Cell-free acylation of rat brain myelin proteolipid protein and DM-20

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Incubation of rat brain myelin with [^3H]palmitic acid in the presence of ATP, CoA and MgCl_2 or [^{14}C]palmitoyl-CoA in a cell-free system resulted in the selective labelling of 'PLP' [proteolipid protein; Folch & Lees (1951) *J. Biol. Chem.* **191**, 807–817] and 'DM-20' [Agrawal, Burton, Fishman, Mitchell & Prensky (1972) *J. Neurochem.* **19**, 2083–2089] which, after polyacrylamide-gel electrophoresis in SDS, were revealed by fluorography. These results provide evidence of the association of fatty acid-CoA ligase and acyltransferase in isolated myelin. Palmitic acid is covalently bound to PLP and DM-20, because 70 and 92 % of the radioactivity was removed from proteolipid proteins after treatment with hydroxylamine and methanolic NaOH respectively. Incubation of myelin with [^3H]palmitic acid in the absence of ATP, CoA, MgCl_2 , or all three, decreased incorporation of fatty acid into PLP to 3, 55, 18 and 2 % respectively. The cell-free system exhibits specificity with respect to the chain length of the fatty acids, since myristic acid is incorporated into PLP at a lower rate when compared with palmitic and oleic acids. The acylation of PLP is an enzymic reaction, since (1) maximum incorporation of [^3H]palmitic acid into PLP occurred at physiological temperatures and decreased with an increase in the temperature; (2) acylation of PLP with [^3H]palmitic acid and [^{14}C]palmitoyl-CoA was severely inhibited by SDS (0.05 %); and (3) the incorporation of fatty acid and palmitoyl-CoA into PLP was substantially decreased by the process of freezing–thawing and freeze-drying of myelin. We have provided evidence that all of the enzymes required for acylation of PLP and DM-20 are present in isolated rat brain myelin. Acylation of PLP in a cell-free system with fatty acids and palmitoyl-CoA suggests that a presynthesized pool of non-acylated PLP and DM-20 is available for acylation.

INTRODUCTION

Folch & Lees [1] discovered the presence of a protein in the chloroform/methanol-soluble fraction of bovine white matter. It was designated 'proteolipid protein' (PLP) because of its avidity for lipids. Proteolipid protein was subsequently shown to contain 2–3 % fatty acids [2–7]. A second proteolipid protein in the central-nervous-system myelin was described by Agrawal *et al.* [8] and designated 'DM-20'. The molecular masses of PLP and DM-20 were found to be 25 and 20.54 kDa respectively. We were able to acylate rat brain myelin PLP and DM-20 after intracerebral injection of [^3H]palmitic acid [9,10] and after incubation of brain slices with [^3H]palmitic acid [11]. We also showed that fatty acids are conjugated to proteolipid proteins by ester bonds. Subsequently, Bizzozero *et al.* [12,13] also demonstrated acylation of PLP and DM-20 *in vitro* and *in vivo*. In the present paper evidence will be given that rat brain myelin contains fatty acid-CoA ligase and acyltransferase, enzymes that acylates PLP and DM-20 in a cell-free system.

MATERIALS AND METHODS

The reagents for polyacrylamide-gel electrophoresis were obtained from Eastman–Kodak, except *NN'*-methylenebisacrylamide and SDS, which were purchased

