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Purification of phospholipid methyltransferase from rat liver microsomal fraction

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Phospholipid methyltransferase, the enzyme that converts phosphatidylethanolamine into phosphatidylcholine with *S*-adenosyl-L-methionine as the methyl donor, was purified to apparent homogeneity from rat liver microsomal fraction. When analysed by SDS/polyacrylamide-gel electrophoresis only one protein, with molecular mass about 50 kDa, is detected. This protein could be phosphorylated at a single site by incubation with [α-^{32}P]ATP and the catalytic subunit of cyclic AMP-dependent protein kinase. A less-purified preparation of the enzyme is mainly composed of two proteins, with molecular masses about 50 kDa and 25 kDa, the 50 kDa form being phosphorylated at the same site as the homogeneous enzyme. After purification of both proteins by electro-elution, the 25 kDa protein forms a dimer and migrates on SDS/polyacrylamide-gel electrophoresis with molecular mass about 50 kDa. Peptide maps of purified 25 kDa and 50 kDa proteins are identical, indicating that both proteins are formed by the same polypeptide chain(s). It is concluded that rat liver phospholipid methyltransferase can exist in two forms, as a monomer of 25 kDa and as a dimer of 50 kDa. The dimer can be phosphorylated by cyclic AMP-dependent protein kinase.

INTRODUCTION

Phospholipid methylation was first described in rat liver by Bremer & Greenberg (1961). This reaction consists in the transfer of methyl groups from *S*-adenosylmethionine to phospholipids. The phospholipids that serve as substrates are PtdEtn, PtdEtnMe and PtdEtnMe$_2$. Interest on this reaction has grown during the last few years as changes in phospholipid methylation have been related to cell activation (Hirata & Axelrod, 1980; Mato & Alemany, 1983; Mato, 1986). However, much less is known about the molecular structure of phospholipid methyltransferase, and the question remains open about how many such enzymes there are in mammalian cells (Mato *et al.*, 1984; Hirata, 1985). The enzyme from rat liver microsomal fraction (referred to below simply as 'microsomes') has been solubilized by treatment with a variety of detergents, the best results being obtained with CHAPS (Pajares *et al.*, 1984). The enzyme has been partially purified from rat liver microsomes (Pajares *et al.*, 1984). Photoaffinity labelling of the partially purified enzyme with the probe 8-azido-*S*-adenosylmethionine suggests that a protein of 25 kDa is the catalytic subunit of the enzyme (Pajares *et al.*, 1984). Furthermore, cyclic AMP-dependent phosphorylation of a 50 kDa protein present in the enzyme preparation leads to activation of the methylation reaction (Varela *et al.*, 1984). Phosphorylation of this 50 kDa protein has also been observed with intact hepatocytes in response to the addition of glucagon (Varela *et al.*, 1985) and vasopressin (Merida *et al.*, 1986), suggesting a modulatory role for this protein *in vivo*. The present paper describes a method for the purification of phospholipid methyltransferase from rat liver microsomes to apparent homogeneity and provides new data on its molecular structure.

EXPERIMENTAL

Materials

S-Adenosyl[*Me*-^{3}H]methionine (15 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were from Amersham International. Products for electrophoresis and reagents for silver staining were from Bio-Rad Laboratories. CHAPS was from Calbiochem. DEAE-Sephacel was from Pharmacia. Ultrogel AcA-34 was from LKB Produkter. LiBr was from Merck. Catalytic subunit of cyclic AMP-dependent protein kinase, leupeptin and benzamidine were from Sigma Chemical Co.

Standard enzyme assay

The enzyme reaction was monitored as described by Pajares *et al.* (1984) by measuring the formation of labelled phospholipids after incubation for 30 min at room temperature of 100 μM-*S*-adenosyl[*Me*-^{3}H]methionine (2.5 μCi/μmol) with 400 μl of purified enzyme in 25 mM-Tris/HCl buffer, pH 8.35, containing 5 mM-2-mercaptoethanol in a final volume of 440 μl. This pH is close to the optimum of about 9 that we have reported for the solubilized partially purified enzyme (Pajares *et al.*, 1984). The reaction was stopped with 2 ml of chloroform/methanol/2M-HCl (6:3:1, by vol.) for lipid extraction (Castano *et al.*, 1980). In those experiments where phospholipid methylation was measured in the presence of exogenous phospholipids, lipids were dissolved in chloroform, pipetted into glass tubes, dried under a stream of N_2 at room temperature and sonicated

Abbreviations used: PtdEtn, phosphatidylethanolamine; PtdEtnMe, phosphatidyl-*N*-monomethylethanolamine; PtdEtnMe$_2$, phosphatidyl-*NN*-dimethylethanolamine; PtdChol, phosphatidylcholine; CHAPS, [3-(cholamidopropyl)dimethylammonio]propane-1-sulphonate.

