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Phosphorylation of partially purified 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine:acetyl-CoA acetyltransferase from rat spleen

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A new improved method for purification of the enzyme 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine:acetyl-CoA acetyltransferase (EC 2.3.1.67) from rat spleen is described. The catalytic subunit of cyclic AMP-dependent protein kinase in the presence of MgATP stimulated about 3-fold the activity of this partially purified enzyme activity. When [γ-^{32}P]ATP was included in the assay mixture, the analysis of phosphoprotein products by SDS/polyacrylamide-gel electrophoresis and autoradiography showed the incorporation of [^{32}P]phosphate into a single protein band of about 30 kDa. Analysis of the phosphorylated amino acids indicated that the phosphate was incorporated into a serine residue. Activation of the acetylation reaction by the protein kinase was reversible. The reversal of the activation was coincident with the loss of the [^{32}P]phosphate incorporated into the 30 kDa protein band, which suggests that the acetyltransferase is regulated by a phosphorylation–dephosphorylation mechanism dependent on cyclic AMP.

INTRODUCTION

Platelet-activating factor (paf-acether) is a potent lipid mediator involved in a great variety of physiological and pathological events. Paf-acether is produced following activation of a variety of inflammatory cells and it can be found in various organic fluids [1]. The final steps in the biosynthesis of this autacoid are known to occur by two independent enzymatic routes: 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine:acetyl-CoA acetyltransferase (EC 2.3.1.67) and dithiothreitol-insensitive CDP-choline:1-*O*-alkyl-2-acetyl-*sn*-glycerol cholinephosphotransferase (EC 2.7.8.16). Although there has recently been reported a complete '*de novo*' pathway for the formation of 1-*O*-alkyl-2-acetyl-*sn*-glycerol precursors of paf-acether synthesis by the latter pathway [2], special attention has been paid to the acetyltransferase as the limiting step in the synthesis of paf-acether [3–6], because it is stimulated by a variety of specific secretagogues that generate inflammatory responses in human neutrophils, eosinophils and peritoneal and alveolar macrophages. By now, some information has been accumulated about molecular aspects of acetyltransferase regulation. Peritoneal macrophages require entry of calcium ions into the cytosol prior to acetyltransferase activation [7]. Microsomal rat spleen acetyltransferase is modulated by calcium ions at submicromolar concentrations [8] and it seems to be activated by reversible phosphorylation, possibly involving protein kinases C [9] or A [10]. Acetyltransferase has been located in an intracellular membrane of the polymorphonuclear leucocyte, probably the endoplasmic reticulum [11], and it has been recently partially purified and characterized from rat spleen [12]. The enzyme has a K_m of 137 μM for its substrate acetyl-CoA and it has an apparent molecular size of about 30 kDa.

In the present paper, we have tried to ascertain whether our proposed mechanism of acetyltransferase modulation in the microsomal fraction [10] is likely to occur in the partially purified enzyme as well. In this paper we describe an improvement of the method for acetyltransferase purification and show that a partially purified preparation of acetyltransferase activity can be converted from a low-activity dephosphorylated form to a high-activity phosphorylated form by a cyclic AMP-dependent mechanism.

MATERIALS AND METHODS

Materials

Lyso-paf-acether was purchased from Bachem Feinchemikalien, Switzerland. Acetyl-CoA and alkaline phosphatase were from Boehringer Mannheim, Germany (1 unit of alkaline phosphatase hydrolyses 1 μmol of *p*-nitrophenyl phosphate/min at pH 10.4 at 37 °C). The catalytic subunit of the cyclic AMP-dependent protein kinase from bovine heart (1 unit of the kinase transfers 1 pmol of phosphate/min from [γ-^{32}P]ATP to hydrolysed and partially dephosphorylated casein at pH 6.5 at 30 °C), heat-stable inhibitor of protein kinase from porcine heart, ATP, dithiothreitol and 2-mercaptoethanol were purchased from Sigma Chemical Co. Cellulose plates for high-voltage electrophoresis were from Merck, Germany. Ultrogel AcA 44 was from LKB, Sweden. Products for electrophoresis, Coomassie Brilliant Blue, reagents for silver staining and reagents for protein determination were from Bio-Rad. [^{3}H]Acetyl-CoA (3.8 Ci/mmol) was from New England Nuclear,

Abbreviations used: paf-acether, platelet-activating factor (1-*O*-hexadecyl/octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine); lyso-paf-acether, lyso-platelet-activating factor; protein kinase A, cyclic AMP-dependent protein kinase; PAGE, polyacrylamide-gel electrophoresis.