from Bio-Rad and Pierce Chemical Co. respectively. X-Omat AR film was obtained from Eastman–Kodak. Fatty-acid-free bovine serum albumin (BSA), 2',7'-dichlorofluorescein, hydroxylamine (NH_2OH), dimethyl sulphoxide, ATP and CoA (sodium salt) were purchased from Sigma. Palmitic acid and the methyl ester of palmitate were purchased from Supelco. Soluene-350, 2,5-diphenyloxazole (PPO) and Instafluor were from Packard Instruments. [$9,10\text{-}^3\text{H}(\text{n})$]Palmitic acid (30 Ci/mmol), [$9,10\text{-}^3\text{H}$]myristic acid (22.4 Ci/mmol), [$9,10\text{-}^3\text{H}(\text{n})$]oleic acid (8.9 Ci/mmol) and [$1\text{-}^{14}\text{C}$]palmitoyl-CoA (58.5 mCi/mmol) were obtained from New England Nuclear. Sephadex LH-60 was obtained from Pharmacia. Silica-gel G plates were purchased from Analtech. 2-Methoxy-2,4-diphenylfuran-3(2*H*)-one (MDPF) was a gift from Dr. M. Weigele, Hoffman–La Roche. Palmitohydroxamate was prepared by the method of Rosenfeld *et al.* [14]. All other reagents and solvents were of analytical grade.

Isolation of myelin

Sprague–Dawley rats (21 days old) were killed by decapitation and the brains were rapidly removed. All operations were carried out at 4 °C. Four rat brains were homogenized in 0.88 M-sucrose (5 %, w/v) in a Dounce glass homogenizer, and myelin was isolated as described [8,10]. Myelin was suspended in water, and the protein

Abbreviations used: PLP, proteolipid protein (Folch & Lees [1]); DM-20, a proteolipid protein so designated by Agrawal *et al.* [8]; BSA, bovine serum albumin; MDPF, 2-methoxy-2,4-diphenylfuran-3(2*H*)-one; RSA, relative specific radioactivity.

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concentration in all samples was determined by the method of Lowry *et al.* [15], with bovine serum albumin as a standard. A portion of the myelin was freeze-dried; another portion of myelin was frozen and thawed five times from -140°C to 23°C to determine the activity of acylating enzymes.

Cell-free acylation

Acylation of PLP and DM-20 was carried out by incubation of $1000\ \mu\text{l}$ ($1000\ \mu\text{g}$ of myelin proteins) of fresh myelin membrane in $20\ \text{mM}$ -Tris/HCl, pH 7.4, containing $2\ \text{mM}$ -ATP, $50\ \mu\text{M}$ -CoA, $10\ \text{mM}$ -MgCl₂ (co-factors), $25\ \mu\text{l}$ of 1% BSA and $250\ \mu\text{Ci}$ of [³H]palmitic acid at 37°C for 1 h in a shaking water bath. In order to determine the transfer of radioactivity from palmitoyl-CoA to PLP and DM-20 by fatty acyltransferase, $1000\ \mu\text{l}$ of myelin membrane ($1000\ \mu\text{g}$ of myelin proteins) in $20\ \text{mM}$ -Tris/HCl, pH 7.4, was incubated with $1\ \mu\text{Ci}$ of [¹⁴C]palmitoyl-CoA with or without ATP at 37°C for 1 h. The reaction was terminated by adding 3 ml of ice-cold solution of $20\ \text{mM}$ -Tris/HCl, pH 7.4, followed by centrifugation at $10000\ g$ for 10 min at 4°C . The pellet was resuspended in the Tris buffer and centrifuged as described above. The pellet was suspended in 1 ml of ice-cold water and freeze-dried.

Lipids were removed from freeze-dried myelin by four successive extractions with diethyl ether/ethanol (3:2, v/v) and diethyl ether [16]. The resulting pellet was dissolved in 2% SDS. After determining protein concentration, an equal volume of a sample buffer, containing $125\ \text{mM}$ -Tris/HCl, pH 6.8, 3% (w/v) dithiothreitol, 8 M-urea, 16% (w/v) sucrose and 0.01% Bromophenol Blue was added to the myelin proteins.