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for 10 s in a Branson B-12 Sonifier equipped with microtip set at position 3. Phospholipid concentration during the enzyme assay was 0.46 mg/ml. This amount is in the same range as that previously used by Schneider & Vance (1979). This procedure gave higher activities than when phospholipids sonicated for 10 min, in the presence or in the absence of 0.3% CHAPS, were added to the enzyme mixture. Measurement of the incorporation of radioactivity into lipids was performed as previously described after the sample had been dried under a stream of N_2 at 60 °C (Castano *et al.*, 1980). Results are expressed as nmol of [^{3}H]methyl group transferred/min per mg of protein. Protein was determined as described by Bradford (1976), with bovine plasma γ-globulin as standard. Products of the reaction of *S*-adenosyl[*Me*-^{3}H]methionine with the enzyme were examined by t.l.c. as described previously (Castano *et al.*, 1980).

Electrophoresis and determination of molecular mass

SDS/polyacrylamide-gel electrophoresis was performed with 10% (w/v) acrylamide slab gels in an SDS/Tris/HCl buffer as described in Bio-Rad Laboratories Bulletin 1024. Sample mixtures contained 5–50 μg of protein, 2% (w/v) SDS, 10% (v/v) glycerol, 0.0015% Bromophenol Blue, 2.5 mM-2-mercaptoethanol, 30 mM-dithiothreitol and 0.1 M-Tris/HCl buffer, pH 6.8, in a final volume of 120 μl. Sample mixtures were boiled for 2 min before application of 100 μl to the polyacrylamide gel. Unless otherwise stated, proteins were silver-stained as described by Bio-Rad Laboratories Bulletin 1089. Standards for determination of molecular mass were: lysozyme (14.4 kDa), soya-bean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31.0 kDa), ovalbumin (45.0 kDa), bovine serum albumin (66.2 kDa) and phosphorylase *b* (92.5 kDa).

Elution of proteins from polyacrylamide gels was carried out with an ISCO model 1750 electrophoretic-sample concentrator. After electrophoresis, proteins were located by comparison with a stained gel. The gel that contained the band was cut out and placed in the concentration well of the sample cup of the apparatus. Then 40 mM-Tris/acetate buffer, pH 8.6, containing 2 mM-EDTA was added to the sample cup and electro-elution was carried out for 5.5 h at 1 W in 0.1 M-Tris/acetate buffer, pH 8.6, containing 2 mM-EDTA. The sample was then removed from the concentration well, freeze-dried and examined again by SDS/polyacrylamide-gel electrophoresis and silver staining as described above.

Protein phosphorylation

Phosphorylation of purified phospholipid methyltransferase by the catalytic subunit of cyclic AMP-dependent protein kinase was carried out basically as previously described (Varela *et al.*, 1984). The reaction mixture (70 μl) contained 25 mM-Tris/HCl buffer, pH 8.35, 5 mM-2-mercaptoethanol, 10 μM-MgATP (containing about 10^6 c.p.m. of [γ-^{32}P]ATP), purified enzyme (5 μg of protein), 60 mM-dithiothreitol and protein kinase (30 units/ml). After incubation for 10 min at 37 °C, the reaction mixture was stopped by the addition of 60 μl of a mixture containing 4% (w/v) SDS, 20% (v/v) glycerol, 0.002% Bromophenol Blue, 5.0 mM-2-mercaptoethanol and 0.1 M-Tris/HCl buffer, pH 6.8, and immediately boiled for 2 min. After this boiling, 100 μl of the mixture was used for separation of phosphoproteins by electrophoresis on SDS/polyacrylamide gels as described above. After electrophoresis, the gel was dried and phosphoproteins were located by autoradiography with a Kodak X-Omat-GRS film and intensifier screen at −70 °C during 6 days. As mentioned by the supplier of the protein kinase, the concentration of dithiothreitol during protein phosphorylation was 60 mM to stabilize enzymic activity.

