

# Structure and molecular arrangement of proteolipid protein of central nervous system myelin

(membrane integration of proteolipid protein/hyposmotic treatment of myelin/trypsinization of myelin membrane/  
characterization of protein fragments)

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**ABSTRACT** Proteolipid protein (PLP) of central nervous system myelin is one of the most hydrophobic integral membrane proteins. It consists of a 276-residue-long polypeptide chain with five strongly hydrophobic sequences of 26, 30, 39, 12, and 36 residues, respectively, linked by highly charged hydrophilic sequences. Hyposmotically dissociated bovine myelin membranes were treated with trypsin. PLP was completely cleaved into smaller fragments, whereas basic myelin protein remained essentially unaltered. The proteins and tryptic peptides of myelin were separated after the removal of the short, water-soluble peptides into three large fragments of 11, 7.3, and 9.0 kDa, respectively. They were characterized by their molecular mass and NH<sub>2</sub>-terminal amino acid sequences, which proved that trypsin cleaved predominantly at Arg-97 yielding the 11-kDa fragment from Gly-1 through Arg-97, at Arg-126 releasing the 7.3-kDa fragment from Gly-127 through Lys-191, and at Lys-191 releasing the 9-kDa fragment from Thr-192 through Phe-276. We propose that PLP is integrated into the lipid bilayer of myelin with the NH<sub>2</sub> terminus and three positively charged hydrophilic loops oriented toward the extracytosolic side of the membrane, whereas one strongly negative hydrophilic loop and the positively charged COOH terminus cover the cytosolic side of the lipid bilayer. Basic myelin protein remains protected against tryptic cleavage, which indicates its apposition to the cytosolic side of the membrane. These cleavage sites of trypsin support the suggested orientation of PLP in the myelin membrane and thereby extend our knowledge about the molecular arrangement of the components of this membrane. In demyelinating processes membrane desintegration could be initiated by proteolysis at the external surfaces of proteolipid protein in a similar way as described here.

Proteolipid protein (PLP), also named lipophilin (1), is the major structural protein of brain white matter. It accounts for more than 50% of myelin membrane proteins of the central nervous system (CNS) and is supposed to maintain the myelin structure and function. PLP has resisted its biochemical characterization because of its insolubility and tendency to aggregate in aqueous solution. These properties explain the sparse sequence data and slow progress in the structural elucidation of PLP ever since its discovery (2). Only small hydrophilic sequences, except a limited hydrophobic sequence at the COOH-terminal end (3-5), were elaborated with the conventional peptide separation techniques.

Recently, new approaches in the separation of hydrophobic peptides (6-8) enabled us to determine the entire amino acid sequence of bovine PLP (9). The polypeptide chain consists of 276 amino acid residues that are arranged in four long and one short hydrophobic segments, which are linked by hydrophilic highly charged sequences. One fatty acid residue

is attached by an ester bond to a threonine residue within a hydrophilic segment (9). During our structural studies, it also became evident that cysteine residues form disulfide bonds between distant hydrophilic sequences. This inevitably leads to a condensed alignment of these hydrophilic loops at the surface of the membrane lipid bilayer, associated with a clustering of the membrane-spanning and embedded hydrophobic sequences.

The knowledge of the primary structure led to the proposal of the alignment in the myelin membrane of CNS with respect to the main dense line and the intraperiod dense line—the equivalents of the adjacent cytosolic surfaces and the external surfaces, respectively, in electron microscopy (8, 9). In this communication, experimental evidence in support of this model is given by tryptic cleavage within the hydrophilic peptide loops oriented toward the external side of the myelin membranes. Hyposmotic shock of myelin preparation renders this surface available for enzymatic attack. Three large tryptic polypeptides were formed. Their characterization allowed the conclusion that the NH<sub>2</sub> terminus and three positively charged hydrophilic segments are located at the extracytosolic side of the lipid bilayer, whereas the negatively charged loop and the positively charged COOH terminus point to the cytosolic side.

These results contribute to our understanding of the molecular architecture of the highly organized myelin membrane system and of factors that may affect its stability and function in demyelinating processes.

## MATERIALS AND METHODS

**Isolation of Myelin.** All procedures were carried out at 4°C. Myelin was isolated by a modification of the procedure described by Norton (10). It permits the preparation of myelin on a large scale. The subcortical white matter from a fresh bovine brain (300 g) was homogenized in 700 ml of 0.17 M sucrose in a Waring Blender for 2 min (medium speed). The suspension was centrifuged at 5000 × g in a JA-14 Beckman rotor for 20 min. The supernatant was discarded, and the pellet was resuspended in 600 ml of 0.17 M sucrose and washed under the same condition twice. The pellet was suspended in 80 ml of 0.17 M sucrose and layered on top of a 0.67 M sucrose cushion (5 ml) in polyethylene tubes. Myelin banded in the interphase of the two-step gradient upon centrifugation at 40,000 × g (18,500 rpm) in a JA-20 Beckman rotor. The myelin band was collected, suspended in 200 ml of water, and centrifuged for 30 min at 5000 × g (6000 rpm). The gradient centrifugation and the hyposmotic shock (11) was repeated once. The myelin band was collected.