Polyacrylamide-gel electrophoresis in SDS

Myelin proteins ($50\ \mu\text{g}$) were separated in a 15% polyacrylamide/SDS slab gel in a Tris/glycine buffer system [17]. Proteins in the gel were stained with Coomassie Blue and then destained as described [8]. Gels were scanned at 570 nm with a Gilford spectrophotometer equipped with a linear-transport mechanism. Gel sections corresponding to PLP were excised and heated with 1.5 ml of Soluene-350 at 50°C for 12 h. After addition of 15 ml of Instafluor, the radioactivity was determined by liquid-scintillation spectrometry [18]. Radioactivity associated with PLP was divided by the weight of the paper corresponding to the peak area of PLP and was designated as the relative specific radioactivity (RSA; d.p.m./unit of PLP). Stained gels were infiltrated with dimethyl sulphoxide and then with dimethyl sulphoxide in PPO (22.2%, w/v) and then fluorographed [19]. The stained gels were bisected; one section was treated with 1 M-hydroxylamine, pH 10, and the other section was treated with 1 M-Tris/HCl buffer, (pH 10) for 18 h. The gels were then fluorographed [19]. After fluorography, the gel sections corresponding to PLP were excised from the dried gel, rehydrated, and the radioactivity was measured as described above.

Bulk labelling of myelin proteins in a cell-free system

Myelin membrane (9 mg of myelin proteins) was incubated at 37°C for 2 h in 3 ml of $20\ \text{mM}$ -Tris/HCl buffer, pH 7.4, containing $2\ \text{mM}$ -ATP, $50\ \mu\text{M}$ -CoA, $10\ \text{mM}$ -MgCl₂ and 3 mCi of [³H]palmitic acid as described above. After incubation, myelin was washed and freeze-dried. Freeze-dried myelin was mixed with the unlabelled myelin (18 mg of myelin proteins) and solubilized in

chloroform/methanol (2:1, v/v) (5 mg of dry myelin/ml). Basic proteins and high- M_r proteins were salted out by adding 0.05 vol. of 1 M-KCl. This mixture was stirred for 30 min, centrifuged at $10000\ g$ for 15 min at 4°C . The supernatant was partitioned with 0.2 vol. of water and then washed three times with the synthetic upper phase (chloroform/methanol/water, 3:48:47, by vol.) as described [1]. The lower phase was made homogeneous by adding methanol, and proteolipid proteins were precipitated by adding 4 vol. of diethyl ether at -20°C and pelleted by centrifugation at $12000\ g$ for 30 min at 4°C . The pellet was dissolved in chloroform/methanol/0.1 M-HCl (10:10:1, by vol.) and applied to a column ($3.2\ \text{cm} \times 60\ \text{cm}$) of Sephadex LH-60 to separate the lipids from the proteins. Proteolipid proteins were eluted with chloroform/methanol/0.1 M-HCl (10:10:1, by vol.). Eluted proteolipid proteins were again precipitated with 4 vol. of diethyl ether at -20°C . The precipitated proteins were pelleted by centrifugation, dissolved in 4% SDS, and 20% of the labelled proteins were coupled to MDPF [20] before SDS/polyacrylamide-gel electrophoresis.

Cleavage of fatty acids covalently bound to the proteins

Proteolipid proteins purified by chromatography on Sephadex LH-60 were treated with 5 ml of methanolic

Fig. 1. Cell-free acylation of PLP and DM-20 with [³H]palmitic acid and [¹⁴C]palmitoyl-CoA

Fresh myelin (1 mg of protein) was incubated for 1 h with $250\ \mu\text{Ci}$ of [³H]palmitic acid (lanes 1–3) as described in the Materials and methods section. Lane 1, presence of ATP, CoA and MgCl₂ at 37°C ; lane 2, absence of ATP at 37°C ; lane 3, presence of ATP, CoA and MgCl₂ at 0°C . Heated myelin (80°C for 10 min) was incubated for 1 h at 37°C with $250\ \mu\text{Ci}$ of [³H]palmitic acid in the presence of ATP, CoA and MgCl₂ (lane 4). Fresh myelin (1 mg of protein) was incubated for 1 h at 37°C with $1\ \mu\text{Ci}$ of [¹⁴C]palmitoyl-CoA in the presence of ATP (lane 5) and in the absence of ATP (lane 6). Lipids migrated out of the gel. Gels were exposed to X-ray film for 3 days (lanes 1–4) and 22 days (lanes 5 and 6). Abbreviations: WP, Wolfgram protein; LBP, large basic protein; SBP, small basic protein. A faint band of DM-20 labelled with [¹⁴C]palmitoyl-CoA is apparent in the fluorogram, but is barely visible in this photograph (lanes 5 and 6).