Analysis of ^{32}P-labelled trypic peptides

Purified phospholipid methyltransferase was phosphorylated as described above. Analysis of ^{32}P-labelled peptides was carried out basically as previously described (Villalba *et al.*, 1985). Briefly, after phosphorylation the enzyme was precipitated with acetone at −20 °C. Acetone was then removed by centrifugation and the pellet dried under a stream of N_2. After being dried, the pellet was resuspended into 50 mM-ammonium bicarbonate buffer, pH 7.7, and dialysed overnight against the same buffer. After dialysis, trypsin was added (trypsin/protein ratio 1:20, w/w) and the mixture was incubated for 1 h at 37 °C. After this incubation trypsin was again added (trypsin/protein ratio 1:40, w/w) and the mixture was incubated for 3 h at 37 °C. After this trypsin treatment the solution was freeze-dried, and this freeze-dried material was dissolved in a solution containing 9.2 M-urea, 5% (w/v) 2-mercaptoethanol, 2% (v/v) Nonidet P-40, 4% (v/v) Ampholines (pH range 5–7) and 1% (v/v) Ampholines (pH range 3.5–10), and phosphopeptides were separated by isoelectrofocusing in 10% acrylamide gels with a non-equilibrium pH gradient from 3.5 to 10.0 as described previously (Thomas *et al.*, 1981). After electrophoresis, gels were cut into 2.5 mm pieces and their radioactivities counted with the aid of 3 ml of Normascint 22 scintillator cocktail (Scharlau). Standards for pI determination were amyloglucosidase (pI 3.50), soya-bean trypsin inhibitor (pI 4.55), lactoglobulin (pI 5.20), bovine carbonic anhydrase B (pI 6.85), horse myoglobin acidic band (pI 6.85), horse myoglobin basic band (pI 7.35), lentil lectin acidic band (pI 8.15), lentil lectin middle band (pI 8.45), lentil lectin basic band (pI 8.65) and trypsinogen (pI 9.30).

Analysis of ^{125}I-labelled tryptic peptides

After electro-elution, purified 25 kDa and 50 kDa proteins were dialysed overnight against water and freeze-dried. The freeze-dried proteins were iodinated basically as described by Bhown *et al.* (1980) in a medium containing 20 μl of 0.2 M-phosphate buffer, pH 7.5, 40 μl of water, 5 μl of Na^{125}I (0.5 mCi/μg) and 100 μl of freshly prepared chloramine-T (1 mg/ml). The amount of protein varied from 10 to 50 μg. The mixture was vortex-mixed for 1 min, 100 μl of sodium metabisulphite (1 mg/ml) was added, to terminate the reaction, and labelled proteins were diluted to 0.5 ml with 25 mM-Tris/HCl buffer, pH 8.3. After 5 min at 4 °C, 200 μl of bovine serum albumin (0.5 mg of protein) was added. Then the sample was applied on to a Bio-Gel P6 column (35 cm × 1 cm) equilibrated in 25 mM-Tris/HCl buffer, pH 8.3, containing 5 mM-2-mercaptoethanol. ^{125}I-proteins were eluted in the same buffer (10 ml/h), pooled and dialysed for 5 days against water. The water was changed at least 20 times. After dialysis, the ^{125}I-proteins were precipitated with acetone at −20 °C. Acetone was removed by centrifugation and the pellet was dried under a stream of N_2 as described above. After

being dried, the pellet was incubated with trypsin as described above. Tryptic peptides were separated, as described by Villalba *et al.* (1985), by h.p.l.c. on a Nucleosil C18 column with a linear gradient over a 67 min period starting with 100% 10 mM-ammonium acetate buffer, pH 6.5, to 70% ammonium acetate/30% acetonitrile. The gradient was started after the column had been washed for 8 min with the starting buffer. At the end of the gradient the concentration of acetonitrile was raised to 100% in 1 min, and the column was washed under these conditions for 20 min. The flow rate was 1.5 ml/min. Chromatographic fractions were collected at 0.5 min intervals and the γ-radiation was counted.

RESULTS AND DISCUSSION

Purification of phospholipid methyltransferase

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

Steps 1–4: solubilization and chromatography on DEAE-Sephacel and Ultrogel AcA-34. Rat liver microsomes were isolated from normally fed Wistar rats (250–300 g) and solubilized in ice-cold 25-mM-Tris/HCl buffer, pH 8.35, containing 5 mM-2-mercaptoethanol and 0.3% CHAPS as previously described (Pajares *et al.*, 1984). The solubilized microsomes were applied to a DEAE-Sephacel column and eluted with a linear NaCl gradient. The active peak was concentrated and further purified by chromatography on Ultrogel AcA-34 (Pajares *et al.*, 1984). CHAPS was only added during the solubilization step.

Step 5: LiBr incubation. The active peak after Ultrogel AcA-34 chromatography was incubated during 7 h in 1.8 M-LiBr in 25 mM-Tris/HCl buffer, pH 8.35, containing 22 mM-2-mercaptoethanol, 10.4 mM-$MgCl_2$, 7.3% (w/v) sucrose, 50 μg of leupeptin/ml and 1.0 mM-benzamidine at 4 °C. After incubation, the sample was centrifuged at 20000 *g* for 10 min, and the clear supernatant was used for a second chromatography through an Ultrogel AcA-34 column. The LiBr step activates the enzyme 5–6-fold (see Table 1). At present we do not have an explanation for this effect. This is not due to the increased addition of 2-mercaptoethanol, from 5 mM to 22 mM, or to changes in pH, since appropriate controls were run to test these possibilities. However, since the enzyme prepared after step 4 is a lipoprotein, it is possible that under these conditions *S*-adenosylmethionine did not have accessibility to all the molecules of enzyme. After LiBr treatment the enzyme is dissociated and *S*-adenosylmethionine might have access to more molecules of enzyme.

