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# Proteolipid Protein (PLP) of CNS Myelin: Positions of Free, Disulfide-Bonded, and Fatty Acid Thioester-Linked Cysteine Residues and Implications for the Membrane Topology of PLP†

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ABSTRACT: Proteolipid protein (PLP), the major integral membrane protein of central nervous system myelin, contains 14 cysteine residues within its 276-residue polypeptide chain. We determined the state of all cysteine residues and localized four of them as free thiols at positions 24, 32, 34, and 168. Four cysteines are connected by disulfide bonds: $Cys^{200}$–$Cys^{219}$ and $Cys^{183}$–$Cys^{227}$. The remaining six cysteine residues at positions 5, 6, 9, 108, 138, and 140 are modified by long-chain fatty acids, mainly palmitic acid, in thioester linkage. The extreme hydrophobicity of PLP can therefore be explained by two structural features: a composition of approximately 50% apolar amino acid residues and a high degree of fatty acid acylation. A differential fluorescent-labeling technique was developed for the structural studies: the cysteine residues belonging to one of the three states were derivatized by *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid (I-AEDANS) either directly (a), after thioester cleavage with hydroxylamine (b), or after disulfide cleavage with dithiothreitol (c). The protein was then proteolytically digested with thermolysin, and the labeled peptides were isolated by reversed-phase HPLC followed by sequence analysis. The results were further confirmed by determination of the fatty acid to protein stoichiometry. The structural data not only demand the revision of our concept of the membrane topology of PLP but will also promote more sophisticated studies on the mechanism of myelination and new functions of PLP.

Myelin proteolipid protein (PLP)[1] is the most abundant protein component and the major integral membrane protein of the myelin of central nervous system. It is one of the most hydrophobic proteins in nature, insoluble in aqueous solution but soluble in organic solvents like mixtures of chloroform and methanol (Folch-Pi & Lees, 1951). PLP awaited its structural elucidation for 30 years due to these unusual hydrophobic properties (Stoffel et al., 1982). The 276-amino acid polypeptide contains approximately 50% hydrophobic amino acids and is distinctly structured into four hydrophobic and five hydrophilic domains. The hydrophobicity is further enhanced by the presence of covalently linked long-chain fatty acids (Stoffyn & Folch-Pi, 1971). So far, only structural functions have been assigned to PLP which are believed to be essential for the wrapping and close and compact apposition of oligodendrocyte plasma membrane processes around the axon. Besides the newly synthesized PLP, MBP, other minor protein constituents, and vast amounts of sphingo- and phospholipids are also targeted during myelination with the formation of the highly ordered myelin membrane system of CNS.

The contribution of the hydrophobic domains of PLP to the high order of the membrane becomes strikingly apparent when point mutations cause minute structural changes which in most cases have lethal effects. Point mutations of the PLP locus have been analyzed in several animal species, e.g., the md rat (Boison & Stoffel, 1989), msd mouse (Gencic & Hudson, 1990), human dysmyelinoses of the type Pelizaeus–Merzbacher (Hudson et al., 1989; Weimbs et al., 1990), and a point mutation (A–G transition) at the splicing acceptor site of intron IV of the jimpy mouse (Nave et al., 1987) which leads to an alternative splicing associated with a frame shift.

The amino acid sequence of PLP shows a most remarkable homology between mammalian species (Stoffel, 1990) and phylogenetically distant species like frog, chicken, and rodents (Schliess & Stoffel, 1991). Several models of the topology of PLP in the lipid bilayer of the myelin membrane have been suggested as working hypotheses. They were either hypothetical models (Laursen et al., 1984; Popot et al., 1991) or derived from results obtained by different techniques such as proteolytic attack of hypoosmotically shocked myelin with subsequent characterization of the main proteolytic fragments (Stoffel et al., 1984), immunochemical analysis with anti-peptide antibodies (Hudson et al., 1989; Stoffel et al., 1989), photoaffinity labeling (Kahan & Moscarello, 1985), and X-ray diffraction (Inouye & Kirschner, 1989). These models altogether lack the essential information about the correct state of the 14 cysteine residues within the complete amino acid sequence of PLP. Cysteine residues of PLP have been claimed to be present partly as free thiols and partly as disulfide bonds (Lees et al., 1969). So far, one disulfide bond between $Cys^{200}$ and $Cys^{219}$ has been reported (Shaw et al., 1989), and $Cys^{108}$ was found to be acylated by a long-chain fatty acid (Bizzozero et al., 1990b).

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[1] Abbreviations: PLP, proteolipid protein; DM-20, isoform of PLP, originating from alternative splicing; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; I-AEDANS, *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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We report here on the state of all 14 cysteine residues of PLP. There are only four cysteine residues present as free thiols in hydrophobic transmembranal domains I and III, four are involved in two disulfide bridges, and surprisingly six cysteine residues form thioester bonds with long-chain fatty acids. This high degree of fatty acylation has been confirmed on the peptide level and furthermore by the determination of the molar ratio of fatty acid to PLP and DM-20 protein, the 26-kDa isoform of PLP, respectively. The results were derived from differential labeling of cysteines in their different states with the fluorescent, thiol-specific alkylating reagent I-AEDANS. The method proved much superior over the radioactive labeling technique due to the easy detection and monitoring.

On the basis of these experimental results together with the well-documented physical properties of PLP, it is feasible to propose a revised model of the membrane topology of the polytopic PLP in the lipid bilayer of myelin.