Fig. 2. Gel filtration on a Sephadex LH-60 column of a chloroform/methanol-soluble fraction of myelin labelled with [^3H]palmitic acid in a cell-free system

Myelin proteins were acylated with [^3H]palmitic acid as described in the Materials and methods section. The chloroform/methanol-soluble fraction was applied to a Sephadex LH-60 column. Proteolipid proteins were eluted with chloroform/methanol/0.1 M-HCl (10:10:1, by vol.). Fractions (3.5 ml) were collected and monitored for protein (○) by absorbance at 280 nm. Radioactivity in 20 μl of each fraction (●) was determined by liquid-scintillation spectrometry. Proteolipid protein fractions indicated between arrows (→) were collected for further experiments.

NaOH (0.5 M) or 5 ml of 1 M-hydroxylamine, pH 10.0, at 25 °C for 16 h. Fatty acids released by mild alkaline hydrolysis were extracted as described [9]. Fatty acids released by hydroxylamine were extracted three times with chloroform/methanol (2:1, v/v). The organic extracts were dried under N_2 and redissolved in a small amount of chloroform/methanol (2:1, v/v). Palmitic acid and palmitohydroxamate were separated on silica-gel G plates by using toluene/acetic acid/water (80:20:1, by vol.) as described [21]. The plates were sprayed with 2',7'-dichlorofluorescein to reveal the spots under long-wavelength u.v. light [9]. The plate was divided into 1 cm sections, and the radioactivity in each section was determined by liquid-scintillation spectrometry. R_F values for palmitohydroxamate and palmitic acid were 0.65 and 0.82 respectively.

RESULTS AND DISCUSSION

Rat brain myelin has been shown to contain Wolfram protein, myelin-associated glycoprotein, PLP, DM-20 and basic proteins (Fig. 1, lanes 1–6). Only PLP and DM-20 were acylated after incubation of rat brain myelin with [^3H]palmitic acid in the presence of ATP, CoA and MgCl_2 in a cell-free system (Fig. 1, lane 1).

These results provided evidence for the presence of both fatty acid-CoA ligase, which activates non-esterified fatty acids into fatty acyl-CoA, and an acyltransferase, which transfers fatty acyl-CoA into PLP and DM-20. It has been shown that rat brain myelin PLP and DM-20 are selectively labelled with palmitic acid *in vivo* [9,13] and *in vitro* [11,12], and this cell-free system exhibits identical specificity. The fatty acid-CoA ligase had an absolute requirement for ATP, since a negligible amount of radioactivity was incorporated into proteolipid proteins in the absence of ATP (Fig. 1, lane 2). In addition, acylation of PLP and DM-20 appears to be enzyme-mediated, since no radioactivity was incorporated into these proteins after incubation of myelin with ATP, CoA and MgCl_2 at 0 °C (Fig. 1, lane 3) or incubation of myelin heated to 80 °C for 5 min with [^3H]palmitic acid, ATP and cofactors (Fig. 1, lane 4). Likewise, PLP and DM-20 were not labelled after incubation of myelin with 187 μCi of radiolabelled lipids under identical conditions (results not shown). These results also rule out the possibility of non-specific binding of radioactivity to proteolipid protein.

