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Cysteine-108 is an Acylation Site in Myelin Proteolipid Protein

Oscar A. Bizzozero*, Louise K. Good and James E. Evans

Biochemistry Dept., E. K. Shriver Center, Waltham MA 02254

*Depts.of Biological Chemistry and Molecular Pharmacology,
Harvard Medical School, Boston MA 02115

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Summary Myelin proteolipid protein (PLP) contains covalently bound long-chain fatty acids. A large proportion of these acyl moieties are bound in thioester linkages, as demonstrated by alkylation of newly formed SH groups upon deacylation. To identify the Cys residue(s) involved in the thioester linkage(s), reduced and carboxyamidomethylated proteolipid protein was labeled with [^{14}C]iodoacetamide upon deacylation with neutral hydroxylamine. The labeled protein was digested with trypsin or pepsin, and peptides analyzed by RP-HPLC. Identification of the isolated radioactive peptides by amino acid analysis, peptide sequencing and/or fast-atom bombardment-mass spectrometry revealed that Cys^{108} in the bovine PLP sequence is an acylated site. The sequence surrounding the palmitoylation site in the myelin PLP is strikingly similar to that found in rhodopsin. Furthermore, as in rhodopsin and other members of the G protein-coupled receptor family, this Cys residue is located within a hydrophilic, basic, and possibly cytoplasmic, domain.

The covalent modification of proteins by fatty acid acylation has been reported to occur in a large variety of pro- and eukaryotic cell types (1,2). Two classes of fatty acid-protein linkages have been identified: thioester or oxyester linkages to the side chains of cysteine, serine and threonine residues, and amide linkages to an amino-terminal glycine residue. The former generally involves palmitic (C16:0) acid whereas the amide linkage involves myristic (C14:0) acid almost exclusively. Identification of the acylation site in palmitoylated proteins is a crucial step towards the understanding of the biological functions of ester-bound fatty acids. However, this information is particularly limited as reflected in the low number of proteins for which the acylated site is known.

Proteolipid protein (PLP) is the major component of central nervous system myelin and was the first to be recognized as an acylated protein (3). It is a hydrophobic membrane protein and contains approximately 2 moles of covalently-bound fatty acid, mainly palmitic, oleic and stearic

Abbreviations: APL, proteolipid apoprotein; cam-, carboxyamidomethyl-; FAB, fast-atom bombardment; MS, mass spectrometry; PLP, major proteolipid protein; RP-HPLC, reversed-phase high performance liquid chromatography; P and T, peptic and tryptic peptides, respectively.

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acids (3-6). Although, it was initially considered that the fatty acids were bound in O-ester linkages (7), recent evidence suggests that at least some of them may be in thioester linkages (8). We have recently provided additional evidence for the occurrence of thioesters in myelin PLP by showing that removal of the covalently bound-fatty acids with hydroxylamine is accompanied by generation of free thiol groups which can be titrated with radioactive iodoacetamide (9). The present study was undertaken to determine the precise location of the thioester within the bovine PLP sequence. The experimental data identify Cys^{108}, within the basic peptide Tyr^{91}-Lys^{150}, as the acylated amino acid residue.

EXPERIMENTAL PROCEDURES

Materials - Chemicals for electrophoresis and the ion retardation resin AG-11A8 were obtained from Bio-Rad (Richmond, CA). Hydroxylamine hydrochloride, iodoacetamide, pepsin (E.C. 3.4.23.1, from porcine stomach mucosa) and TPCK-treated trypsin (E.C. 3.4.21.4, from bovine pancreas) were from Sigma (St. Louis, MO). All other chemicals used were of the highest purity available. [1-^{14}C]Iodoacetamide (24.1 mCi/mmol) was purchased from DuPont-New England Nuclear (Boston, MA) and was utilized without further purification.