**Trypsin Cleavage.** Myelin (100 mg) was suspended in 5 ml of 0.1 M NH<sub>4</sub>HCO<sub>3</sub> buffer (pH 8.4), 0.5 mg of trypsin was added, and the suspension was stirred at room temperature for 6 hr. The reaction was stopped by adjusting the pH to 4.0

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Abbreviations: PLP, myelin proteolipid protein; BMP, basic myelin protein; CNS, central nervous system.

with acetic acid. The suspension was centrifuged at  $5000 \times g$  at  $4^\circ\text{C}$ , and the pellet was lyophilized. Myelin treated with 0.01 M HCl/5% KCl for 16 hr at room temperature also was subjected to trypsin treatment as described above for hypototically shocked myelin.

**Isolation of Tryptic Fragments.** For delipidation, the lyophilized trypsin-treated myelin was dissolved in 4 ml of acidified chloroform/methanol [2:1 (vol/vol), 1% in aqueous HCl] and dialyzed against 2:1 chloroform/methanol for 4 hr in a Visking dialysis tubing (Serva, Heidelberg). The polypeptides were precipitated by the addition of 4 ml of petroleum ether ( $30\text{--}60^\circ\text{C}$ ) and centrifuged.

**Reductive Carboxymethylation.** The pellet was dissolved in 5 ml of 0.4 M Tris·HCl, pH 8.6/0.08% NaDodSO<sub>4</sub>, and 0.30 mmol (50 mg) of dithiothreitol was added for reduction at  $40^\circ\text{C}$  for 2 hr. For carboxymethylation, 0.8 mmol of iodoacetamide was added, and the reaction was continued for 30 min at  $40^\circ\text{C}$ . The reaction mixture was dialyzed for 24 hr against 0.01 M NH<sub>4</sub>HCO<sub>3</sub> buffer using Spectropor dialysis tubing (exclusion size, 2000 Da) and subsequently was lyophilized.

**Separation of Tryptic Fragments.** Of the lyophilized peptide mixture, 20 mg was extracted twice with 3 ml of 0.01 M HCl at room temperature to remove the water-soluble peptides (6–8). The extract contained a minimal amount of peptides with molecular masses below 2000 Da. The residue was dissolved in 500  $\mu\text{l}$  of concentrated formic acid and was separated by gel permeation high-performance liquid chromatography with a mixed-bed column (30  $\times$  2 cm) packed with 5- $\mu\text{m}$ -particle-size 1:1:1 Si 100/Si 60/Si 50 (Merck) with 90% formic acid as solvent as described (6–8). The column was calibrated with the peptides and proteins as indicated in Fig.

4. Three fragments generated by trypsin treatment of the myelin membrane were eluted in distinct peaks at retention times of 9.82 min (15 kDa), 10.32 min (11.5 kDa), and 10.96 min (8 kDa). They were further purified by repeated HPLC under the same conditions for automated Edman degradation (11).

**Automated Sequence Determination.** Edman degradation (12) was performed in a Beckman Sequenator, model 890 C, using the 0.2 M Quadrol program as described (6–8).

**NaDodSO<sub>4</sub>/Polyacrylamide Gel Electrophoresis.** Reduced and carboxymethylated polypeptide and protein samples of bovine myelin were subjected to NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis with 15–20% acrylamide gels.

**Electron Microscopy.** Aliquots of untreated myelin and of osmotically shocked myelin before and after trypsin treatment were fixed in buffered glutaraldehyde and stained with buffered 2% osmium tetroxide. A Philips electron microscope (model 300) was used.