## MATERIALS AND METHODS

*Fluorescence Labeling of the Acylated Cysteine Residues.* Bovine brain was homogenized in a Waring Blendor in 20 volumes of chloroform/methanol (2:1) containing 1 mM EDTA and 5 mM iodoacetamide. The suspension was stirred for 1 h at 4 °C and filtered. The proteolipid proteins and some of the lipids were precipitated by addition of five volumes of diethyl ether and sedimented by centrifugation. The pellet was washed twice with ether, dried in a stream of nitrogen, and dissolved in chloroform/methanol/0.1 M HCl (10:10:1). Proteins were rapidly separated from lipids and excessive reagent by gel filtration chromatography at Sephadex LH-60 (Pharmacia) in the same solvent. The protein-containing fraction was precipitated by the addition of five volumes of diethyl ether, the precipitate collected by centrifugation, washed three times with ether, and dissolved to a final concentration of approximately 1 mg/mL in an aqueous solution containing 100 mM Tris-HCl, pH 7.4, 0.5% SDS, 6 M urea, and 10 mM iodoacetamide. The solution was incubated at 37 °C for 2 h. Subsequently, excessive reagent was removed by gel filtration over Bio-Gel P-30 (Bio-Rad) in 50 mM Tris-HCl, pH 7.4, 0.1% SDS, and 0.5 mM EDTA. A solution of 1 M $NH_2OH$–HCl, pH 7.4, solid urea, and *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid (I-AEDANS, Fluka) was added to a final concentration of 0.4 M, 6 M, and 3 mM, respectively, and the resulting mixture was incubated at 37 °C. After 2 h, the disulfide bonds were reduced to their thiols by addition of DTT at a final concentration of 9 mM and 2 h incubation at 37 °C and carboxyamidomethylated by the addition of iodoacetamide (final concentration, 25 mM; 2 h; 37 °C). The solution was again desalted by gel filtration chromatography over Bio-Gel P-30 in 50 mM Tris-HCl, pH 7.4, and 0.1% SDS for subsequent protease digestion.

*Fluorescence Labeling of the Cysteine Residues Involved in Disulfide Bridges.* The procedure was identical to that described above including the delipidation step by Sephadex LH-60 chromatography. The protein was subsequently dissolved in a solution containing 0.5% SDS, 0.4 M $NH_2$-OH–HCl, pH 7.4, 6 M urea, and 10 mM iodoacetamide and incubated for 5 h at 37 °C. DTT was then added to a final concentration of 30 mM. The reduction proceeded for 4 h at 37 °C. Excessive DTT was removed by gel filtration chromatography over Bio-Gel P-30 in 0.1% SDS, 50 mM Tris-HCl, pH 7.4, 1 mM DTT, and 0.5 mM EDTA. The thiols were labeled by the addition of I-AEDANS, urea, and Tris-HCl, pH 8.5, to final concentrations of 7 mM, 6 M, and 100 mM, respectively, and the solution was incubated for 2 h at 37 °C. Following desalting by gel filtration over Bio-Gel P-30 in 50 mM Tris-HCl, pH 7.4, 0.1% SDS, the labeled protein was digested proteolytically.

*Fluorescence Labeling of the Free Thiols.* Extraction with chloroform/methanol and delipidation were carried out as described above but in the presence of 2 mM I-AEDANS instead of iodoacetamide. Two aliquots of the protein solution were further processed separately. Aliquot A was dissolved in a solution of 100 mM Tris-HCl, pH 7.4, 0.5% SDS, 6 M urea, and 5 mM I-AEDANS and incubated at 37 °C for 1 h. Aliquot B was treated in the same way, but I-AEDANS was replaced by iodoacetamide. Both portions were then reduced by addition of DTT at a final concentration of 20 mM for 2 h at 37 °C, iodoacetamide was added to a final concentration of 50 mM, and the incubation was continued at 37 °C for 2 h. The protein solution was desalted by gel filtration chromatography as described above and subjected to proteolytic cleavage.

*Protease Digestion.* Thermolysin (20 mg/mL) (Boehringer Mannheim) in the presence of 2 mM $CaCl_2$ was added to the labeled PLP solution and the digestion carried out at 37 °C for 48 h. The resulting peptides were processed for subsequent HPLC separation.

*HPLC Analysis of the Proteolytic Peptides.* SDS was removed from the peptide solution by precipitation at 4 °C in the presence of 80 mM $KH_2PO_4$. Complete removal of SDS, desalting, and concentration of the peptides were achieved by adsorption to Sep Pak C18 cartridges (Waters), washing with water, and elution of the peptides with a solution of 30% acetonitrile in water. Samples were dried in a speed-vac concentrator, dissolved in a small volume of water, and characterized by reversed-phase gradient HPLC using a Macherey-Nagel 300-5 C18 column (0.4 × 25 cm) with the eluent solutions A [0.1% trifluoroacetic acid (Sigma) and 10 mM triethylamine (Fluka) in water (Milli-Q, Millipore)] and B [0.1% trifluoroacetic acid and 10 mM triethylamine in 50% water/50% acetonitrile (Baker) at a flow rate of 1 mL/min]. The gradient program is described in the legend to Figure 1. Peptides were monitored with a diode-array UV–vis detector (Shimadzu model SPD-M6A) and a fluorescence detector (Kratos-Analytical, Spectroflow 980) connected in series at 300-nm excitation wavelength. Fluorescent peptides were collected and rechromatographed using different chromatographic conditions.

*Characterization of the Peptides.* Peptides were hydrolyzed with 6 M HCl in the gas phase at 165 °C for 1 h, amino acids were derivatized with phenylisothiocyanate (Pierce), and the phenylthiocarbamoyl derivatives were analyzed by reversed-phase HPLC. For N-terminal sequence analysis, peptides were covalently coupled to Sequelon AA membranes (Millipore) and applied to the gas-phase sequencer (Applied Biosystems model 477A) equipped with an HPLC system for on-line phenylthiohydantoin separation.

*Quantitation of the Protein to Fatty Acid Stoichiometry.* Bovine brain was extracted with chloroform/methanol (2:1), 1 mM EDTA, and 5 mM iodoacetamide and subsequently delipidated by gel filtration chromatography as described above. The proteolipid proteins were further purified by cation-exchange chromatography on a CM-Trisacryl column (IBF Biotechnics) in chloroform/methanol/water (4:4:1) by the method of Helnyk (1983). The three proteolipid protein fractions (DM-20, PLP-I, and PLP-II) were collected. The ratio of protein to protein-bound fatty acids was determined:

protein by quantitative amino acid analysis with norleucine (Sigma) as an internal standard. After hydrolysis with 6 N HCl at 110 °C for 24, 48, 72, and 110 h, the resulting amino acids were derivatized with phenylisothiocyanate and analyzed by reversed-phase HPLC. The peak areas of the phenylthiocarbamoyl derivatives of Gly, Thr, Ala, Tyr, Val, Phe, and Lys were related to the corresponding areas of an amino acid standard mixture (Pierce), and the exact amount was determined by graphic extrapolation to a hydrolysis time of zero.