Preparation of Reduced and Carboxyamidomethylated (RCM)-APL - Bovine white matter proteolipids were extracted with chloroform-methanol (2:1 v/v) (10) and proteolipid apoprotein (APL) was prepared by Sephadex LH-60 column chromatography (11). Protein, free of non-covalently bound lipids, was precipitated with 4 vol. of cold acetone and the suspension centrifuged at 4,000 x*g* for 10 min. The pellet was dried under nitrogen and dissolved in 50 mM Tris-HCl buffer pH 8.0, containing 1% sodium dodecyl sulfate (SDS). To reduce disulfide linkages, dithiothreitol (160 ug/mg protein) was added and the solution was incubated at 37 °C for 16 h. Finally, iodoacetamide (2 mg/mg protein) was added and incubation was continued in the dark for another 2 h. Unreacted iodoacetamide was removed by dialysis against 10 mM Tris-HCl buffer pH 7.5 , 0.1% SDS at 4 °C for several days. Proteolipid protein was determined by a modification of the Lowry procedure (12) using bovine serum albumin as standard.

Treatment of RCM-APL with [^{14}C]Iodoacetamide and Hydroxylamine - RCM-APL, dissolved in 0.5 % SDS, was incubated concomitantly with a 50 fold molar excess of [^{14}C]iodoacetamide (1-2 mCi/mmol) and 0.2 M hydroxylamine-HCl pH 7.5, for 4 h at 37 °C in the dark . Excess reagents were removed by dialysis against water for two days.

Enzymatic Digestion of RCM-APL - To remove SDS, the labeled protein was dialyzed against water followed by ion-retardation column chromatography through an AG-11A8 resin (13). Protein (1 mg) was incubated with 40 µg of TPCK-treated trypsin in 20 mM amonium acetate buffer pH 8.0 containing 0.1 % Triton X-100 at 37 °C for 16 h. To terminate digestion, 4 vol. of chloroform-methanol (2:1 v/v) were added. The mixture was stirred and centrifuged, and the upper phase was collected and dried under nitrogen. Peptides were dissolved in 100 µl of 0.1% trifluoroacetic acid in 50% methanol and analyzed by reversed-phase HPLC on a Beckman Ultrapore C-8 column (25 cm x 4.6mm, 300 Å) using a gradient of acetonitrile in 0.1% trifluoroacetic acid. Fractions of 1 ml were collected and radioactivity determined by liquid scintillation counting. Radioactive peaks were collected and re-purified on the same column, but using a shallower gradient. Digestion of RCM-APL was also carried out with pepsin at pH 2.0 for 16 h and the resulting peptides were processed as above.

Mass Spectrometry - Fast atom bombardment-mass spectrometry (FAB-MS) of isolated peptides was carried out on a VG 70-250 SE high resolution mass spectrometer. Peptides were dissolved in a matrix of 1,4-dithiothreitol : dithioerythritol (5:1) (14). The accelerating voltage was 8 kV and the resolution 1 : 2,500. Repetitive scans were acquired from m/z 250 to 2,500 with a cycle time of 22 sec. An Ion Tech FAB gun was used with xenon beam at 8 kV and 1 mA. The MS was calibrated on cluster ions from a mixture of CsI and RbI.

Amino acid analysis and peptide sequencing - Samples were hydrolyzed with 6N HCl at 110°C for 24 h. Amino acids were derivatized (ABI-420A derivatizer) with phenylisothiocyanate (PITC) and analyzed on-line via HPLC. In some experiments, amino acid analysis was carried out by the procedure of Chang (15). Peptide sequencing was carried out by automated Edman degradation on an ABI protein sequencer. Phenylthiohydantoin derivatives of amino acids were identified by HPLC.