Incubation of myelin with [^{14}C]palmitoyl-CoA in the presence (Fig. 1, lane 5) or the absence (Fig. 1, lane 6) of ATP resulted in acylation of PLP. This confirms that rat

Fig. 3. SDS/polyacrylamide-gel electrophoresis and re-electrophoresis of purified proteolipid proteins

Lane 1, proteolipid proteins (50 μ g of protein) purified on Sephadex LH-60. Lanes 2 and 3, samples of PLP and DM-20 respectively were subjected to re-electrophoresis. The arrowhead ($\blacktriangleright$) indicates aggregated PLP. This band was immunostained with antiserum to PLP. Gels were exposed to X-ray film for 10 days (lanes 1 and 2) and 56 days (lane 3).

brain myelin contains an acyltransferase that catalyses the transfer of palmitic acid from palmitoyl-CoA to proteolipid proteins. The incorporation of [14 C]-palmitoyl-CoA into PLP and DM-20 was decreased to 6% when myelin was incubated at 0 $^{\circ}$ C. No radioactivity was incorporated into these proteins when myelin was heated to 80 $^{\circ}$ C for 5 min (results not shown). Recently a number of proteins have also been shown to be acylated in a cell-free system [22–28].

A chloroform/methanol-soluble fraction of myelin contains a substantial amount of lipids in addition to PLP and DM-20. These lipids are not dissociated from proteolipid proteins even after precipitation with diethyl ether [9]. In order to separate proteolipid proteins from lipids, precipitated proteolipid proteins were subjected to gel filtration on Sephadex LH-60 in acidified chloroform/methanol. It is apparent from Fig. 2 that radiolabelled proteolipid proteins were clearly separated from radiolabelled lipids. Examination of purified proteolipid proteins (marked with arrows in Fig. 2), followed by fluorography, revealed that radioactivity was retained with PLP and DM-20 (Fig. 3, lane 1), but that some radioactivity also migrated to the position of lipids. Further purification of PLP and DM-20 by re-electrophoresis showed the presence of radioactivity with PLP (Fig. 3, lane 2) and DM-20 (Fig. 3, lane 3) and the absence of radioactivity in the region of lipids. These results provide further evidence that the incorporation of palmitic acid into PLP and DM-20 in a cell-free system is not due to radiolabelled phospholipids.

Fatty acids conjugated to protein by thiol ester and an *O*-ester bond can be readily hydrolysed by

Fig. 4. Effect of hydroxylamine on proteolipid proteins labelled with [3 H]palmitic acid or [14 C]palmitoyl-CoA

Sephadex LH-60-purified proteolipid proteins (50 μ g) were labelled with [3 H]palmitic acid (lanes 1 and 2). Myelin (1 mg of protein) was incubated with 1 μ Ci of [14 C]-palmitoyl-CoA (lanes 3 and 4). Gels were treated with 1 M-Tris/HCl, pH 10.0, for 16 h (lanes 1 and 3) or with 1 M-hydroxylamine, pH 10.0, for 16 h (lanes 2 and 4). Gels were exposed to X-ray film for 3 days (lanes 1 and 2) or 13 days (lanes 3 and 4).

hydroxylamine [9,21,29,30] and methanolic NaOH [9,30,31]. These reactions have been utilized to demonstrate covalent linkage of fatty acids to proteins. About 70% of the radioactivity in PLP and DM-20, due to [3 H]palmitic acid (Fig. 4, lanes 1 and 2) or [14 C]palmitoyl-CoA (Fig. 4, lanes 3 and 4), was removed. These results were confirmed by treatment of the purified protein with hydroxylamine, followed by t.l.c. A mixture of palmitohydroxamate (17%) and palmitic acid (77%) were released after treatment of PLP and DM-20 with hydroxylamine (Fig. 5a). About 92% of the radioactivity was released after treatment of PLP and DM-20 with methanolic NaOH. The released radioactivity was associated with non-esterified palmitic acid after t.l.c. (Fig. 5b). These results suggest that fatty acids are bound by thioester or ester bonds to PLP acylated in a cell-free system. These results are similar to acylation of PLP *in vivo* [9,13] and *in vitro* [11,12].

