty acid methyl esters by methylation of fatty acids has been described in other systems (Zatz *et al.*, 1981). It is not known whether the same enzyme catalyses phospholipid methylation and fatty acid methylation. Furthermore, the question has arisen as to whether the conversion of PtdEtn into PtdCho is catalysed by one or two methyltransferases. Several workers using solubilized methyltransferase from rat liver microsomes (microsomal fraction) have, on the basis of kinetic data, concluded that probably a single enzyme is responsible for the conversion of PtdEtn into PtdCho (Rehbinder & Greenberg, 1965; Tanaka *et al.*, 1979; Schneider & Vance, 1979). However, Sastry *et al.* (1981) have concluded that, in rat liver microsomes, two enzymes are needed for the conversion of PtdEtn into PtdCho. The first enzyme incorporates the first methyl group and the second enzyme the remaining two methyl groups.

This conclusion was based on kinetic studies, Mg²⁺-dependence and optimum pH for the various substrates. Audubert & Vance (1983) have recently shown that the claim of two methyltransferases based only on kinetic data was not justified, since the conversion of PtdEtn into PtdEtnMe, PtdEtnMe into PtdEtnMe₂ and PtdEtnMe₂ into PtdCho have the same optimum pH and *K_m* for *S*-adenosylmethionine. To solve this question about the number of methyltransferases in rat liver, our approach has been to purify and to photoaffinity-label with [*methyl*-³H]-8-azido-*S*-adenosylmethionine the enzyme lipid methyltransferase from rat liver microsomes. The present data provide compelling evidence that only one enzyme catalyses the three-step methylation of PtdEtn into PtdCho and that this enzyme also catalyses the methylation of fatty acids. These results are of interest in view of the numerous reports implicating lipid methylation in a variety of physiological processes (reviewed by Mato & Alemany, 1983).

Experimental procedures

Materials

[*methyl*-³H]*S*-Adenosylmethionine (7.8 Ci/mmol) and [*methyl*-³H]8-azido-*S*-adenosylmethionine (80 Ci/mmol) were from New England Nuclear. Ultrasphere-Si columns were from Beckman. Products for electrophoresis and reagents for silver staining were from Bio-Rad. Lipid standards were from Sigma. Chaps was from Calbiochem and Triton X-100 was from Merck. DEAE-Sephacel

Abbreviations used: PtdEtn, phosphatidylethanolamine; PtdEtnMe, phosphatidyl-*N*-monomethylethanolamine; PtdEtnMe₂, phosphatidyl-*NN*-dimethylethanolamine; PtdCho, phosphatidylcholine; Chaps, 3-[(cholamidopropyl)dimethylammonio]propanesulphonic acid; SDS, sodium dodecyl sulphate; h.p.l.c., high-pressure liquid chromatography.

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and Blue-Sepharose CL6B were from Pharmacia. Ultrogel ACA-34 was from LKB. NCS tissue solubilizer was from Amersham International.

Standard enzyme assay

The enzyme reaction was monitored by measuring the formation of labelled lipids after incubation for 30 min at room temperature of 100 μM -[methyl- ^3H]S-adenosylmethionine (250 $\mu\text{Ci}/\mu\text{mol}$) with 400 μl of purified enzyme in 25 mM-Tris/HCl (pH 8.35)/5 mM- β -mercaptoethanol in a final volume of 440 μl . The reaction was stopped with 2 ml of chloroform/methanol/2M-HCl (6:3:1, by vol.) for lipid extraction (Castaño *et al.*, 1980). In those experiments in which lipid methylation was measured in the presence of exogenous lipid, the enzyme was sonicated for 10 s with phospholipid (20 $\mu\text{g}/\text{ml}$) in a Branson B-12 sonifier equipped with microtip set at position 3. Measurement of the incorporation of radioactivity into lipids was performed as previously described after drying the sample with N_2 at 60°C (Castaño *et al.*, 1980). The reaction was linear with time for at least 30 min under these conditions. Results are expressed as pmol of [^3H]methyl group transferred/min per mg of protein. Protein was determined as described by Bradford (1976).