RESULTS

To determine the cysteine residue(s) involved in the thioester linkage with the fatty acid, RCM-APL was treated with neutral hydroxylamine in the presence of [^{14}C]iodoacetamide. Under these conditions 2 moles of fatty acid are released and approximately 1.3 moles of SH groups are concomitantly alkylated with the labeled reagent (9). After digestion of the radioactive protein with trypsin, approximately 70% of the [^{14}C]-labeled peptides partitioned in the water-methanol phase derived from a two phase chloroform-methanol-water system. The remainder of the radioactivity was found at the interface, and probably corresponds to undigested material. RP-HPLC of the

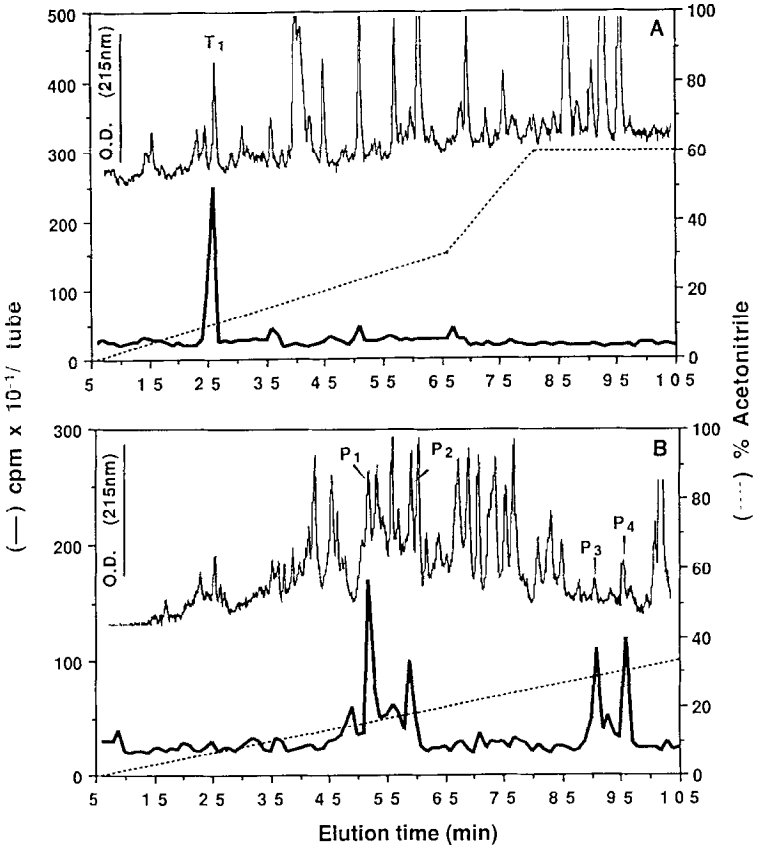
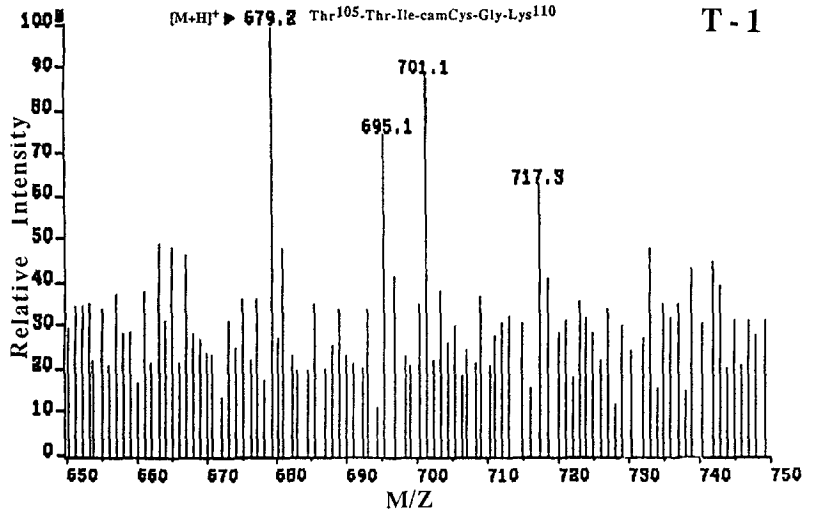
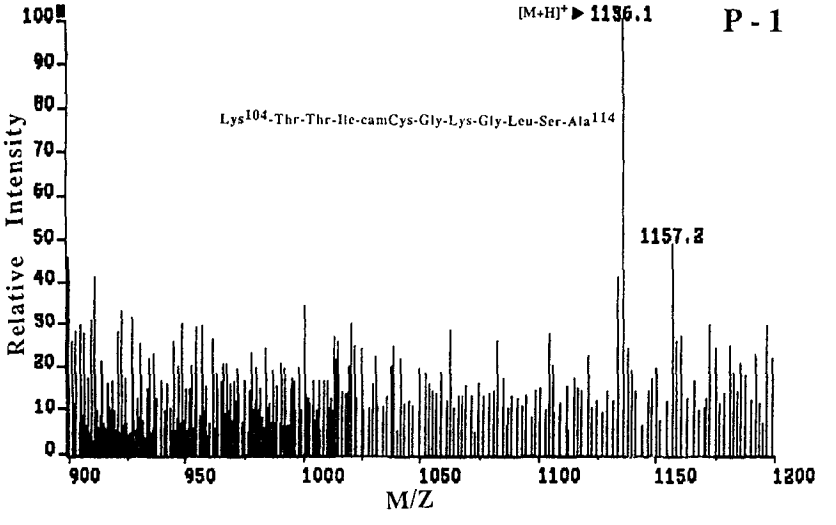
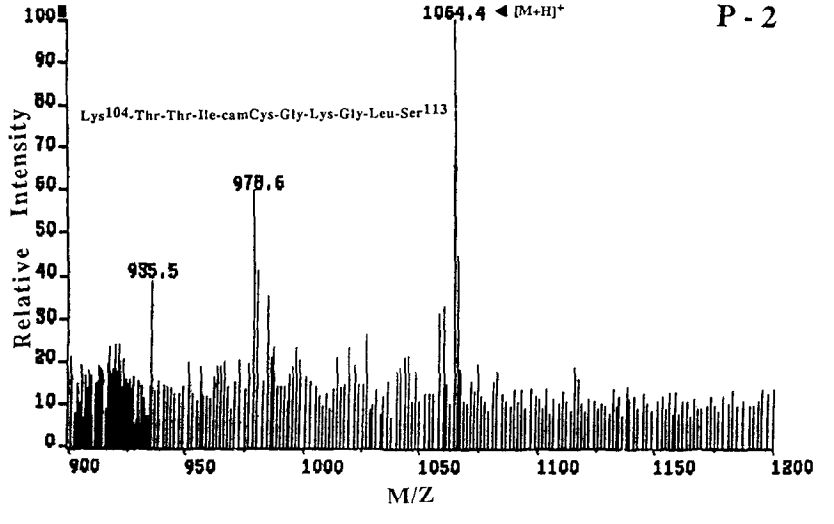