Step 6: second Ultrogel AcA-34 chromatography. The LiBr-treated and centrifuged sample was applied on to an Ultrogel AcA-34 column equilibrated with 25 mM-Tris/HCl buffer, pH 8.35, containing 5 mM-2-mercaptoethanol and 90 mM-LiBr. A single main peak of activity was eluted under these conditions (Fig. 1). In contrast with the results obtained with the first Ultrogel chromatography, where the enzyme was eluted close to the void volume (Pajares *et al.*, 1984), after LiBr treatment the enzyme was eluted with a molecular mass close to 50 kDa. To measure enzymic activity after LiBr treatment, the addition of exogenous phospholipids was necessary. Active fractions after step 6 were pooled, concentrated by ultrafiltration through an Amicon PM 10 filter (55 mm diameter) and used for enzyme assay, product analysis, electrophoresis and phosphorylation.

Properties of phospholipid methyltransferase

The activity of the purified enzyme with each phospholipid substrate is shown in Table 2. The purified enzyme can use PtdEtn, PtdEtnMe or $PtdEtnMe_2$ as substrate. Since after SDS/polyacrylamide-gel electrophoresis the purified enzyme comprises only one protein of molecular mass 50 kDa (see below), the present results support the concept that, in the rat liver, a single enzyme catalyses the conversion of PtdEtn into PtdChol. The enzymic activity of the purified enzyme is highest with $PtdEtnMe_2$ and lowest with PtdEtn. These results agree with previous data (Schneider & Vance, 1979) indicating that the first methylation reaction is the rate-limiting step for the synthesis of PtdChol by the transmethylation pathway. Products of the reaction of the purified enzyme

Table 1. Results from a typical purification of phospholipid methyltransferase

Samples were incubated with 100 μM-*S*-adenosylmethionine under standard assay conditions as described in the Experimental section. 'Homogenate' refers to crude extract obtained by homogenization of rat liver as described by Pajares *et al.* (1984). Phospholipid methyltransferase was assayed in the presence of exogenous phospholipids in steps 5 and 6. Equal amounts of PtdEtn, PtdEtnMe and $PtdEtnMe_2$, to give a final concentration of 0.46 mg of phospholipid/ml, were added as described in the Experimental section. No significant activity was observed in steps 5 and 6 if exogenous phospholipids were not added.

Purification step	Volume (ml)	Protein (mg/ml)	Activity (nmol of [^{3}H]methyl group transferred/min per mg)	Purification (fold)	Total activity recovered (nmol/min)	Yield (%)
1. Homogenate	600	28	0.062	1	1041.6	100
2. Solubilized microsomes	270	4.9	0.316	5.1	418.1	40.1
3. DEAE-Sephacel	14	9.1	0.230	3.7	29.3	2.8
4. 1st Ultrogel AcA-34	10	4	1.4	23	56	5.4
5. LiBr incubation	60	0.5	9.8	158	294	28.2
6. 2nd Ultrogel AcA-34	50	0.005	273	4403	68.2	6.5