Product identification

Products of the reaction of [methyl- ^3H]S-adenosylmethionine with the enzyme were examined by h.p.l.c. using two Beckman pumps equipped with an Ultrasphere-Si column (5 μm -diameter particles; 250 mm $\times$ 4.6 mm) and a u.v. (214 nm) recorder. After lipid extraction, samples were dried under a stream of N_2 , dissolved in chloroform and a 20 μl sample was injected on the column. The column was eluted with a linear gradient over a 15 min period starting with 96% (v/v) propan-2-ol/hexane (1:1, v/v) and 4% (v/v) water to 93% propan-2-ol/hexane (1:1, v/v) and 7% (v/v) water. The flow rate was 1 ml/min. Fractions were collected at 1 min intervals and counted for radioactivity. Recovery of the radioactivity applied on the column was 80–90%. PtdEtn, PtdEtnMe, PtdEtnMe₂, PtdCho and fatty acid methyl esters were separated from each other under these conditions in 40 min. The column was washed with 50 ml of starting solvent before injecting a new sample. Authentic lipid standards were used to calibrate the column.

Electrophoresis and determination of M_r

SDS/polyacrylamide-gel electrophoresis was performed with 10% (w/v)-acrylamide slab gels in a Tris/HCl/SDS buffer as described in *Bio-Rad Laboratories Bulletin 1024*. Proteins were silver-stained as described in *Bio-Rad Laboratories*

Bulletin 1089. Standards for M_r determination were: lysozyme (14 400), soya-bean trypsin inhibitor (21 500), carbonic anhydrase (31 000), ovalbumin (45 000), bovine serum albumin (66 200) and phosphorylase b (92 500).

Photoaffinity-labelling of lipid methyltransferase

Aliquots (20 μl) of lipid methyltransferase (about 30 μg of protein) in 25 mM-Tris/HCl, pH 7.0, were placed in a glass tube at 4°C in the presence of 20 μl of [methyl- ^3H]8-azido-S-adenosylmethionine (2 μCi) and incubated for 10 min. Photoaffinity labelling was performed at pH 7.0, since it has been reported that the affinity of the microsomal enzyme for S-adenosylmethionine is higher at this pH (Audubert & Vance, 1983). Irradiation was carried out for 10 min with a short-wavelength u.v. light (General Electric G1T58) located 10 cm from the surface of the solution. After irradiation, the sample was treated with 80 μl of trichloroacetic acid (10%, w/v). After 10 min incubation at 4°C the sample was centrifuged at 6000g, the supernatant removed and the pellet dissolved in 100 μl of sample buffer for SDS/polyacrylamide-gel electrophoresis containing β -mercaptoethanol. After boiling for 2 min, the sample was applied to 10% SDS/10%-polyacrylamide-gel electrophoresis and then electrophoresed as described above. After electrophoresis, the gel was washed three times at 3 h intervals with 10% (v/v) acetic acid and then cut into 2.5 mm pieces. Each piece received 1 ml of NCS/water (9:1, v/v) and, after incubation for at least 4 h at 37°C, was counted for radioactivity (Mato & Marin Cao, 1979).

Results and discussion

Purification procedure

The results from a typical purification are summarized in Table 1. All procedures were carried out at 4°C.

Step 1: solubilization. Rat liver microsomes were isolated as previously described (Alemany *et al.*, 1982) from normally fed Wistar rats (250–300 g) and resuspended at a density of about 5 mg of protein/ml in ice-cold 25 mM-Tris/HCl (pH 8.35)/5 mM- β -mercaptoethanol (buffer A) containing 0.3% Chaps. After 30 min incubation at 4°C the microsomes were centrifuged at 105 000g for 2 h at 4°C and the supernatant (solubilized microsomes) applied to a DEAE-Sephacel column.

Step 2: DEAE-Sephacel. The solubilized microsomes were applied to a DEAE-Sephacel column equilibrated with buffer A and eluted with a linear gradient of 0–0.6 M-NaCl in the same buffer (Fig. 1). A single peak of activity eluted at about 200 mM-NaCl. No other peaks of activity were observed when the gradient was continued up to 2 M-NaCl.

Table 1. Results from a typical purification of lipid methyltransferase

Samples were incubated with 100 μ M-S-adenosylmethionine under standard assay conditions as described in the Experimental procedures section. 'Homogenate' means the crude extract obtained by homogenation of rat liver as described by Alemany *et al.* (1982).