For quantitative fatty acid analysis, aliquots of the protein fractions were hydrolyzed in 6 N HCl at 110 °C for 6.5 and 24 h, respectively, in ampoules together with 20 μg of 17:0 fatty acid methyl ester (Sigma) as an internal standard. The resulting free fatty acids were extracted with 2 × 1 mL of hexane, and the solvent was evaporated. The fatty acids were converted into their methyl esters in 1 mL of a solution of $BF_3$ (25%) in methanol at 80 °C for 1 h. Water (2 mL) was added, and the fatty acid methyl esters were extracted twice with 1 mL of hexane, concentrated in a stream of nitrogen, and analyzed by gas–liquid chromatography (Carlo Erba Instruments, model GC 8130 equipped with a fused-silica capillary column, J&W Scientific, DB-225, 30 m × 0.25 mm i.d.). Peak areas were related to the corresponding areas of the fatty acid methyl ester standard mixture (Supelco).

## RESULTS

Myelin proteolipid protein has unusual solubility properties. It is easily soluble in organic solvents like mixtures of chloroform and methanol. Therefore, organic extraction of the whole brain is the preferred isolation procedure. The organic phase contains, besides myelin membrane lipids, PLP as the major protein component and, in addition, other minor myelin protein components. This proteolipid fraction was applied in the experiments described below.

We investigated which of the 14 cysteine residues of the PLP molecule are present as free thiols, involved in disulfide bonds, or acylated with long-chain fatty acids. Cysteines belonging to either one of these three functional states were differentially fluorescently labeled separately with a thiol-specific alkylating reagent. Peptides containing these labeled cysteines were released by proteolytic digestion and separated by HPLC, and the position of the derivatized cysteine residues within the peptide sequence in the polypeptide sequence was determined. The strategy of the differential-labeling techniques is schematically outlined in Figure 1.

There is always a risk of disulfide exchanges in proteins that contain free cysteines as well as cystines. We therefore captured any free sulfhydryl groups by alkylation immediately upon its formation or exposure to solvents.

*Sites of Fatty Acid-Acylated Cysteine Residues.* PLP was isolated by extraction of bovine brain with chloroform/methanol (2:1) in the presence of iodoacetamide to block free cysteine residues of the proteolipid proteins. Iodoacetamide specifically reacts with thiol groups in chloroform/methanol as demonstrated by Lees et al. (1969). After delipidation by gel filtration in acidified chloroform/methanol, the protein was precipitated by the addition of ether and transferred into an aqueous solution containing 0.5% SDS, 6 M urea, and again iodoacetamide. This denaturation step guarantees that any thiol group of cysteine residues which might be buried in organic solvent become exposed to the alkylating reagent iodoacetamide. Excessive reagent was subsequently removed by gel filtration, and the protein was incubated in a buffer,

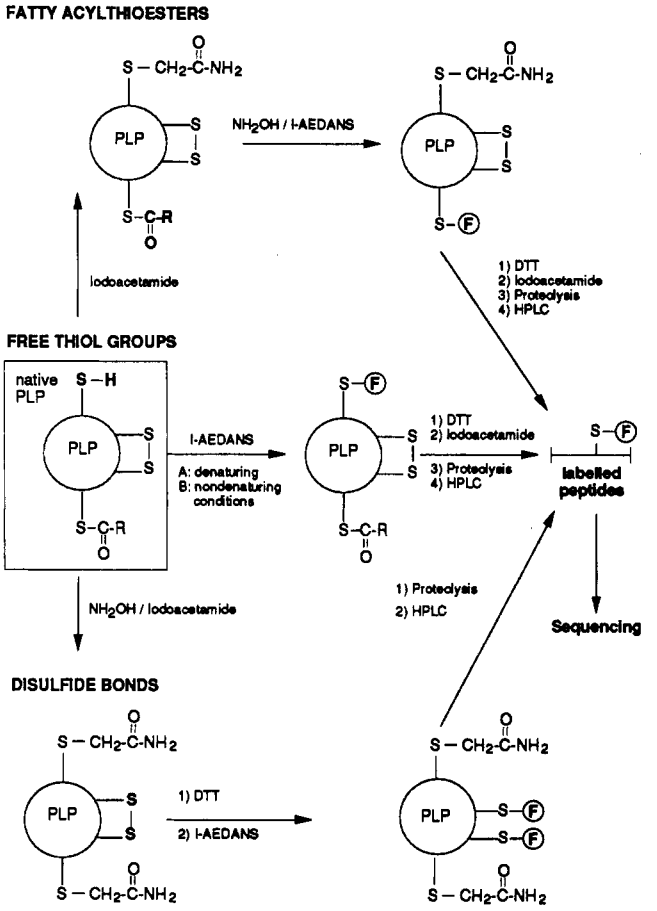


FIGURE 1: Schematic presentation of the differential labeling experiments. All experiments started with the native PLP (box) with the cysteine residues in the three functional states: free thiols, disulfide bridges, and acylated with fatty acids. Cysteines belonging to each of these states are fluorescently labeled differentially with the thiol-specific reagent I-AEDANS (indicated by the circled "F"). Proteolytically derived labeled peptides are separated by HPLC and sequenced. Details are described in the text.

pH 7.4, containing 0.4 M hydroxylamine and the fluorescent sulfhydryl reagent *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid (I-AEDANS) (Hudson & Weber, 1973). Thioester bonds are cleaved under these conditions by hydroxylamine, releasing fatty acids as hydroxamates, whereas free thiols are labeled by I-AEDANS concomitantly. Hence, PLP modified in this way bears the fluorescencent label specifically at the cysteine residues being acylated in the native protein. After reduction of the disulfide bridges and carboxyamidomethylation, the protein was cleaved by thermolysin. The proteolytic peptides were separated by reversed-phase HPLC. Labeled peptides were detected by their UV absorption at 340-nm wavelength, which corresponds to the absorption maximum of the labeling group (Figure 2-I), and by their fluorescence at 300-nm excitation wavelength (not shown). Peptides corresponding to the seven major peaks were collected, rechromatographed, and analyzed by automated Edman degradation. The obtained sequences of PLP assign the six cysteine residues to positions 5 and 6 (peptides LECC, LECC, LLECC), 9 (peptide ARC), 108 (peptide ICGKG), 138, and 140 (peptides VCHC, VCHC) in the native PLP form.