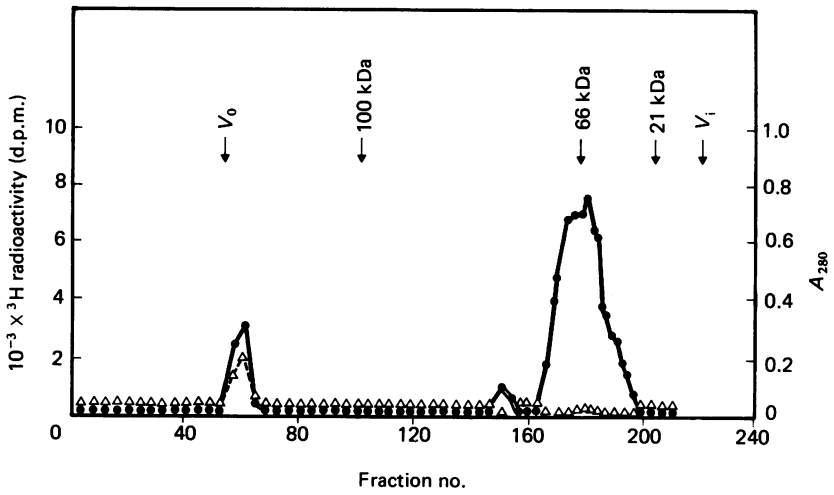

Fig. 1. Chromatography of the sample incubated with LiBr on Ultrogel AcA-34

The Ultrogel column (2.4 cm × 90 cm) was equilibrated in 25 mM-Tris/HCl buffer, pH 8.35, containing 5 mM-2-mercaptoethanol and 90 mM-LiBr at a rate of 15 ml/h. The sample was eluted in the same buffer. Fractions (2.5 ml) were collected and phospholipid methyltransferase activity (●) was assayed in the presence of exogenous phospholipids as described in the Experimental section. Phospholipid concentration during the enzyme assay was 0.46 mg/ml. The phospholipid mixture contained equal amounts of PtdEtn, PtdEtnMe and $PtdEtnMe_2$. No activity was observed in the absence of exogenous phospholipids. Protein (A_{280}, △) was also monitored. Markers for determination of molecular mass were bovine serum albumin (66 kDa), a protein that contaminates bovine serum albumin (whose molecular mass as determined by SDS/polyacrylamide-gel electrophoresis, is 100 kDa) and soya-bean trypsin inhibitor (21 kDa). V_0 was determined with Dextran Blue (approx. 2 MDa), and V_i with *S*-adenosyl[*Me*-^{3}H]methionine. Fractions 164–196 were collected, pooled and concentrated by ultrafiltration through an Amicon PM 10 filter (55 mm diameter).

Table 2. Activity of purified phospholipid methyltransferase for each phospholipid substrate

Phospholipid methyltransferase was assayed in the presence of exogenous phospholipids as described in the Experimental section. Purified enzyme (step 6 of Table 1) was used in all experiments. The final concentration of phospholipid added was 0.46 mg/ml. Phospholipid mixture means a mixture of equal amounts of PtdEtn, PtdEtnMe and $PtdEtnMe_2$. Abbreviation: N.D., not detectable.

Substrate	Activity (nmol/min per mg of protein)
PtdEtn	79.1
PtdEtnMe	116.2
$PtdEtnMe_2$	235.4
Phospholipid mixture	253.0
None	N.D.

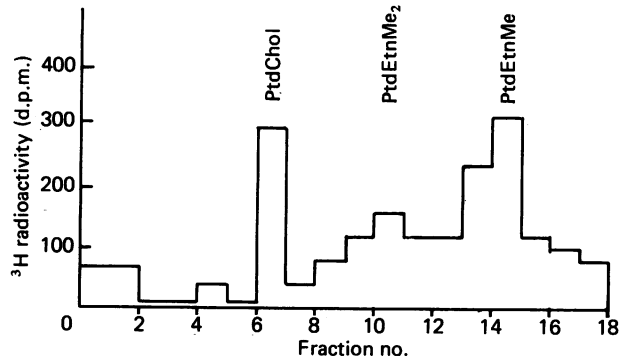

Fig. 2. Analysis of methylated phospholipids by t.l.c.

The purified enzyme was incubated with *S*-adenosyl[*Me*-^{3}H]methionine and a mixture of equal amounts of PtdEtn, PtdEtnMe and $PtdEtnMe_2$ to give a final concentration of 0.46 mg of phospholipid/ml. Further details are provided in the Experimental section. The amount of radioactivity applied to the t.l.c. plate was about 2000 d.p.m. About 95% of this radioactivity was recovered after chromatography, and about 76% of the radioactivity chromatographed with standards of PtdEtnMe, $PtdEtnMe_2$ or PtdChol. Background radioactivity (60 d.p.m.) has been subtracted from all fractions.

with a mixture of PtdEtn, PtdEtnMe and $PtdEtnMe_2$ were analysed by t.l.c. As shown in Fig. 2, the labelled material migrated as PtdEtnMe, $PtdEtnMe_2$ and PtdChol. These results further support our hypothesis that a single enzyme catalyses the conversion of PtdEtn into PtdChol.

After SDS/polyacrylamide-gel electrophoresis and silver staining of the pooled active fractions eluted from the second Ultrogel column (step 6), only one protein, of molecular mass about 50 kDa, was detected (Fig. 3). Previously we had shown (Pajares *et al.*, 1984) the presence of a 25 kDa protein that was labelled by incubation of the partially purified enzyme with the probe 8-azido-*S*-adenosylmethionine. These results contrast with the present data showing that the purified enzyme consists of a single protein of molecular mass 50 kDa. The following experiments were carried out to explain this apparent discrepancy. After the first Ultrogel chromatography (step 4), and consistent with our previous observations (Varela *et al.*, 1984), the