Figure 1. RP-HPLC elution profile of carboxyamidomethylated-PLP peptides. Radiolabeled RCM-APL was digested with trypsin (A) or pepsin (B), and the resulting water-soluble peptides were analyzed by HPLC on a wide pore C-8 column as described under Experimental Procedures. UV absorption at 215 nm, gradient profile (broken line) and distribution of radioactivity are shown for each HPLC run. Gradient: Buffer A, 0.1% trifluoroacetic acid, Buffer B, 0.1% trifluoroacetic acid in acetonitrile. O.D., optical density.


T - 1
[M+H]+ ▶ 679.2 Thr105-Thr-Ile-camCys-Gly-Lys110
701.1
695.1
717.3
Relative Intensity
M/Z


P - 1
[M+H]+ ▶ 1136.1
Lys104-Thr-Thr-Ile-camCys-Gly-Lys-Gly-Leu-Ser-Ala114
1157.2
Relative Intensity
M/Z


P - 2
1064.4 ◀ [M+H]+
Lys104-Thr-Thr-Ile-camCys-Gly-Lys-Gly-Leu-Ser113
978.6
935.5
Relative Intensity
M/Z

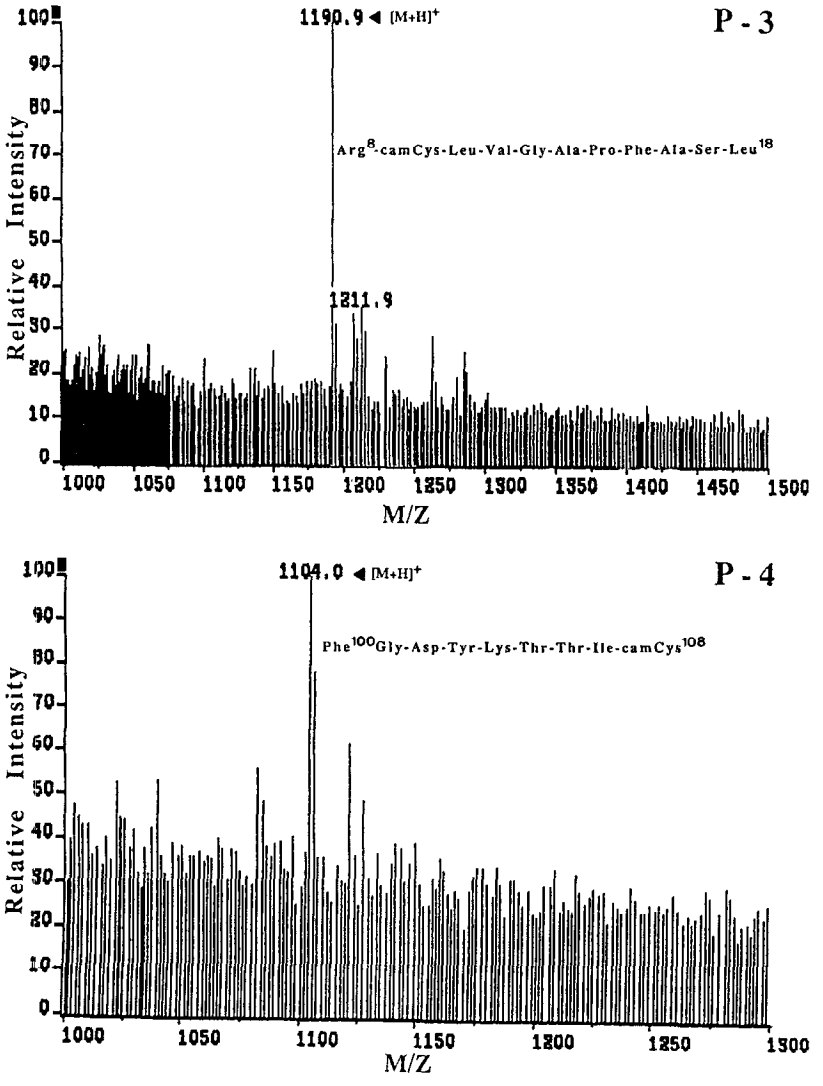


Figure 2. FAB-MS of carboxyamidomethylated-PLP peptides. Isolated tryptic (T) and peptic (P) peptides were analyzed by positive FAB-MS as described under Experimental Procedures. Peptide sequences are those corresponding to the protonated molecular ion ($[M+H]^+$). Numbering of the peptides as in Fig.1.

water-soluble peptides on a wide pore C-8 column showed a single major radioactive peak (T_1) that elutes at 8.5% acetonitrile (Fig.1A). The amino acid composition of this tryptic peptide is shown in Table 1. Five cycles of automated Edman degradation gave the sequence Thr-Thr-Ile-camCys-Gly, which corresponds to the first five amino acid of the tryptic carboxyamidomethylated (cam)-hexapeptide Thr^{105}-Lys^{110}. To confirm the identity of the isolated peptide, we determined its molecular weight by FAB-MS. Positive ion FAB-spectra are shown in Fig.2. T_1 yielded a protonated molecular ion ($[M+H]^+$) at m/z 679.2, which is in agreement with the calculated molecular mass of the cam-peptide Thr^{105}-Lys^{110} (679.3). An ion at m/z 695.1 corresponding to $[M+H]^+$ for the oxidized form of the cam-peptide Thr^{105}-Lys^{110} was also observed. Peaks at m/z