Two peptides (VCHC and LECC) contain two cysteine residues, but only the first one is alkylated by I-AEDANS as determined by Edman degradation. The iodoacetamide derivative of cysteine appears in the sequencing cycles of the second cysteine residues. In peptides VCHC, LECC, and

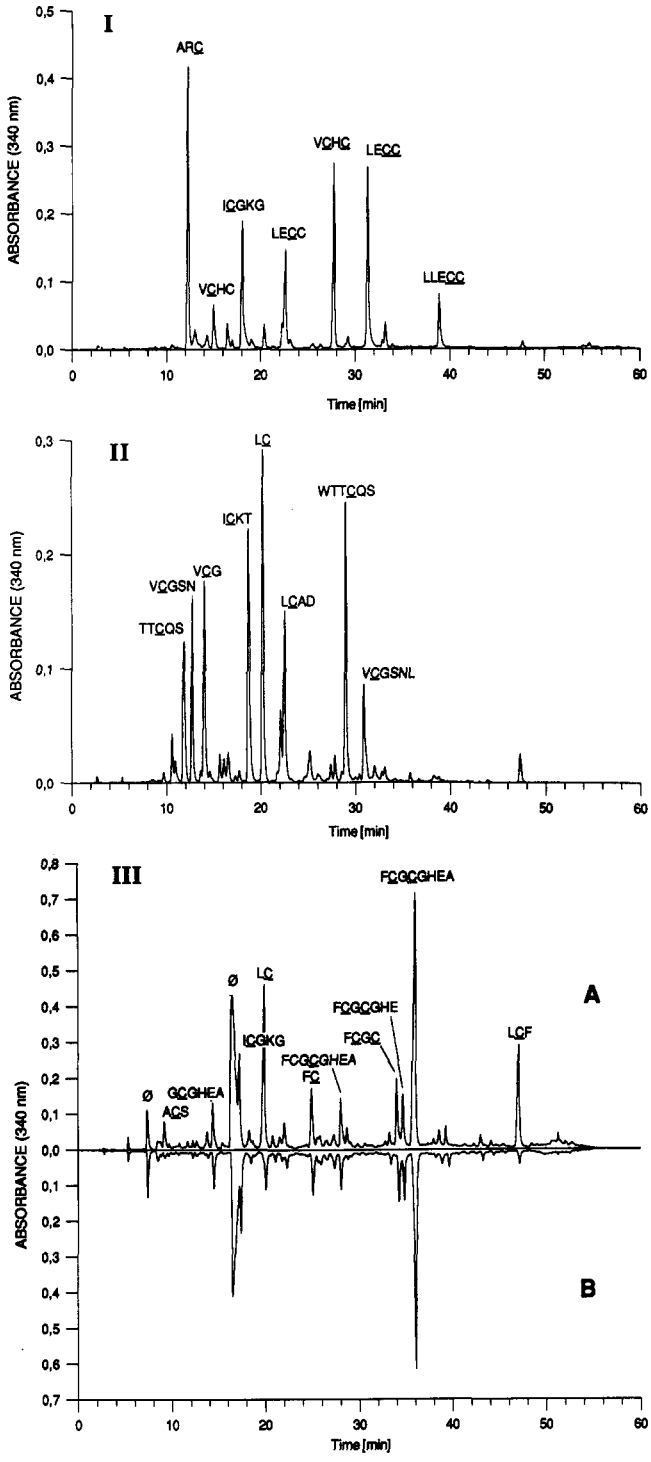


FIGURE 2: Reversed-phase HPLC separation of the proteolytical peptides labeled at the sites of fatty acid acylation (I), of half-cystines involved in disulfide bonds (II), and of free cysteines (III). In panel III, the chromatograms of portions A and B are combined for comparison. In portion A the protein was denatured during the labeling step, whereas in portion B it was not (see text). The UV absorption was monitored at 340 nm. The following two-step linear gradient was used with the eluent solutions A and B as described under Materials and Methods: 5% buffer B in A to 40% B during 45 min and then to 100% B during 10 min. The amino acid sequences of the peaks are listed above each peak in the one-letter code. Fluorescent-labeled cysteine residues are underlined.

LLECC, however, both cysteines are fluorescence-labeled, and these peptides predominate over the single-labeled ones. This heterogeneity could result from a partial cleavage of the thioester bonds during the experimental procedure or by the in vivo existence of both the acylated and the free thiol form.

*Localization of the Half-Cystines Involved in Disulfide Bridges.* The approach was similar to that described above: free cysteine residues were alkylated with iodoacetamide during the chloroform/methanol extraction step and after transfer into aqueous solution containing SDS and urea. Fatty acids were now cleaved from the protein by hydroxylamine treatment in the presence of iodoacetamide, which blocks the newly released thiols. After reduction of the disulfide bonds with a large excess of DTT, the free sulfhydryl groups generated from the cysteine residues linked by disulfide bridges in the native protein were labeled by alkylation with I-AEDANS.