## RESULTS

**Protein Structure and Proposed Assembly in the Lipid Bilayer of Myelin.** The polypeptide chain of the most abundant protein of CNS myelin consists of 276 amino acid residues that are aligned in a hydrophilic NH<sub>2</sub> terminus and COOH terminus and four long hydrophobic sequences of 26, 29, 40, and 36 amino acid residues and one short hydrophobic sequence of 17 residues separated by four hydrophilic loops (6–9). Fig. 1 represents the proposed folding and orientation of the hydrophilic and hydrophobic sequences of PLP on the basis of these sequence data and the linkage of the NH<sub>2</sub>-

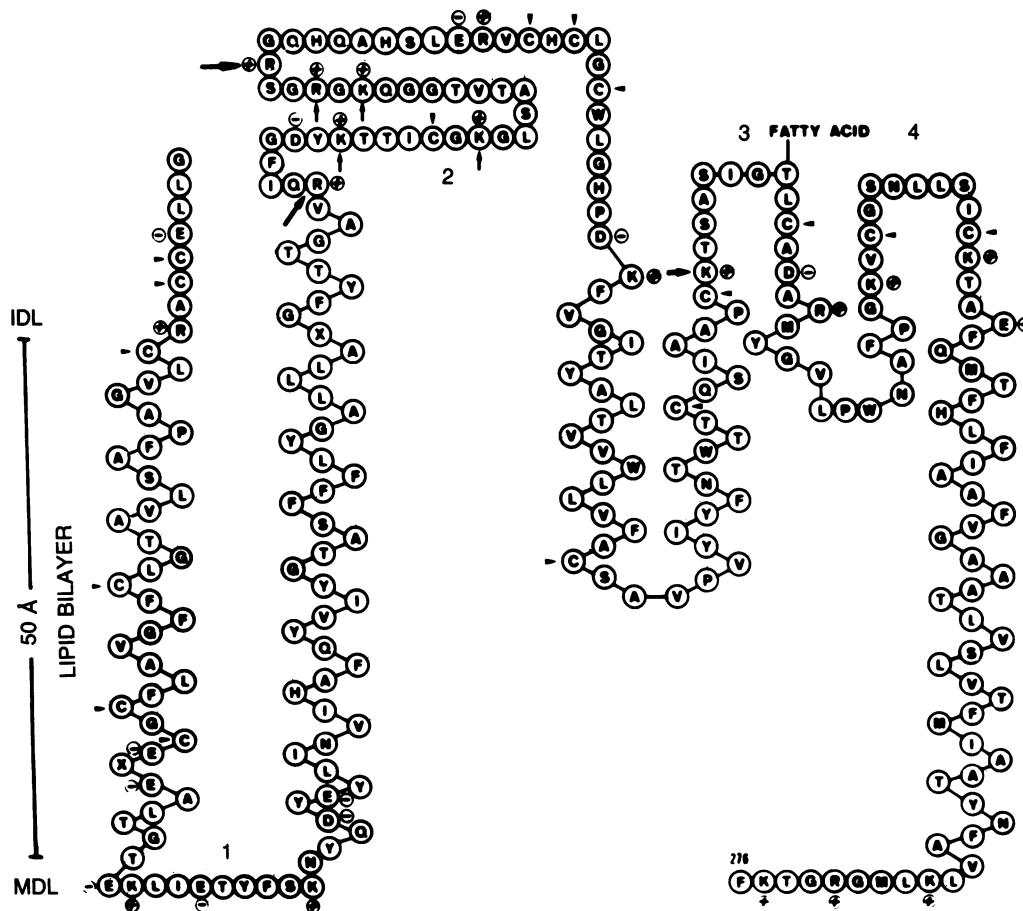


FIG. 1. Proposed assembly of proteolipid protein in the myelin membrane. The NH<sub>2</sub> terminus and three positively charged hydrophilic amino acid sequences are oriented toward the extracytosolic side (IDL), with the positively charged COOH terminus and one negatively charged loop oriented toward the cytosolic side of the myelin membrane (main defense line, MDL).

terminal cysteine-rich sequence to one of the two cysteine residues (Cys-219 or -227) of the penultimate hydrophilic sequence and of Cys-219 or -227 to one of the three cysteine residues in position 138, 140, or 143. Unambiguous evidence for the orientation of the hydrophilic loop and, therefore, support for the proposed topography should come from the proteolytic attack on the polypeptide chain exposed on the external side of the myelin membrane and the identification of the resulting proteolytic fragments.

**Dissociation of Myelin Membrane System by Hyposmotic Shock and Trypsin Treatment.** The tightly spirally wrapped layers of the myelin membrane were dissociated by hypotonic shock. This step proved to be essential for an effective access of trypsin, which was chosen because of the favorable distribution of lysine and arginine residues in the hydrophilic loops. Fig. 2 shows electron microscopic pictures of hypototically shocked myelin before (Fig. 2 *Upper*) and after (Fig. 2 *Lower*) trypsinization. The dissociation of the myelin layers within the intraperiod dense lines, which corresponds to the space between adjacent external membrane sides, is apparent (arrow in Fig. 2 *Upper*). The stacked mem-