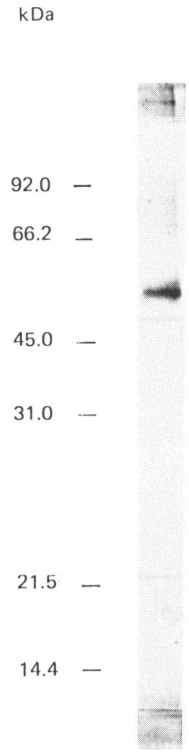

Fig. 3. Electrophoresis of the purified enzyme in SDS/10%-polyacrylamide gels

A portion of the pooled active fractions (about 8 μg of protein) eluted from the second Ultrogel column (step 6) was dialysed overnight against water and concentrated by freeze-drying. The dried sample was then dissolved into 30 μl of 25 mM-Tris/HCl buffer, pH 8.35, containing 5 mM-2-mercaptoethanol and used for electrophoresis. After electrophoresis proteins were detected by silver staining. The molecular masses of the protein standards are shown.

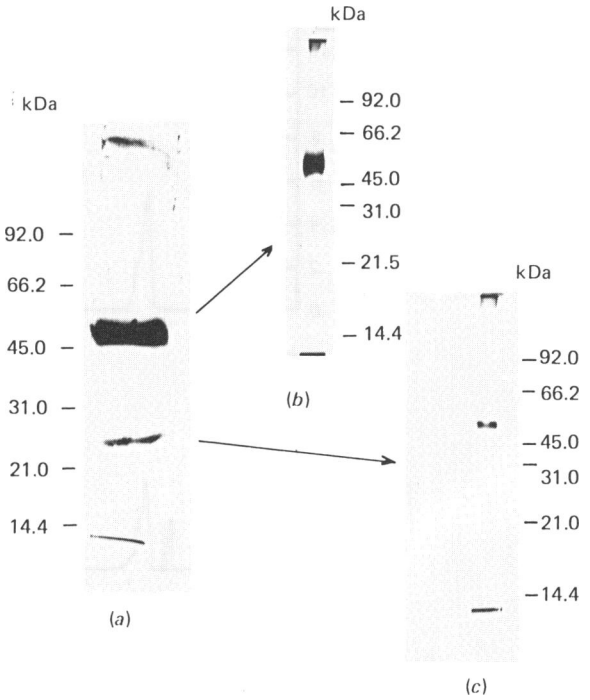

Fig. 4. Electro-elution of the 50 kDa and 25 kDa proteins of the enzyme purified after step 1 of the present procedure

After step 4 of the present purification procedure the enzyme consists mainly of two proteins of molecular mass 50 kDa and 25 kDa (*a*). Both proteins were electro-eluted and concentrated as described in the Experimental section. After electro-elution, both proteins were examined again by SDS/polyacrylamide-gel electrophoresis. (*b*) Electrophoresis of the 50 kDa protein after electro-elution. (*c*) Electrophoresis of the 25 kDa protein after electro-elution. The molecular masses of the protein standards are shown. The amount of protein loaded in lanes (*a*), (*b*) and (*c*) was respectively 50 μg, 5 μg and 5 μg. In (*a*) proteins were detected with Coomassie Blue R-250, and in (*b*) and (*c*) by silver staining.

enzyme preparation mainly consists of two proteins, of molecular masses 50 kDa and 25 kDa (Fig. 4*a*). Both proteins were purified by electro-elution and examined again by SDS/polyacrylamide-gel electrophoresis and silver staining. After electro-elution, the 25 kDa protein migrated with a molecular mass of 50 kDa (Fig. 4*b*), whereas the 50 kDa protein maintained its molecular-mass position (Fig. 4*c*). These results indicate that the 25 kDa protein can form a 50 kDa dimer. To confirm this hypothesis we have made peptide maps of the 25 kDa and 50 kDa proteins purified by electro-elution. After electro-elution, both proteins were iodinated, then treated with trypsin, and the ^{125}I-peptides were separated by h.p.l.c. As shown in Fig. 5, ^{125}I-peptide maps of both proteins are identical. The same results were obtained when the ^{125}I-peptides were separated by isoelectrofocusing (results not shown). These results indicate that both the 25 kDa and 50 kDa proteins are composed of the same polypeptide chain(s).