In all steps, solutions were 6 M in urea and 0.5% SDS in order to keep the protein in a denatured state with all cysteine residues accessible to reagents. The labeled PLP was then cleaved by thermolysin, and the peptides were separated by reversed-phase HPLC (Figure 2-II). Four cysteine residues in positions 183 (peptides TTCQS and WTTCQS), 200 (peptides LC and LCAD), 219 (peptides VCGSN, VCG, and VCGSNL), and 227 (peptide ICKT) are involved in disulfide bridges, suggesting the existence of two intramolecular disulfide bonds. The disulfide bridge between $Cys^{200}$ and $Cys^{219}$ has also been established earlier (Shaw et al., 1989). Therefore, the remaining cysteines in the positions 183 and 227 form a second disulfide bridge since PLP is not linked by intermolecular disulfide bonds.

*Localization of the Free Cysteines.* The free cysteine residues of PLP were labeled by I-AEDANS already during the chloroform/methanol extraction step, and the protein was subsequently separated from lipids by gel filtration. In order to investigate if cysteine residues are inaccessible to the alkylating reagent in organic solution due to incomplete denaturation, two aliquots of the PLP solution were analyzed separately. Aliquot A was transferred to a denaturing aqueous solution (6 M urea, 0.5% SDS) containing I-AEDANS to ensure the complete labeling of cysteine residues which might have remained as free thiols. Aliquot B was treated exactly like aliquot A but in the presence of iodoacetamide instead of I-AEDANS so that no further fluorescence labeling could occur. Both aliquots were subsequently reduced with DTT, which in addition to the disulfide bonds also cleaves the remaining thioester linkages. Both samples were alkylated with iodoacetamide, desalted by gel filtration, and digested with thermolysin. Peptides of aliquots A and B were separated by HPLC. The elution patterns are compared in Figure 2-III. Fluorescent-labeled peptides which harbored cysteine residues 24 (peptides LC and LCF), 32 and 34 (peptides GCGHEA, FC, FCGC, FCGCGHEA, FCGCGHE, and FCGCGHEA), 108 (peptide ICGKG), and 168 (peptide ACS) were identified. This indicated that cysteines 24, 32, 34, and 168, which are all located within intramembrane domains of PLP, are present as free thiols in native PLP. $Cys^{108}$, which has also been detected in acylated form, is present as a minor peak in peptide ICGKG (Figure 2-III). This might be due either to partial hydrolysis of the thioester bond during the isolation and characterization procedure or to the existence of both forms in vivo with a preponderance of the acylated form.

Two peaks (LC and LCF) corresponding to peptides containing $Cys^{24}$ of the PLP are major peaks in A of Figure 2-III but appear as very small peaks in B. We conclude that the position of $Cys^{24}$ is shielded by the hydrophobic domains of the PLP or by membrane lipids and might therefore be hardly accessible for the charged and bulky alkylating reagent I-AEDANS even in chloroform/methanol solution.

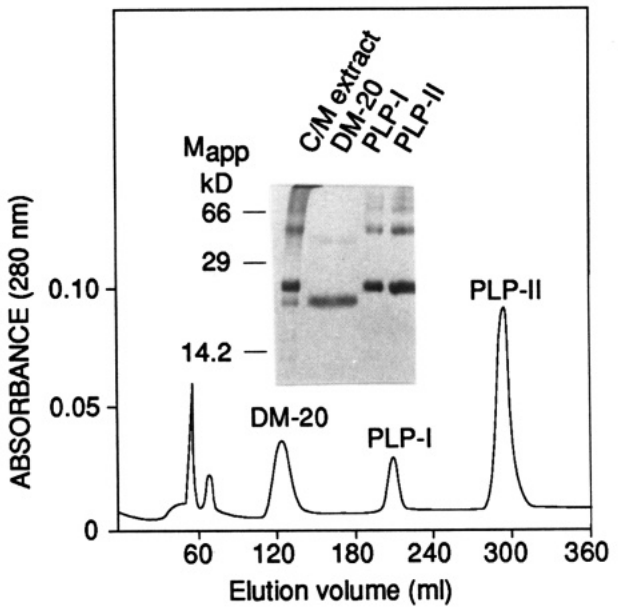


FIGURE 3: Separation of the delipidated proteins of the chloroform/methanol extract of bovine brain by cation-exchange chromatography. The CM-Trisacryl column (16 cm × 2.5 cm i.d.) was equilibrated with chloroform/methanol/water (4:4:1). Proteins were eluted with 50 mL of the same solvent, 100 mL of 10 mM ammonium acetate, and the rest with 50 mM ammonium acetate in the same solvent at a flow rate of 1 mL/min. Proteins were detected by their absorbance at 280 nm. The three major peaks were collected (DM-20, PLP-I, and PLP-II) and subjected to quantitative amino acid analysis and fatty acid analysis. The inset shows the band pattern of the eluted peaks and of the chloroform/methanol extract on a SDS–PAGE analysis (15%) stained with Coomassie blue.

Peptide ACS gives only a small signal among the peptides of aliquot A separated by HPLC and is almost absent among those of aliquot B. This suggests that cysteine 168, which is also located within a hydrophobic membrane region, is much less accessible for I-AEDANS than cysteine 24 even under the denaturing conditions of 6 M urea and 0.5% SDS.

Peaks marked by Ø in Figure 2-IIIA are nonpeptide contaminants of unknown origin and structure. No derivatives of amino acids can be detected upon automated Edman degradation and amino acid composition analysis; the UV spectra differ significantly from those of all other detected labeled peptides as revealed by on-line UV–vis photodiode array detection.