Purification step	Volume (ml)	Protein (mg/ml)	Activity (pmol of [³ H]methyl group transferred/min per mg)
Homogenate	140	28	40
Solubilized microsomes	51	4.1	252
DEAE-Sephacel	4	18	79
Ultrogel ACA-34	15	1.2	435
Blue Sepharose	9	0.05	8178

Fig. 1. Chromatography of solubilized rat liver microsomes on DEAE-Sephacel

The DEAE-Sephacel column (1.5 cm $\times$ 25 cm) was equilibrated in Tris/HCl (pH 8.35)/5 mM- β -mercaptoethanol at a rate of 35 ml/h. After application of the sample (50 ml) the column was eluted with a linear gradient 0–0.6 M-NaCl in the same buffer. Fractions (7 ml) were collected and lipid methyltransferase assayed in the presence of exogenous PtdEtnMe₂ as described in the Experimental procedures section. Fractions 55–68 were pooled.

Fig. 2. Chromatography of the concentrated peak obtained after DEAE-Sephacel on Ultrogel ACA-34

The Ultrogel column (1.5 cm $\times$ 90 cm) was equilibrated in 25 mM-Tris/HCl (pH 8.35)/5 mM- β -mercaptoethanol at a rate of 6 ml/h. The sample (4 ml) was eluted in the same buffer. Fractions (3 ml) were collected, and lipid methyltransferase was assayed in the presence of exogenous PtdEtnMe₂ as described in the Experimental procedures section. Fractions 19–24 were pooled.

The same profile of activity was observed whether the enzyme was assayed in the absence of exogenous lipid or with PtdEtn, PtdEtnMe or PtdEtnMe₂ (20 μ g/ml). Active fractions were concentrated about 20-fold by ultrafiltration through Amicon (PM 30; 55 mm-diameter) membrane.

Step 3: Ultrogel ACA-34. The Amicon-membrane-concentrated sample was applied on an Ultrogel ACA-34 column equilibrated with buffer A and eluted in the same buffer. Lipid methyltransferase activity eluted as a single peak of activity close to the void volume of the column (Fig. 2). The same profile of activity was observed whether the enzyme activity was assayed in the absence or presence of exogenous lipid (PtdEtn, PtdEtnMe or PtdEtnMe₂). Active fractions were pooled and made 150 mM with respect to NaCl.

Step 4: Blue Sepharose CL6B. The active fraction from the Ultrogel column made 150 mM with respect to NaCl was applied to a Blue Sepharose CL6B column equilibrated in buffer A

containing 150 mM-NaCl. The column was washed with the same buffer until the A_{280} of the eluate was close to zero. Then the column was eluted with buffer A containing 0.04% Triton X-100. Lipid methyltransferase activity was eluted as a single peak of activity (Fig. 3). Once again, the same profile of activity was observed in the absence or presence of exogenous lipid. Active fractions were pooled, concentrated about 3-fold by Amicon membrane ultrafiltration and used immediately for enzyme assay, product analysis by h.p.l.c., photoaffinity labelling and electrophoresis. The purified enzyme is a lipoprotein that contains about 0.4 mg of phospholipid/mg of protein. Phospholipid composition is about 60% PtdCho, 25% PtdEtn and 5% lyso-PtdCho and/or lyso-PtdEtn. These values are expressed as molar percentages of total phospholipid. The purified enzyme also contains about 50 μ g of free fatty acids/mg of protein.

Photoaffinity labelling

Phospholipid methyltransferase from rat liver microsomes has been partially purified (about 9-fold) by Schneider & Vance (1979). These authors used Triton X-100 to solubilize the enzyme. The disadvantage of this procedure is that Triton X-100 rapidly inactivates the enzyme. Since the enzyme purified by this procedure was inactive in the absence of exogenous substrate, an assay including exogenous lipid substrate and detergent was necessary to measure the increase in specific

activity with purification. Chaps solubilizes the enzyme and keeps it active for several days. Furthermore, the enzyme purified by this procedure retains lipids that serve as substrate. Therefore to calculate the increase in specific activity by our procedure (about 200-fold) the addition of exogenous lipid substrate is not obligatory. Sonication of the purified enzyme with PtdEtnMe or PtdEtnMe₂ (20 μ g/ml) increases lipid methyltransferase activity about 40%. This result agrees with previous results by other authors indicating that exogenous PtdEtnMe or PtdEtnMe₂ stimulates lipid methylation in rat liver microsomes (Bremer & Greenberg, 1961; Reh binder & Greenberg, 1965; Schneider & Vance, 1979; Hoffman & Cornatzer, 1981; Audubert & Vance, 1983).