Table 1- Amino Acid Composition (residues/100) of Tryptic (T) and Peptic (P) Peptides derived from Carboxyamidomethylated-PLP

	Peptide[a]				
	T_1	P_1	P_2	P_3	P_4
Asx	1.7	0.6	0.5	2.7	6.5 (1)
Glx	3.1	0.9	2.5	1.3	2.8
Ser	4.5	4.7 (1)	8.0 (1)	4.9 (1)	4.8
Gly	20.7 (2)	17.1 (2)	14.5 (2)	15.7 (1)	12.6 (1)
His	0.4	N.D.	N.D.	0.9	2.4
Arg	1.5	0.6	0.7	8.1 (1)	N.D.
Thr	30.2 (2)	15.8 (2)	10.7 (2)	2.7	26.2 (2)
Ala	2.7	8.7 (1)	2.5	14.4 (2)	2.9
Pro	0.7	0.6	2.4	6.3 (1)	1.9
Tyr	0.6	3.4	10.0	3.1	6.8 (1)
Val	3.8	0.6	3.5	7.6 (1)	0.5
Met	N.D.	N.D.	N.D.	0.9	N.D.
Ile	11.3 (1)	10.9 (1)	10.0 (1)	3.6	10.2 (1)
Leu	N.D.	12.4 (1)	11.2 (1)	15.7 (2)	0.4
Phe	0.7	4.7	4.0	9.0 (1)	10.1 (1)
Lys	17.8 (1)	19.0 (2)	19.5 (2)	2.7	11.6 (1)

[a]. Numbering of peptides as in Fig. 1. Amino acid analysis of T_1 was carried out as described under Experimental Procedures. Amino acid analysis of P_1-P_4 was carried out by the method of Chang (15). Values in parentheses indicate the number of residues found by sequencing or MS analysis. N.D.: Not Detected.

701.1 and 717.3 correspond to the above species as their sodium adducts. In addition to the molecular ion, numerous ions corresponding to sequence specific fragments of this peptide were observed (not shown), further strengthening its identification.

To investigate whether additional acylation sites could be identified, digestion was also carried out with pepsin. As with trypsin, a high proportion (>67%) of the radioactive peptic peptides partitioned into the upper, water-methanol, phase. RP-HPLC analysis of the peptic peptides showed 4 major radioactive peaks (P_1-P_4) (Fig.1B). Mass spectrometry (Fig.2) and amino acid analysis (Table 1) of the four labeled peptic peptides showed that three (P_1, P_2 and P_4) contain cam-Cys^{108}. P_1, the most abundant peptide, corresponds to the cam-undecapeptide Lys^{104}-Ala^{114} with a protonated molecular ion $[M+H]^+$ at m/z 1136.1 (calculated 1135.8). P_2 corresponds to the cam-decapeptide Lys^{104}-Ser^{113} with a $[M+H]^+$ at m/z 1064.4 (calculated 1064.3). Additional molecular ions at m/z 978.6 and 935.5 correspond to cam-peptides Lys^{104}-Leu^{112} (calculated m/z 978.0) and Thr^{105}-Ser^{113} (calculated 936.4), respectively, which derive from partial hydrolysis of the parent peptide. P_4, the most hydrophobic of the radioactive peptic peptides, corresponds to the cam-nonapeptide Phe^{100}-camCys^{108} with a $[M+H]^+$ at m/z 1104.0 (calculated 1104.5). Thus, using two different enzymes, Cys^{108} is clearly identified as the major acylated cysteine in APL. The remaining peptic peptide (P_3) was identified as the cam-undecapeptide Arg^8-Leu^{18} ($[M+H]^+$ at m/z 1190.9), which has a cam-Cys at position 9. Thus, this residue may be an additional acylation site, but this possibility remains to be confirmed.