*Protein to Fatty Acid Stoichiometry.* Two of the six cysteine residues ($Cys^{138}$ and $Cys^{140}$ which we found to be fatty-acylated in PLP are located in the doamin which is delected in the splicing variant DM-20. This isoform should therefore contain only four thioester-linked fatty acids instead of six as the PLP itself. Our results were also verified by the molar ratios of fatty acids to protein of PLP and DM-20. This ratio has been previously estimated in the range of 1–3 molecules of fatty acid per molecule of PLP. However, these analyses are based upon the total delipidated chloroform/methanol extract containing a mixture of PLP, DM-20, and an unknown amount of minor proteins (see Discussion). We obtained a more accurate stoichiometry with the proteolipid proteins purified from the chloroform/methanol extract by cation-exchange chromatography using a CM-Trisacryl column in organic solution by the method of Helynck et al. (1983). Three major peaks appeared, one of which corresponds to the DM-20 isoform and two peaks to PLP (designated PLP-I and PLP-II; see Figure 3). Some minor peaks eluted near the solvent front. The nature of the charge difference between the two PLP forms is unknown. The protein fractions of the three peaks and the chloroform/methanol extract before cation-exchange chromatography were investigated separately for their ratio of protein to protein-bound fatty acids as described under Materials and Methods.

Table I

| | fatty acid content (nmol/mL)[a] | protein content (nmol/mL) | stoichiometry (mol of fatty acid/mol of protein) |
|---|---|---|---|
| C/M extract | 918 ± 14 | 256 ± 10 | 3.6 ± 0.2 |
| DM-20 | 17.6 ± 0.2 | 4.83 ± 0.13 | 3.6 ± 0.1 |
| PLP-I | 9.2 ± 0.4 | 2.33 ± 0.16 | 3.9 ± 0.3 |
| PLP-II | 42.8 ± 0.8 | 9.49 ± 0.25 | 4.5 ± 0.2 |

[a] Protein to fatty acid stoichiometry of the proteolipid fractions obtained by cation-exchange chromatography (DM-20, PLP-I, and PLP-II) and of delipidated proteins of the chloroform/methanol extract (C/M-extract) before chromatographic separation. Fatty acids were determined as fatty acid methyl esters after acid hydrolysis and subsequent esterification. The error is expressed as the mean error of the mean value of four independent determinations. The protein concentration was determined by quantitative amino acid analysis after acidic protein hydrolysis. For each individual protein, duplicate hydrolysis experiments were done with hydrolysis times of 24, 48, 72, and 110 h, respectively. The amounts of seven amino acids were determined and divided by the known number of the amino acid residues in the protein, and the result is expressed as the mean value together with the mean error. For the calculation of the protein concentration in the C/M extract, a protein composition of 90% PLP and 10% DM-20 was estimated from SDS–PAGE.

The results shown in Table I indicate that there are 3.6 fatty acid molecules attached to the DM-20 polypeptide (theoretically four) and 3.9 and 4.5 fatty acid residues (theoretically six) are released from one molecule of PLP-I and PLP-II, respectively. Whether this deviation from the theoretical values is due to an incomplete acylation of the cysteine residues in vivo or to a loss of fatty acids during the isolation procedure of the proteins cannot be decided.

It is obvious that the PLP-II fraction which represents the major portion of PLP is acylated to a greater extent than DM-20 (4.5 versus 3.6 mol of fatty acid/mol of protein), strongly supporting the result that $Cys^{138}$ and $Cys^{140}$, which are part of the deleted sequence $Val^{116}$ to $Lys^{150}$ in DM-20, are sites of fatty acylation.

The minor PLP fraction (PLP-I) seems to be less acylated than PLP-II. Whether this is the reason for its different chromatographic behavior in the cation-exchange chromatography is questionable because the acylation of a cysteine residue only eliminates one weakly acidic thiol group.

## DISCUSSION

We completed the primary structure of proteolipid protein by defining the state of its 14 cysteine residues. The differential labeling studies with the thiol-specific fluorescent probe I-AEDANS, proteolytic cleavage, separation, and amino acid sequencing of the fluorescent-labeled peptides described here revealed four cysteines in positions 24, 32, 34, and 168 as free thiols.

Two pairs of cysteine residues form disulfide bridges, $Cys^{183}$ with $Cys^{227}$ and $Cys^{200}$ with $Cys^{229}$. Surprisingly, six cysteine residues in positions 5, 6, 9, 108, 138, and 140 turned out to be acylated with long-chain fatty acids.

This unexpected finding is at variance with early reports which describe a fatty acid to PLP ratio in the range of 1–3 mol of fatty acids/mol of PLP (Braun & Radin, 1969; Sherman & Folch-Pi, 1970; Gagnon et al., 1971; Cockle et al., 1980). The discrepancy of this stoichiometry measured in these early studies to our analyses can be explained by (a) the proteins of the crude chloroform/methanol extract have been analyzed,

(b) the exact molecular mass of PLP was unknown, and calculations based on the apparent molecular mass were derived from the mobility in SDS–PAGE suggesting an erroneously low molecular mass (c) methods for protein quantifications used were insufficient for stoichiometric analyses; and (d) protein isolation procedures included a reduction step with mercaptoethanol or DTT and extension dialysis in acidic media, conditions well known to cause hydrolysis of labile thioester linkages.

In the present study, we elaborated a rapid and mild isolation and separation procedure for PLP and DM-20 essential for structural studies and the quantitation of the protein and fatty acid thioesters bonded in the two isoforms. Hydroxylamine released the fatty acids as hydroxamates at six cysteines of PLP. These residues were derivatized with I-AEDANS. Six peptides containing fluorescent-labeled cysteines were released by proteolysis with thermolysin, separated, sequenced, and assigned to the PLP amino acid sequence. Only four fluorescent-labeled peptides were obtained from DM-20. Table I summarizes the ratios of fatty acid to protein. PLP yielded a ratio of 4.5 and DM-20 of 3.6 mol of fatty acid/mol of protein. The deviation from the ideal value derived from the peptide analysis may be explained by partial hydrolysis of thioesters during protein isolation or by incomplete acylation at the different sites of the native PLP or by a combination of both. In vitro labeling experiments suggested that a pool of only partially acylated PLP might be present in myelin (Bizzozero et al., 1987; Yoshimura et al., 1987; Ross & Braun, 1988; Bizzozero & Good, 1991). Also, the fatty acylesterase recently described in myelin (Bizzozero et al., 1992) might contribute to a partial deacylation and obscure the stoichiometry. The four cysteine residues determined as free thiols are located in hydrophobic and putative transmembrane domains of PLP. $Cys^{32}$ and $Cys^{34}$, located at the border of the transmembrane domain, are readily labeled with I-AEDANS in organic solvents in contrast to $Cys^{24}$ and $Cys^{168}$, which are located in the center of hydrophobic domains. Drastic denaturing conditions are required for the complete derivatization of $Cys^{24}$ which leaves $Cys^{168}$ incompletely alkylated by the spacious and charged I-AEDANS. However, $Cys^{168}$ is alkylated by iodoacetamide completely. No labeled peptides containing $Cys^{168}$ are found in the determination of the acylation sites or the disulfide-bonded cysteine residues.