The enzyme that we have purified has an M_r , as determined by gel filtration on Ultrogel ACA-34, of about 300000. However, several bands of smaller M_r are visible after SDS/polyacrylamide-gel electrophoresis of the active fractions eluted from the Blue Sepharose CL6B column (Fig. 4).

Fig. 3. Chromatography of the Ultrogel-purified sample on Blue-Sepharose CL6B

The column (2 cm $\times$ 4 cm) was equilibrated in 25 mM-Tris/HCl (pH 8.35)/150 mM-NaCl/5 mM- β -mercaptoethanol at a rate of 3 ml/h. The sample (15 ml) was applied in the same buffer and washed until the A_{280} was close to zero. At the time indicated by the arrow, the column was eluted with 25 mM-Tris/HCl (pH 8.35)/5 mM- β -mercaptoethanol/0.04% Triton X-100. Fractions (3 ml) were collected, and lipid methyltransferase was assayed, as described in the Experimental procedures section, in the presence of exogenous PtdEtnMe₂. Fractions 33–40 were pooled.

Fig. 4. Electrophoresis of the enzyme at different stages of purification in SDS/10% polyacrylamide-gel electrophoresis and after labelling by photoaffinity of the purified enzyme with [methyl-³H]8-azido-S-adenosylmethionine (a) Microsomes; (b) solubilized microsomes; (c) DEAE-Sepharose; (d) Ultrogel ACA-34; (e) Blue Sepharose CL6B; and (f) photoaffinity labelling of the purified enzyme. Electrophoresis and photoaffinity labelling of the purified enzyme were performed as described in the Experimental procedures section. The smaller peak of radioactivity moving with the Bromophenol Blue marker was also observed when [methyl-³H]8-azido-S-adenosylmethionine alone was incubated under standard photolabelling conditions and then subjected to electrophoresis. The M_r values of the protein standards are shown.

The same pattern of bands was observed if the sample was not boiled with β -mercaptoethanol before electrophoresis. A single band incorporated radioactivity by photoaffinity labelling of the purified enzyme with [methyl- ^3H]8-azido-S-adenosylmethionine (Fig. 4). On the basis of its mobility on SDS/polyacrylamide-gel electrophoresis, this band has an M_r of about 25000 and coincides with one of the bands that is revealed by silver staining (Fig. 4). These results, together with the observation that the purified enzyme does not require exogenous lipid for activity, strongly suggest that the enzyme is purified as an aggregate with other proteins and lipids. Photolabelling was also performed with total microsomal proteins, and several bands, including one with an M_r of about 25000, were labelled (results not shown). These results suggest the existence of different methyltransferases in rat liver microsomes. Thiol methyltransferase (Borchardt & Cheng, 1978) and carboxymethyltransferase (S. Alemany, M. A. Pajares, D. Marin Cao & J. M. Mato, unpublished work) activities are present in rat liver microsomes. Therefore this result cannot be used to suggest the existence of more than one lipid methyltransferase in rat liver microsomes. Incubation of the purified enzyme with [methyl- ^3H]8-azido-S-adenosylmethionine under the present conditions (incubation at 4°C and pH 7.0) did not result in significant transfer of [^3H]methyl group from the azido probe to lipids (result not shown). Therefore the incorporation of significant non-labelled adduct of S-adenosylhomocysteine to proteins, which could have affected our observations, seems very unlikely.