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and [γ-^{32}P]ATP (3000 Ci/mmol) was from Amersham International. All other reagents and biochemicals were of the highest analytical grade available.

Enzyme purification

Acetyltransferase was purified as previously reported [12] with a slight modification. Briefly, rat spleen microsomes were obtained by a two-step centrifugation, solubilized with 0.4% deoxycholate at 4 °C and ultracentrifuged at 105000 *g*. Solubilized microsomes were incubated with $(NH_4)_2SO_4$ at a concentration of 145 g/l. The precipitated proteins were collected by centrifugation, resuspended in 20 mM-Tris/HCl buffer and dialysed against the same buffer. The $(NH_4)_2SO_4$-treated microsomes were applied to an Ultrogel AcA-22 column. Fractions containing enzyme activity were applied to a DEAE-Sepharose CL 6B column and eluted with a linear gradient of NaCl. The active peak was loaded into a 6-carboxyhexyl–Sepharose 4B column and developed with a linear gradient of NaCl. The last step of purification previously described [12] (thiopropyl–Sepharose 6B chromatography) was replaced by a treatment with 2 M-KBr plus 0.04% deoxycholate in 20 mM-Tris/HCl buffer, pH 7.4, for 7 h at 4 °C. After centrifugation at 10000 *g* for 15 min the supernatant was applied to an Ultrogel AcA 44 column equilibrated with 20 mM-Tris/HCl buffer, pH 7.4, containing 5 mM-2-mercaptoethanol and 100 mM-KBr and eluted with the same buffer. The peak of activity with molecular size about 25–50 kDa was pooled, dialysed against the same buffer without KBr, concentrated and utilized in the phosphorylation experiments. Experiments in parallel were carried out with the partially purified enzyme obtained from the 6-carboxyhexyl–Sepharose 6B without this latter treatment.

Assay of acetyltransferase activity

The acetylation reaction was performed in 20 mM-Tris/HCl buffer, pH 7.4, containing 5 mM-2-mercaptoethanol, 10 μg of lyso-paf-acether, 0.5 μCi of [^{3}H]acetyl-CoA and 200 μl of the fractions obtained in the Ultrogel AcA-44 chromatography. This medium was used only with the purpose of determining the enzyme activity peaks. The enzyme reaction was carried out for 15 min at 37 °C, stopped by the addition of 3.7 ml of chloroform/methanol (1:2, v/v) and treated as indicated below.

For the assays with protein kinase A, the medium contained 20 mM-Tris/HCl, pH 7.4, 17.5 mM-dithiothreitol and 60 units of protein kinase A in a final volume of 1 ml. After incubation with protein kinase A, protein kinase inhibitor (90 μg/ml) was added immediately before starting the enzyme assay. The acetylation reaction was carried out with the addition of 10 μg of lyso-paf-acether, 0.5 μCi of [^{3}H]acetyl-CoA and 100 μM-acetyl-CoA. The mixture was incubated for 15 min at 37 °C and stopped by the addition of 3.7 ml of chloroform/methanol (1:2, v/v) for lipid extraction [4]. Products of the enzyme reaction were separated by t.l.c. and the paf-acether formed was quantified as previously described [4]. Protein concentration was determined by the method of Bradford [13].