LiBr treatment largely affects phospholipid methyltransferase structure. Before LiBr treatment, the enzyme shows a large molecular mass, as determined by gel filtration on Ultrogel AcA-34, and contains endogenous phospholipids that serve as substrates. After LiBr treatment, the enzyme has a much smaller molecular mass and requires exogenous phospholipids for activity. Since before electrophoresis samples are boiled in the presence of 2.5 mM-2-mercaptoethanol and 30 mM-dithiothreitol, it is not likely that association is due to disulphide-bond formation. Furthermore, it is important to note that the 25 kDa protein is not associating as a consequence of the absence of detergents, since before electrophoresis samples are boiled in 2% SDS and the acrylamide gel contains 0.2% SDS. It can be argued that association of the 25 kDa protein might not occur in biological membranes and that the 50 kDa form of the enzyme might not exist *in vivo*. However, there is evidence indicating that the 50 kDa form of phospholipid methyltransferase does exist in intact cells. When hepatocytes prelabelled with [^{32}P]phosphate and stimulated with glucagon or vasopressin are immunoprecipitated with an antiserum that recognizes both the 25 kDa and the 50 kDa forms of the enzyme, only the 50 kDa protein appears to be phosphorylated (Varela *et al.*, 1985; Merida *et al.*, 1986). Since the 25 kDa protein is not phosphorylated, association must have occurred in the intact cell. Similarly, in a partially purified active

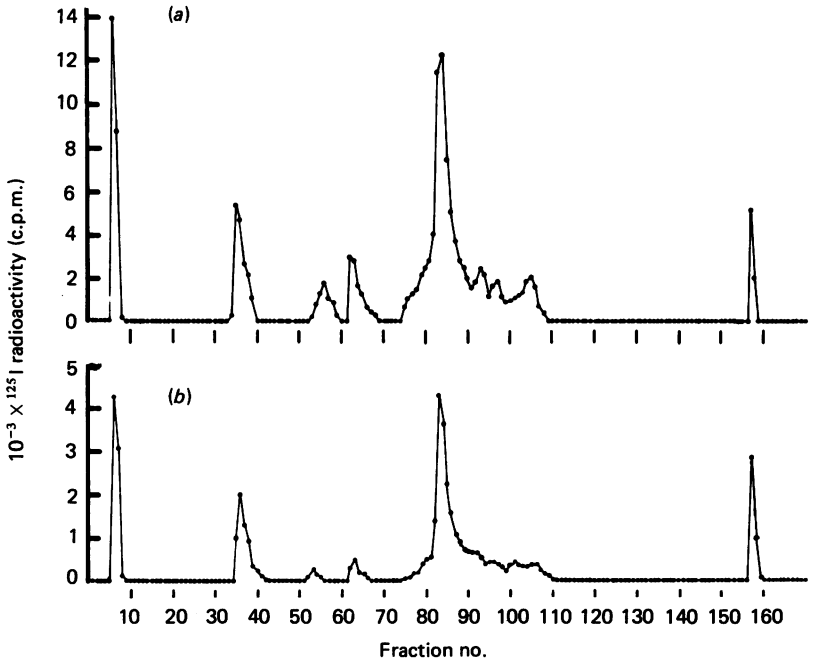

Fig. 5. Comparison of the peptide maps of the 25 kDa and 50 kDa proteins purified by electro-elution

After step 4 of the present purification procedure, phospholipid methyltransferase consists mainly of two proteins of molecular mass 25 kDa and 50 kDa (see Fig. 4). Both proteins were purified by electro-elution and ^{125}I-labelled as described in the Experimental section. After iodination, both proteins were digested with trypsin and tryptic ^{125}I-peptides were separated by h.p.l.c. as described in the text. In (*a*) 50 μg of the 50 kDa protein was treated with trypsin; in (*b*) 10 μg of the purified 25 kDa protein was treated with trypsin. Radioactivity in the first peak (fractions 6–7) of both parts of the Figure is due to ^{125}I-iodine. The relative amounts of radioactivity present in the other peaks were similar in both (*a*) and (*b*).

phospholipid methyltransferase, which when analysed by SDS/polyacrylamide-gel electrophoresis contains both the 25 kDa and the 50 kDa proteins, only the 50 kDa protein is phosphorylated by cyclic AMP-dependent protein kinase (Varela *et al.*, 1984). Since this preparation is a lipoprotein, containing PtdChol and PtdEtn (Pajares *et al.*, 1984), these results indicate that association is possible in a situation similar to that of the native membrane form.

Previously we have shown that only the 25 kDa protein of phospholipid methyltransferase reacted with 8-azido-*S*-adenosylmethionine (Pajares *et al.*, 1984). This result contrasts with the present observation, which indicates that the 25 kDa form of phospholipid methyltransferase forms a 50 kDa dimer. If this is correct, the 50 kDa form should also bind *S*-adenosylmethionine. The concentration of 8-azido-*S*-adenosylmethionine used by Pajares *et al.* (1984) was about 0.140 μM. At this low concentration of substrate, if the affinity for this molecule is higher in the 25 kDa form than in the 50 kDa form of the enzyme, only the 25 kDa form would be labelled. Experiments should be carried out at higher doses of 8-azido-*S*-adenosylmethionine to test this possibility. Unfortunately, at present only the labelled compound is commercially available, and experiments with 10–100 μM concentrations of 8-azido-*S*-adenosylmethionine are not possible.