Incubation of myelin with ATP, CoA, MgCl_2 and [3 H]-palmitic acid at 37 $^{\circ}$ C (Fig. 6) for 2 h (Fig. 7) resulted in the maximum incorporation of radioactivity into PLP. The transfer of fatty acid into PLP was decreased to 3, 55, 18 and 2% in the absence of ATP, CoA, MgCl_2 or all three, respectively, when compared with the RSA of PLP in the complete incubation mixture (Table 1). The high activity observed in the absence of CoA may be due to a trace amount of acyl-CoA thioester associated with the myelin membrane. These results suggest that the incorporation of fatty acids into PLP is absolutely dependent on the presence of ATP. Replacement of MgCl_2 by

Fig. 5. Cleavage of [³H]palmitic acid from PLP purified by Sephadex LH-60 chromatography and identification of released fatty acids by t.l.c. on a silica-gel G plate

PLP purified by Sephadex LH-60 column chromatography was treated with 1 M-hydroxylamine, pH 10.0 (a) or with methanolic NaOH (b). An authentic sample of palmitic acid (50 μ g) and palmitohydroxamate (50 μ g) was added to the released fatty acids. They were separated by t.l.c., spots were revealed and radioactivity was determined as described in the Materials and methods section. Radioactivity applied to the t.l.c. plate was 7900 d.p.m. (a) and 8600 d.p.m. (b). Abbreviations: PH, palmitohydroxamate; PA, palmitic acid.

MnCl₂ or EDTA, however, significantly decreased the incorporation of fatty acid into PLP.

The incorporation of [³H]myristic acid, [³H]palmitic acid and [³H]oleic acid into PLP proceeds at a linear rate for 2 h (Fig. 7). However, the cell-free system does exhibit specificity with respect to the chain length of fatty acids, for myristic acid is incorporated into PLP at a lower rate when compared with palmitic acid and oleic acid (Fig. 7).

The following observations strongly suggest that acylation of PLP in a cell-free system is an enzymic reaction. First, maximum incorporation of [³H]palmitic acid into PLP was observed at physiological temperatures (Fig. 6), and it decreased with an increase in the temperature. Second, acylation of PLP with [³H]palmitic acid and [¹⁴C]palmitoyl-CoA was severely inhibited in the presence of SDS (Table 2). Finally, the incorporation of

Fig. 6. Effect of temperature on acylation of PLP

The composition of the incubation mixture was the same as that described in Table 1. Results are means $\pm$ s.d. for three separate experiments.

Fig. 7. Kinetics of labelling of PLP with different fatty acids

The composition of the incubation mixture was the same as that described in Table 1. Results are means $\pm$ s.d. for three to five separate experiments. ●, [³H]Myristic acid; ■, [³H]palmitic acid; ▲, [³H]oleic acid.

both [³H]palmitic acid and [¹⁴C]palmitoyl-CoA into PLP was substantially reduced by the process of freeze-thawing and freeze-drying of myelin.

Table 1. Requirements for rat brain myelin fatty acid-CoA ligase and acyltransferase for acylation of PLP in a cell-free system

Fresh myelin (500 μ g of protein) was incubated at 37 °C for 2 h in 500 μ l of 20 mM-Tris/HCl buffer, pH 7.4, containing 2 mM-ATP, 50 μ M-CoA, 10 mM-MgCl₂, 50 μ Ci of [³H]palmitic acid and 10 μ l of 1% fatty-acid-free BSA. The RSA of PLP was calculated as described in the Materials and methods section. Results are the means $\pm$ S.D. for three to five separate experiments. The RSA of PLP in the complete incubation mixture was 1828 $\pm$ 336 d.p.m./unit of PLP (n = 5).