Phosphorylation of acetyltransferase

The standard phosphorylation assay of acetyltransferase by the catalytic subunit of protein kinase A was carried out at 37 °C in a reaction mixture (60 μl) containing 20 mM-Tris/HCl, pH 7.4, 5 mM-2-mercaptoethanol, 10 μM-MgATP, 1 μCi of [γ-^{32}P]ATP, 40 μl of purified enzyme (20–30 μg of protein), 60 mM-dithiothreitol and the catalytic subunit of protein kinase A (60 units/ml). The phosphorylation reaction was started by addition of radioactive ATP and was terminated with 60 μl of a 'SDS-stop' solution containing 4% SDS, 20% glycerol, 0.02% Bromophenol Blue and 0.16 M-Tris/HCl, pH 6.8, immediately followed by boiling for 5 min. Samples were then centrifuged at 10000 *g* for 2 min in a Beckman Microfuge (model 11). The separation of [^{32}P]phosphate-labelled proteins was carried out by 12.5% acrylamide slab gels in the presence of SDS in Tris/HCl/SDS buffer as described by Bio-Rad Laboratories (Bulletin 1024). In the same gel were electrophoresed samples of partially purified acetyltransferase in the absence of both protein kinase A and [γ-^{32}P]ATP in order to identify by silver staining the protein bands. Portions of the gel for the identification of phosphoproteins were dried by vacuum and heat. [^{32}P]Phosphate-labelled proteins were located by autoradiography using a Kodak XOmat-GRS film and intensifier screens (Dupont Cronex Lightning Plus XL) after exposure for 5 days at −80 °C. Autoradiograms were quantified by densitometry using a Beckman DU-8 spectrophotometer. Standards for molecular weight determination were: phosphorylase b (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

Estimation of the amount of protein in the different bands of SDS/PAGE

Densitometric scanning of Coomassie Brilliant Blue-stained gels was carried out in a DU-8 spectrophotometer (Beckman Instruments) at 550 nm. For this purpose, the amount of protein contained in each lane was measured prior to its application to the gel (usually 20–40 μg). After the electrophoresis, the protein content in each band was estimated assuming that a linear relationship existed between the amount of protein in a band and the quantity of dye bound and that all the bands showed a similar affinity for the dye [14].

Analysis of phosphoamino acids

After phosphorylation of the partially purified acetyltransferase with protein kinase A and [γ-^{32}P]ATP and electrophoresis, the piece of gel containing the 30 kDa protein was cut and subjected to hydrolysis in 6 M-HCl/1 mM-2-mercaptoethanol in a final volume of 0.5 ml for 2 h at 110 °C in evacuated sealed tubes. After hydrolysis, phosphoamino acids were separated by high voltage electrophoresis on cellulose plates by the procedure of Hunter & Sefton [15]. Phosphoamino acids were located by autoradiography using intensifier screens at −80 °C for 12 days. Phosphoserine, -threonine and -tyrosine were detected with ninhydrin.

RESULTS AND DISCUSSION

Acetyltransferase purification procedure

We have modified the procedure of purification of the acetyltransferase by using a chaotropic agent [16] like KBr in order to dissociate the aggregates of protein and lipids found in the first gel filtration chromatography

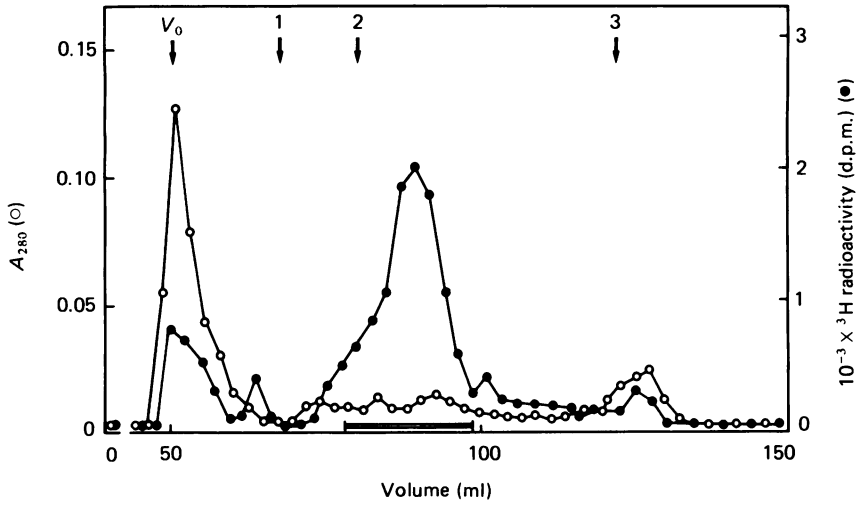

Fig. 1. Ultrogel AcA 44 column chromatography of the KBr-treated acetyltransferase

The Ultrogel column (1.6 cm × 90 cm) was equilibrated with 20 mM-Tris/HCl buffer, pH 7.4, containing 5 mM-2-mercaptoethanol and 100 mM-KBr. The sample incubated with KBr plus deoxycholate was applied and the column eluted with the equilibration buffer at a flow rate of 6 ml/h. Fractions (2 ml) were collected and 100 μl samples were assayed for protein (A_{280}, ○) and acetyltransferase activity (as radioactivity incorporated, ●). Fractions corresponding to between 25 and 50 kDa (bar) were pooled, dialysed and concentrated by ultrafiltration through an Amicon PM 10 filter. Arrows indicate markers used for the calibration of the column: V_0, Dextran 2000 (2 MDa); 1, bovine serum albumin (66 kDa); 2, ovalbumin (45 kDa); 3, cytochrome *c* (13 kDa).