DISCUSSION

Structural studies on myelin PLP have been difficult because of its hydrophobic properties which lead to aggregation and resistance to proteolysis. This problem, in addition to the instability of the protein-fatty acid linkage and poor recovery of hydrophobic peptides from HPLC columns, has made it impossible to determine the fatty acid binding site by direct analysis of radiolabeled acyl-peptides. Consequently, we have used an alternative approach that involves the identification of the cysteine residue which is alkylated upon removal of the fatty acid. Because cam-peptides are less hydrophobic than their acylated counterparts, they can be recovered from silica-bonded HPLC columns with higher yields. Furthermore, the linkage between the protein and the radioactive moiety is chemically stable, and consequently, the label is not lost during peptide purification. Using this approach, we have clearly identified Cys^{108} in bovine myelin PLP as an acylated amino acid. The assignment of Cys^{108} as an acylated amino acid is consistent with the fact that this residue is not detected either as a free sulfhydryl or as part of a disulphide bridge (16). Furthermore, it may explain why the tryptic peptide Thr^{105}-Lys^{110} is isolated only after strong acid treatment of the apoprotein (17), which would cleave the fatty acid-protein linkage. Even though a labeled peak containing Cys^{9} could be identified by MS, it is unlikely that this residue is an acylation site. Shaw et al. (16) have shown that Cys^{9} can be alkylated only after reduction of the protein with 2-mercaptoethanol, suggesting its participation in a disulphide linkage. Incomplete alkylation of the protein during the first carboxyamidomethylation or contamination of the radioactive (P_3) peptide with the unlabeled Cys^{9}-containing peptide may be among the reasons for such finding.

During the course of determining the primary structure of bovine PLP, Stoffel and co-workers (7) identified Thr^{198} as an acylated amino acid. Because of the experimental design used in our study we could neither confirm nor rule out whether Thr^{198} is also acylated. Recently, Ross and Braun (8) suggested that the fatty acid was located within the long Staphyloccocus aureus V-8 protease peptide Cys^{5}-Glu^{37} (M_r 3,321), which contains six cysteines. However, Tyr^{103}-Glu^{135}, another Staphyloccocus aureus V-8 protease peptide, has a strikingly similar molecular mass (M_r 3,412). Since peptide identification was based solely on its electrophoretic mobility on SDS-gels, it is possible that the peptide that they found is the one containing Cys^{108}.

The integral membrane protein rhodopsin, like myelin PLP, contains covalently bound fatty acids (18). Acylation of these two proteins has many features in common : a) the addition of the fatty acid occurs late after translation and is independent of protein synthesis (18,19), b) there is fatty acid specificity (20, 21), c) the acyl moiety turns over very rapidly (22, 23), and d) the attachment of the fatty acid is not mediated by an enzyme (20,24). Furthermore, the amino acid sequence surrounding the palmitoylation site of the myelin PLP (Thr^{105}-Thr-Ile-Cys*-Gly-Lys^{110}) is almost identical to that found in rhodopsin (Thr^{319}-Thr-Leu-Cys*-Cys*-Gly-Lys^{325}) (25). Moreover, as in rhodopsin, *beta*-adrenergic receptor and other G protein-coupled receptors (26), the acylated cysteine in PLP is located within a hydrophilic, positively charged segment. In one of the topographical models of PLP previously proposed, this basic segment (Tyr^{91}-Lys^{150}) is shown exposed to the cytoplasm (27). Thus, palmitoylation of Cys^{108} could promote the association of this extensive hydrophilic segment with the plasma membrane.

At present, the precise role of PLP acylation in myelin in unknown, but in aqueous medium the fatty acids contribute to the stabilization of the tertiary structure of the protein (28). Because of the strong similarities between acylation of PLP and that of G protein-coupled receptors, and the dynamic characteristics of this reaction, it is possible that PLP-bound fatty acids could play a role in myelin maintenance by participating in the transduction of intracellular signals.

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