Vacher et al. (1984) titrated the free thiol groups of PLP in organic solvents and aqueous solution with a variety of thiol–disulfide interchanging reagents. Values between 4.4 and 4.8 were obtained. These authors also noticed the different reactivity of half of the thiol groups. Shaw et al., (1989) labeled free thiols of PLP with [$^{14}$C]iodoacetamide and detected alkylated $Cys^{32}$ and $Cys^{34}$ in two chymotryptic peptides.

Shaw et al. (1989) described $Cys^{200}$ and $Cys^{219}$ as part of two connected peptides, which is in agreement with our analysis. Our results clearly show that only four out of 14 cysteine residues are linked by disulfide bonds, $Cys^{200}$, $Cys^{219}$, $Cys^{183}$, and $Cys^{227}$. Therefore, $Cys^{183}$ and $Cys^{227}$ must form the second bridge. The two disulfide bonds are located in proximity in a hydrophilic loop which therefore should be oriented toward the extracytosolic side of the membrane.

The experimental approach described here demonstrates that PLP is acylated at six cysteine residues in positions 5, 6, 9, 108, 138, and 140. Previous studies have shown that PLP contains thioester-linked fatty acids (Ross & Braun, 1988; Bizzozero & Good, 1990a). Bizzozero et al. (1990b) found only $Cys^{108}$ as an acylation site of PLP. Some decisive experimental steps different from those described here might explain the discrepancy of our results to those of the previous report. Bizzozero et al. reduced and carboxyamidomethylated PLP and used this derivatized product as substrate for the hydroxylamine cleavage of the thioester bonds and simultaneous alkylation with [$^{14}$C]iodoacetamide. The reduced and alkylated PLP substrate was obtained by reducing delipidated chloroform/methanol extracted proteins from bovine brain with a 30-fold excess of DTT over a period of 16 h at 37 °C, followed by alkylation and dialysis for several days at 4 °C. Sulfhydryls like DTT are well known as strong nucleophiles which cleave thioester bonds (O'Brien et al., 1987; Ovchinnikov et al., 1988; Curstedt et al., 1990; Veit et al., 1991). Moreover, during the dialysis over this extended time, further thioester hydrolysis could occur with oxidation of liberated thiol groups.

Several suggestions regarding the membrane integration and orientation of PLP in the lipid bilayer of the myelin membrane have been made on a theoretical basis, a few using experimental approaches. In all these models, the features of the amino acid sequence of PLP, which is well structured into hydrophobic and hydrophilic domains, have been adapted to the lipid bilayer of the myelin membrane either solely on theoretical grounds or by matching the results of biochemical and or immunological targeting experiments with the amino acid sequence of PLP. Essential insight should, however, come from the knowledge of the functional states of the 14 cysteine residues of PLP with regard to the topology of these structural motifs.

Therefore, our results describing the exact positional and functional assignment of all cysteine residues present either as free thiols, disulfide-bonded, or fatty acid thioester-bonded should prepare firmer grounds for a putative model of the membrane topology of PLP. The results are summarized in Figure 4.

The four free thiol groups of $Cys^{24}$, $Cys^{38}$, $Cys^{40}$, and $Cys^{168}$ are all located in hydrophobic domains. Cysteine residues residing on the extracytoplasmatic side of a membrane typically form disulfide bonds placing the loop containing the disulfide bonds formed between $Cys^{183}$–$Cys^{227}$ and $Cys^{200}$–$Cys^{219}$ to this side of the membrane. Cysteine residues exposed to the reductive cytosolic environment should be present as free thiols. The residual six cysteine residues of PLP were found to be acylated with long-chain fatty acids, predominantly palmitic acid. Palmitoylation of proteins is a posttranslational process. It has been suggested that PLP might be modified even after the protein is fully integrated into the plasma membrane (Townsend et al., 1982; Bizzozero et al., 1983; Townsend & Benjamins, 1983). A similar late posttranslational acylation has been demonstrated for ras p21 (Grand & Owen, 1991), the transferrin receptor (Omary & Trowbridge, 1981), and rhodopsin (St. Jules & O'Brien, 1986). So far, acylated cysteine residues of membrane proteins have been found exclusively on the cytoplasmic side of membranes, e.g., of the glycoprotein of the human transferrin receptor (Jing & Trowbridge, 1987), of the human tissue factor (Bach et al., 1988), and of a variety of ras and ras-related proteins (Grand & Owen, 1991; Palme et al., 1992). It is reasonable to assume that this also holds for PLP, which places the acylated thiols 5, 6, and 9 and therefore the N-terminus on the cytoplasmic side of the myelin membrane and likewise the hydrophilic loop 3 with $Cys^{108}$, $Cys^{138}$, and $Cys^{140}$ which links the second and third putative transmembrane $\alpha$-helices. The acyl transfer to PLP has been suggested to occur nonenzymatically and autocatalytically with acyl-CoA esters as donor molecules (Bizzozero & Lees, 1986; Ross & Braun, 1988) analogous to