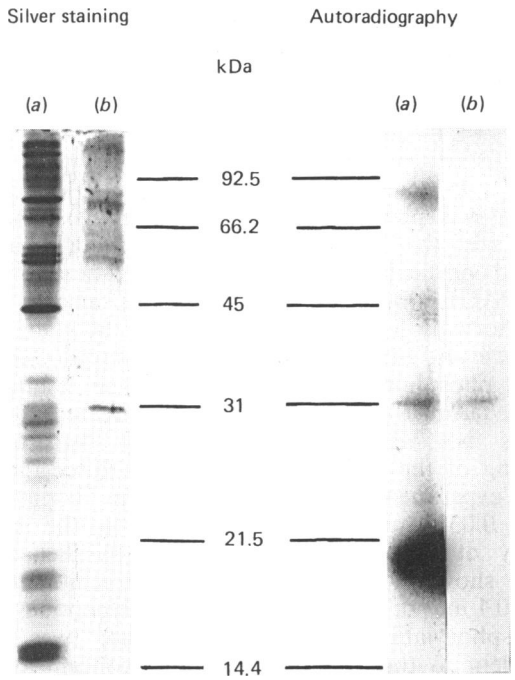

Fig. 2. SDS/PAGE and autoradiogram of partially purified acetyltransferase

Microsomes (*a*) and partially purified acetyltransferase (*b*) were incubated with protein kinase A in presence of [γ-^{32}P]ATP, subjected to SDS/PAGE and the proteins were silver-stained (left) as described in the Materials and methods section. An autoradiogram is shown (right) of the phosphorylated proteins analysed on 12.5% SDS/PAGE.

step (Ultrogel AcA 22) previously described [12]. The active peak after 6-carboxyhexyl–Sepharose chromatography was concentrated and incubated with 2 M-KBr and 0.04% deoxycholate. This high salt concentration

Table 1. Effect of the addition of the catalytic subunit of protein kinase on acetyltransferase activity

Purified acetyltransferase was incubated for 5 min at 37 °C in the presence of the additives indicated in the Table. At the end of this period, protein kinase inhibitor (90 μg/ml) was added and the acetyltransferase reaction was started by addition of the enzyme substrates. Results are expressed as the mean ± S.D. of three experiments in duplicate; 100% corresponds to 0.75 μmol of paf-acether/min per mg of protein.

Addition	Enzyme activity (% of control)
None	100
MgATP (10 μM)	86 ± 8
Protein kinase A (60 units/ml)	110 ± 11
MgATP (10 μM) + protein kinase A (60 units/ml)	280 ± 19

inhibited weakly the enzyme and the presence of the detergent was essential for the dissociating effect. The KBr-treated and centrifuged sample was applied to an Ultrogel AcA 44 column. As shown in Fig. 1, the highest peak of protein was found in the void volume, indicating that a great part of the enzyme remains as a protein coated with phospholipids and with a large molecular mass, but the main peak of activity was eluted at a molecular size of 25–50 kDa. Active fractions after this step were pooled, concentrated, dialysed and used for the phosphorylation experiments, although parallel experiments were carried out as well with the enzyme without KBr treatment. With this new step, the purification of the acetyltransferase was about 2500-fold, the specific activity was 0.77 μmol/min per mg of protein, and the yield 2.5%, as compared with 1.6% in the previous