Incubation mixture	RSA of PLP (% of that for the complete mixture)
Complete	100
– ATP	3 $\pm$ 0.2
– MgCl ₂	18 $\pm$ 3
– CoA	55 $\pm$ 5
– ATP, – MgCl ₂ , – CoA	2 $\pm$ 0.3
– MgCl ₂ + MnCl ₂ (10 mM)	11 $\pm$ 2
– MgCl ₂ + EDTA (10 mM)	6 $\pm$ 0.1

Table 2. Effect of SDS, freeze-thawing and freeze-drying on the acylation of PLP in a cell-free system

Fresh myelin (500 μ g of protein) was incubated at 37 °C for 1 h in 500 μ l of 20 mM-Tris/HCl buffer, pH 7.4, containing 2 mM-ATP, 50 μ M-CoA, 10 mM-MgCl₂, 10 μ l of 1% (w/v) fatty-acid-free BSA and 50 μ Ci of [³H]-palmitic acid. Fresh myelin (500 μ g of protein) was also incubated in 500 μ l of 20 mM-Tris/HCl buffer, pH 7.4, containing 10 mM-MgCl₂ and 0.5 μ Ci of [¹⁴C]palmitoyl-CoA. The reaction was stopped by adding 3 ml of acetone. The RSA of PLP was determined as described in the Materials and methods section. Results are means $\pm$ S.D. for three different experiments.

Incubation mixture	RSA of PLP (% of that for the complete mixture)	
	[³ H]Palmitic acid	[¹⁴ C]Palmitoyl-CoA
Complete*	100	100
+ SDS		
0.05 %	7.5 $\pm$ 1.1	21.4 $\pm$ 0.6
0.5 %	4.4 $\pm$ 0.8	18 $\pm$ 2.6
Freeze-thaw		
Once	69 $\pm$ 10	74 $\pm$ 3.8
Five times	47 $\pm$ 7	49 $\pm$ 10
Freeze-dried	36 $\pm$ 4	16.4 $\pm$ 3.7

* The complete incubation mixture with [³H]palmitic acid and [¹⁴C]palmitoyl-CoA gave RSA values of 893 $\pm$ 208 and 84 $\pm$ 4 d.p.m./unit of PLP respectively.

We have provided evidence that all enzymes required for acylation of PLP are present in myelin isolated from rat brain. The results of the present study and those published by others [27] substantiate our hypothesis that enzymes for the acylation of PLP are present within the myelin sheath [9]. Acylation of proteolipid proteins in a

cell-free system suggest that a presynthesized pool of non-acylated PLP is available for acylation. Although PLP and DM-20 are not glycoproteins, they have been shown to be associated with rough endoplasmic reticulum and Golgi complex before their incorporation into the myelin membrane [32,33]. In addition, incorporation of palmitate into proteolipid proteins [11,12] and other membrane proteins [34–37] has been shown to occur in the presence of cycloheximide. These results suggest that palmitoylation of proteins is not coupled to protein synthesis, but is a relatively late post-translational modification. Townsend & Benjamins [38] also showed that incorporation of [³H]palmitic acid into proteolipid proteins is not inhibited after incubation of brain slices with monensin. They suggested that acylation of PLP occurs past the Golgi complex. Therefore, it is feasible that proteolipid proteins may be exclusively acylated in the myelin membrane instead of oligodendroglial cells *in vivo*.

The presence of fatty acid-CoA ligase and acyltransferase raises the question whether non-esterified fatty acids or their acyl-CoA derivatives are preferentially utilized for the acylation of PLP and DM-20 in myelin membrane *in situ*. If proteolipid proteins are acylated by non-esterified fatty acids in myelin membrane *in vivo*, it is obligatory to have a supply of ATP. ATP might be available to myelin membrane through oligodendroglial cells, even though ATP will have to be transported over a long distance between the cell body and the myelin membrane. Alternatively, the axon may serve as a potential source of ATP to the myelin membrane. However, ATP in general does not permeate plasma membranes; therefore, it will be necessary to determine whether serine-198 in rat brain PLP, like threonine-198 in bovine PLP [7], is acylated *in vivo* and in a cell-free system before the physiological significance of myelin-associated fatty acid-CoA ligase and acyltransferase can be determined. Purification and characterization of these enzymes, and synthesis and turnover of acylated proteins, may shed some light on the biological significance of acylated membrane proteins.

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