Phosphorylation of purified phospholipid methyltransferase was also determined by incubating the enzyme with the catalytic subunit of cyclic AMP-dependent protein kinase and [γ-^{32}P]ATP. As shown in Fig. 6, phospholipid methyltransferase was phosphorylated under these conditions. To obtain phosphorylation, it was necessary to remove LiBr by dialysis. After its removal, no methylation activity could be detected with the dialysed enzyme. Addition of 0.3% CHAPS, exogenous phospholipids or a combination of both did not stabilize or re-activate the enzyme. Therefore at present the effect of protein phosphorylation on phospholipid methyltransferase activity could not be tested. Previously we have shown (Villalba *et al.*, 1985) that the 50 kDa protein of the partially purified active enzyme is phosphorylated at a single site by the catalytic subunit of cyclic AMP-dependent protein kinase, and that phosphorylation stimulates the enzyme activity about 4-fold (Varela *et al.*, 1984). As shown in Fig. 7, the site of phosphorylation of the purified enzyme (after step 6) is the same as the site of phosphorylation obtained with the partially purified native enzyme (step 4). These results strongly indicate that phosphorylation is a property of phospholipid methyltransferase and not a consequence of inactivation by the removal of LiBr or detergents. In favour of this conclusion is also the observation that the 50 kDa protein of phospholipid methyltransferase is phosphorylated in response to glucagon (Varela *et al.*, 1985) and vasopressin (Merida *et al.*, 1986).

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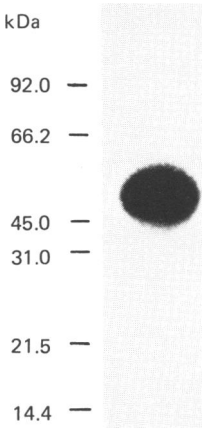

Fig. 6. Autoradiograph showing the phosphorylation of purified phospholipid methyltransferase by the catalytic subunit of cyclic AMP-dependent protein kinase

A portion of the pooled active fractions (about 5 μg of protein) eluted from the second Ultrogel AcA-34 column (step 6) was dialysed overnight against water and concentrated by freeze-drying. The dried sample was then dissolved into 30 μl of 25 mM-Tris/HCl buffer, pH 8.35, containing 5 mM-2-mercaptoethanol and phosphorylated during 10 min at 37 °C with the catalytic subunit of cyclic AMP-dependent protein kinase as described in the Experimental section. After phosphorylation, the enzyme was subjected to SDS/polyacrylamide-gel electrophoresis. The gel was then dried and autoradiographed for 6 days at −70 °C. The molecular masses of the protein standards are shown.

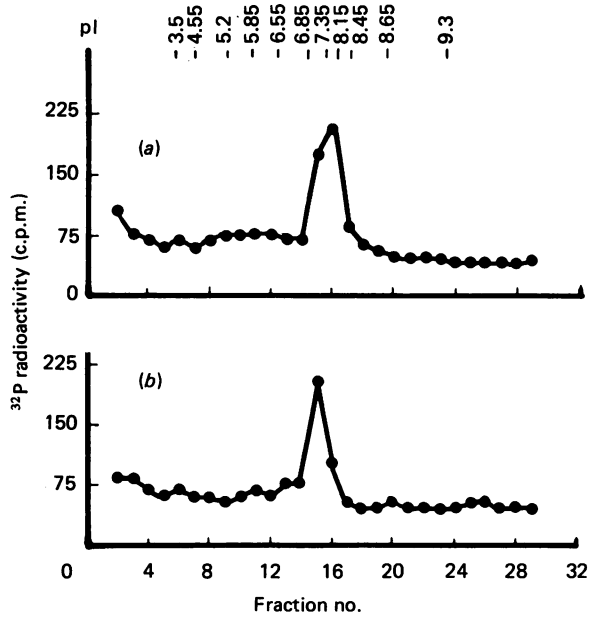

Fig. 7. Comparison of the site of phosphorylation after steps 4 and 6 of the present purification procedure

Samples of phospholipid methyltransferase purified after steps 4 and 6 (see Table 1) were used for phosphorylation. Phosphorylation was carried out for 10 min as described in the Experimental section. After phosphorylation, proteins were treated with trypsin and ^{32}P-labelled tryptic peptides were separated by isoelectrofocusing as described in the text. (*a*) and (*b*) show the results obtained with the enzyme purified after steps 6 and 4 respectively. Approximately the same amount of radioactivity was applied in both (*a*) and (*b*). The pI values of the protein standards are shown.

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