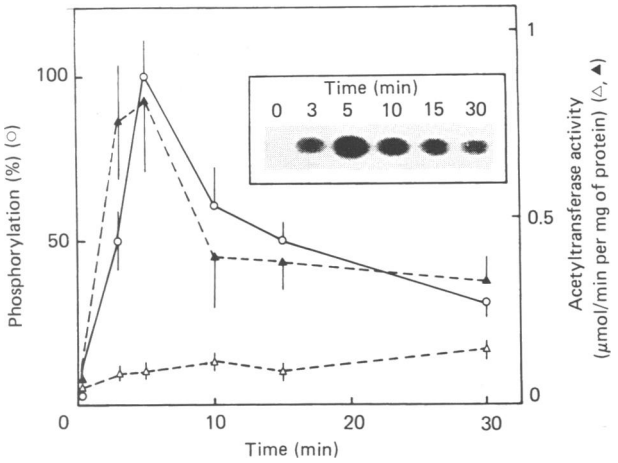

Fig. 3. Time-course of the protein kinase A-stimulated protein phosphorylation and acetyltransferase activation

Phosphorylation was carried out with the partially purified acetyltransferase. At the times indicated 60 μl aliquots were withdrawn and the reaction stopped. Proteins were analysed on 12.5% SDS/PAGE and the gels autoradiographed. The phosphorylation was determined densitometrically from the autoradiogram (arbitrary units, ○). The inset shows the autoradiography of the 30 kDa protein band subjected to densitometric scanning. In parallel experiments, aliquots of partially purified acetyltransferase were taken for determination of acetyltransferase activity in the presence (▲) or absence (△) of protein kinase A. Results are expressed as means ± S.D. of three independent experiments.

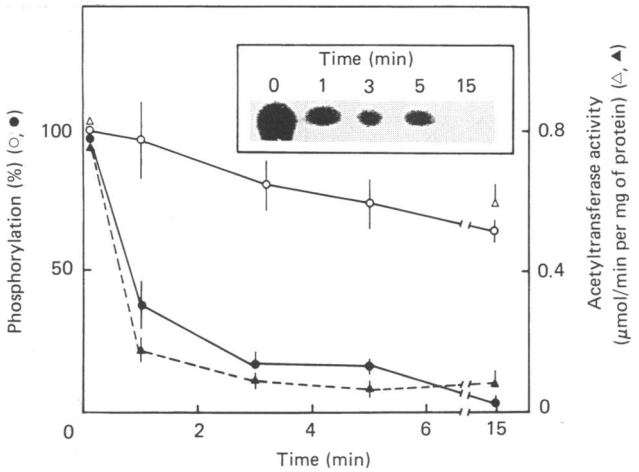

Fig. 4. Reversibility of the acetyltransferase phosphorylation

Phosphorylation was carried out for 5 min with the partially purified acetyltransferase in the presence of 50 mM-KF as described in the Materials and methods section. At this time, the sample was divided into two portions. One received alkaline phosphatase (20 units/ml) (closed symbols). The second sample did not receive any addition (open symbols). At the times indicated, aliquots were taken and the reaction stopped. Phosphorylation was determined as described in the legend to Fig. 3. The inset shows autoradiography of the 30 kDa protein treated with alkaline phosphatase. In parallel experiments aliquots were taken for determination of acetyltransferase activity in the presence (▲) or absence (△) of alkaline phosphatase. Results are expressed as means ± S.D. of three independent experiments.

procedure [12]. The analysis by gel electrophoresis in the presence of SDS indicated the presence of a main band of protein of 30 kDa and others of 57 kDa, 62 kDa and 80 kDa (Fig. 2, left). In experiments in non-denaturing gel electrophoresis followed by band elution and acetyltransferase activity assay, only the 30 kDa polypeptide band showed enzymic activity. Isoelectric focusing of this preparation in 10% acrylamide gel using a non-equilibrium pH gradient from 3.5 to 10.0 showed the presence of two major proteins of pI 4.70 and pI 5.75. All these data indicate an improvement in the purification method previously described [12]. The use of 2 M-LiBr plus 0.04% deoxycholate had the same effect on the enzyme and could be used in place of KBr. By contrast, 2 M-guanidinium chloride or 4 M-urea inactivated the enzyme activity and were unable to dissociate the large aggregates, according to the results of the SDS/PAGE (results not shown).