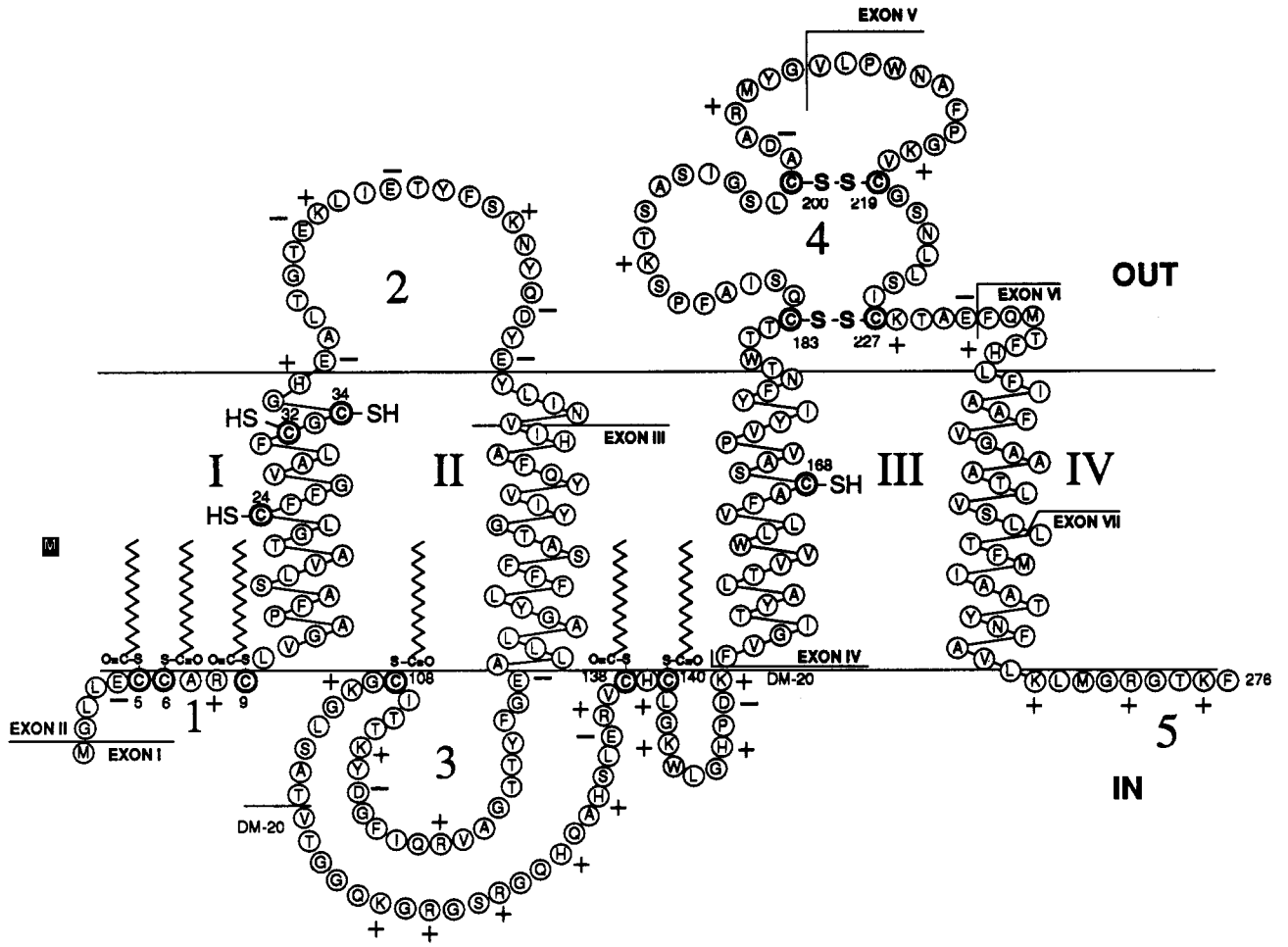


FIGURE 4: Proposed model of the topology of the proteolipid protein in the plasma membrane of oligodendrocytes and the myelin membrane as suggested by the amino acid sequence and the determination of the state of all 14 cysteine residues (circles). The region which is deleted in DM-20 is indicated by bars.

the acylation of Semliki forest virus E2 protein (Berger & Schmidt, 1984), bacterial rhodopsin (O'Brien et al., 1987), and $P_o$ glycoprotein of peripheral nerve myelin (Agrawal & Agrawal, 1989). The cytosolic orientation of the fatty-acylated cysteines is further supported by the rapid turnover catalyzed by a fatty acylesterase (Bizzozero et al., 1992). If we combine the compartmentation of the substrate fatty acyl-CoA esters and the esterase with the complete primary structure including the six acyl thioester groups, the model presented in Figure 4 best explains our experimental results and the theoretical considerations. The previous PLP models are no longer compatible with the model presented here. The positive immunoreaction of the large hydrophilic domain 3 with antipeptide antibodies against partial sequences of this domain demonstrated with oligodendrocytes in two laboratories might be due to the permeabilization of the membrane under the conditions used. The model evolving from our experiments described here agrees with the $N_{in}$–$C_{in}$ model, one of the two models favoring a four $\alpha$-helix topology of PLP, as described by Popot et al. (1991) based on theoretical considerations.

The functional aspects of the acylation of cysteine residues of a number of regulatory proteins, e.g., ras p21 or G-protein-coupled receptors, suggest functions of PLP other than those of a structural element of the myelin membrane involved in the compacting and wrapping process of the multilayer membrane system, particularly in view of their unusually high number of fatty acids. Their acylation and deacylation should considerably influence the interaction of these domains when acylated and integrated into the lipid bilayer with adjacent membrane components or in the deacylated form with membrane cytosolic proteins. DM-20, which misses the two acylation sites $Cys^{138}$ and $Cys^{140}$ due to alternative splicing (Figure 4), could have a reactivity different from that of PLP, causing an additional tuning of the regulation. The acylation of the two adjacent cysteines 5–6 and 138–140 mimick a diglyceride structure which is known to activate protein kinase C.

Our results, which aimed at the assignment of the correct topology of PLP in the myelin membrane, will be helpful for present studies on the biosynthesis, membrane integration, and membrane targeting but also for our understanding of mutational events leading to dysmyelinoses and of the complex processes involved in the demyelinating diseases like multiple sclerosis.

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