Incubation of the purified enzyme with [methyl- ^3H]S-adenosylmethionine in the absence of exogenous lipid results in the formation of labelled PtdEtnMe, PtdEtnMe₂, PtdCho and fatty acid methyl ester (Fig. 5). We have previously shown the synthesis of methyl esters of fatty acids by incubation of microsomes with S-adenosylmethionine (Alemany *et al.*, 1982). Furthermore, both the methylation of phospholipids and fatty acids were activated by MgATP and Ca^{2+} in the presence of calmodulin (Alemany *et al.*, 1982). These results, together with the observation that only one protein band is photolabelled in the presence of [methyl- ^3H]8-azido-S-adenosylmethionine, strongly indicate that only one enzyme catalyses the three-step methylation of PtdEtn to PtdCho and the synthesis of fatty acid methyl esters. Attempts to separate the purified enzyme into more than one component by chromatography on S-adenosylhomocysteine-agarose have been unsuccessful (S. Alemany, M. A. Pajares, D. Marin Cao & J. M. Mato, unpublished work). It could be argued that rat liver contains other lipid methyl-

Fig. 5. Analysis of methylated lipids by h.p.l.c. The purified enzyme was incubated with [methyl- ^3H]S-adenosylmethionine as described in the Experimental procedures section. The methylated lipids were isolated and analysed by h.p.l.c. under standard assay conditions. 1, 2, 3 and 4 indicate the elution time of, respectively, fatty acid methyl esters, PtdEtnMe, PtdEtnMe₂ and PtdCho.

transferases that are specific for certain lipids and that these enzymes were lost during purification. It could also be argued that we cannot exclude the possibility that one of the other proteins that are still present in the purified enzyme preparation is a second lipid methyltransferase that is not labelled by photoaffinity because of a much lower affinity for the S-adenosylmethionine. However, it is important to note that previous reports suggesting the existence of more than one lipid methyltransferase were based on kinetic data and on different optimum pH values for the various lipid substrates (reviewed by Mato & Alemany, 1983) and that at present there is strong evidence indicating that the claim of two methyltransferases in rat liver based only on these data is not justified (Audubert & Vance, 1983). Furthermore, it has been recently shown that the conversion of PtdEtn into PtdEtnMe, PtdEtnMe into PtdEtnMe₂, and PtdEtnMe₂ into PtdCho have the same K_m for S-adenosylmethionine (Audubert & Vance, 1983). Therefore it seems unlikely that a second methyltransferase was not photolabelled because of a much lower affinity for S-adenosylmethionine. When lipid methylation is measured with the enzyme modified by photolabelling in the presence of $100\ \mu\text{M}$ -S-adenosylmethionine as described in

Fig. 6. pH-activity profile for lipid methyltransferase. Portions of the purified enzyme were incubated with S-adenosylmethionine as described in the Experimental procedures section at various pH values. The buffer was 50mM-Tris/HCl.

the Experimental procedures section, no changes were observed in the h.p.l.c. profile of methylated lipids when compared with that shown in Fig. 5. We conclude that photolabelling is not specifically inhibiting any single methylation reaction (e.g. PtdEtn to PtdEtnMe). This observation strengthens our conclusion that only one enzyme converts PtdEtn into PtdCho. The enzyme purified by the present procedure has an optimum pH of about 9 (Fig. 6), which is in the same range as that reported for the microsomal enzyme (Audubert & Vance, 1983). The optimal pH was independent of the presence of exogenous PtdEtn, PtdEtnMe or PtdEtnMe₂ (results not shown), which agrees with previous results by other authors (Audubert & Vance, 1983) indicating that the conversion of PtdEtn into PtdEtnMe, PtdEtnMe into PtdEtnMe₂, and PtdEtnMe₂ into PtdCho have the same optimal pH. Finally, the enzyme that we have purified is not a thiol methyltransferase (Borchardt & Cheng, 1978), since the product of

this reaction would be volatile and our assay includes drying the sample at 60°C with N₂ before measuring the incorporation of radioactivity into lipids or h.p.l.c. analysis. Furthermore, thiol methyltransferase activity [measured as described by Borchardt & Cheng (1978)] was not detected with the purified enzyme (result not shown). In conclusion, the present results are very suggestive of the existence of one methyltransferase that catalyses the conversion of PtdEtn into PtdCho and fatty acid methylation in rat liver microsomes. This is of interest in view of the numerous reports of the existence of two lipid methyltransferases (reviewed by Mato & Alemany, 1983).

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