Phosphorylation of acetyltransferase

Incubation of partially purified and KBr-treated acetyltransferase with 10 μM-MgATP and the catalytic subunit of protein kinase A results in about 3-fold activation of the acetylation reaction (Table 1). In the absence of kinase or MgATP no effect was observed. When [γ-^{32}P]ATP was added to the microsomal fraction and the proteins were separated by 12.5% acrylamide slab gel electrophoresis in the presence of SDS, several protein bands with [^{32}P]phosphate incorporation were revealed by autoradiography (Fig. 2, right). However, using the purified KBr-treated enzyme in the phosphorylation reaction with [γ-^{32}P]ATP, only a protein of about 30 kDa was phosphorylated (Fig. 2, right). This size agrees with that previously reported using the partially purified enzyme labelled with [^{3}H]lyso-paf-acether [12] in SDS/PAGE. Occasionally, there was also a small phosphorylation of a protein of about 60 kDa, which was not investigated further for the lack of reproducibility of this phosphorylation. Again, the same results were obtained using the acetyltransferase not treated with KBr (results not shown). Estimation by densitometric scanning of the 30 kDa polypeptide in three independent experiments showed a mean content of 0.132 ± 0.033 nmol, which according to the specific activity of the [γ-^{32}P]ATP used in parallel experiments showed a phosphorylation stoichiometry of 0.76 ± 0.1 mol of ^{32}P/mol of 30 kDa polypeptide.

Phosphorylation of acetyltransferase was time-dependent. With protein kinase A at a concentration of 60 units/ml, a maximum was observed about 5 min after the addition of the kinase (Fig. 3). Phosphorylation of the 30 kDa protein followed a similar pattern to that of acetyltransferase activation with the kinase in the presence of MgATP; activity seems to rise faster than phosphorylation (Fig. 3), but this could simply be due to the inherent difficulties of the acetyltransferase and phosphorylation assays. These results provide evidence that purified acetyltransferase can be converted from a low-activity dephosphorylated form to a high-activity phosphorylated form. As regards the loss of radioactivity and acetyltransferase activity with time, we investigated the participation of a dephosphorylation mechanism. In the presence of fluoride (50 mM-KF) the incorporation of

[^{32}P]phosphate is maintained up to 15 min (Fig. 4). On the other hand, the addition of alkaline phosphatase (20 units/ml) to the incubation mixture at the time of maximum phosphorylation (5 min) induced a very rapid loss of the [^{32}P]phosphate incorporated into the 30 kDa protein, which paralleled the inactivation of the enzyme (Fig. 4). This suggests that the stimulation by protein kinase of the acetylation reaction is reversed by dephosphorylation. The results agree with our previous findings on acetyltransferase using rat spleen microsomes and protein kinase A [10].

Analysis of phosphoamino acids

Analysis of the phosphorylated amino acids after HCl hydrolysis of the phosphorylated protein indicates that most of the radioactivity was incorporated into phosphoserine whereas it was not possible to observe incorporation of radioactivity into the portions comigrating as either phosphothreonine or phosphotyrosine standards. When the phosphoamino acids were visualized with ninhdyrin, scraped from the plate and counted, the radioactivity corresponding to phosphoserine was 350 c.p.m. (100%), to phosphothreonine 50 c.p.m. (14%) and to phosphotyrosine 22 c.p.m. (6%). This result is in accordance with the well-known specificity of protein kinase A [18].

The suggestion that phosphorylation is involved in control of the acetyltransferase could be tested by investigating whether secretagogues such as zymosan or the cationophore A23187, which are able to increase the intracellular levels of calcium ions and cyclic AMP in macrophages and polymorphonuclear neutrophils [19], or compounds such as prostacyclin and forskolin, that increase the levels of intracellular cyclic AMP in platelets [20], are able to induce the phosphorylation of the same 30 kDa protein.

In summary, the present results suggest that the acetyltransferase, which has an apparent molecular size of about 30 kDa, can be phosphorylated by protein kinase A. The phosphoprotein formed is also of about 30 kDa. The results agree with our previous data in rat spleen microsomes indicating a phosphorylation–dephosphorylation mechanism dependent on cyclic AMP [10] and with our recent description of the partial purification of the acetyltransferase [12].

Note added in proof

While this paper was under review, it was reported [21] that acetyl-CoA:1-alkyl-*sn*-glycero-3-phosphocholine O^2-acetyltransferase (i.e. 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine:acetyl-CoA acetyltransferase, the enzyme studied in our paper) can be activated by phosphorylation by calcium/calmodulin-dependent protein kinase.

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