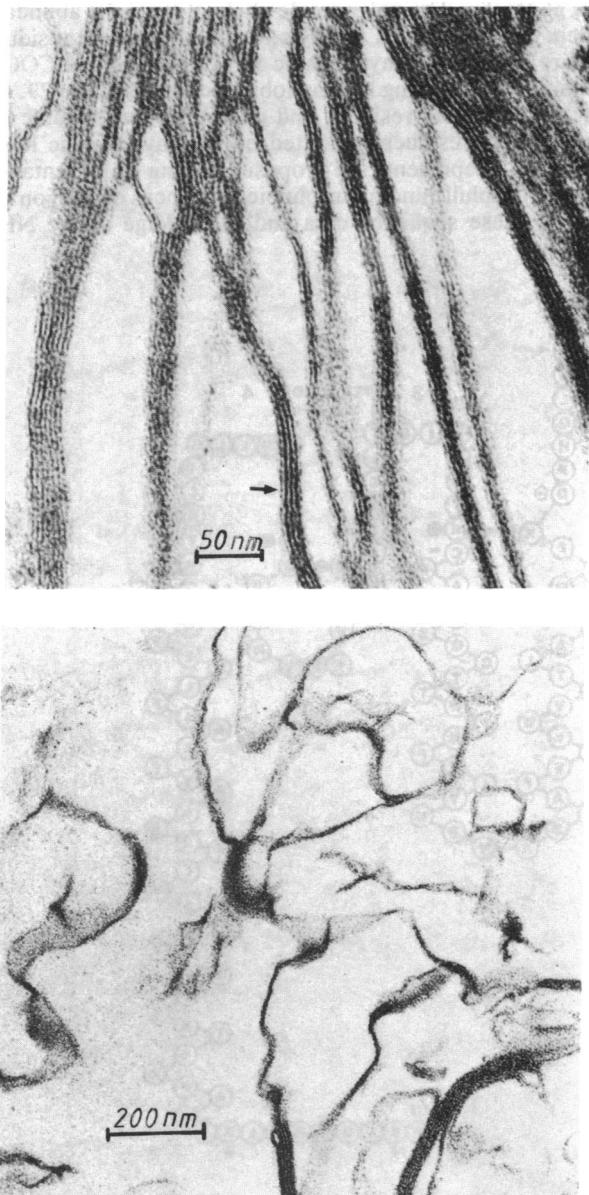


FIG. 2. Electron microscopy of hypototically shocked bovine myelin before (*Upper*) and after (*Lower*) trypsin treatment.

brane appears to be fully detached after trypsin treatment (Fig. 2 *Lower*).

**NaDodSO<sub>4</sub>/Polyacrylamide Gel Electrophoresis.** Bovine myelin exhibited a protein pattern that is similar in many species (Fig. 3, lane 1). The main components are basic myelin protein (BMP) (18.7 kDa); myelin protein with a molecular mass  $\approx$  20 kDa, designated DM20 (23 kDa); PLP with an apparent molecular mass of 26 kDa and its dimeric form and aggregates in the high molecular mass range; and the Wolfgram proteins, designated WP (58–60 kDa). When myelin was exposed to an acidic medium, PLP aggregated (Fig. 3, lane 2). Trypsin treatment of the hypototically shocked myelin preparation (Fig. 3, lane 3) led to the loss of the intensive PLP and DM20 bands but also of the bands corresponding to aggregated PLP. New polypeptide bands, designated A and B in lane 3 of Fig. 3, that were not well resolved appeared in the molecular mass range around and below 10 kDa. The band of BMP (designated C in lane 3 of Fig. 3) remained unaltered. Trypsin treatment of the acid-exposed myelin membranes, on the other hand, led to the complete degradation of PLP, DM20, and BMP (Fig. 3, lane 4).

**Isolation and Characterization of Tryptic Fragments.** After delipidation of the trypsin-treated myelin membranes by dialysis against acidic chloroform/methanol, small water-soluble peptides (<2000 Da) were removed by brief extraction with 0.01 M HCl. The residual polypeptide mixture was separated by preparative gel permeation HPLC. Fig. 4 *a* and *b* shows the resolution of the chromatographic procedure with marker proteins and peptides: ovalbumin, myoglobin, cytochrome *c* and its cyanogen bromide (CNBr) fragments, the tripeptide Lys-Tyr-Ser, and tryptone. Three fractions with retention times of 9.82, 10.32, and 10.96 min, corresponding to molecular masses of approximately 17 kDa, 11.0 kDa, and 8.5 kDa, respectively, were isolated from trypsin-treated myelin (Fig. 4*c*). These fractions were collected (between arrows in Fig. 4*d*) and rechromatographed twice, yielding sufficiently pure fractions for sequence determina-

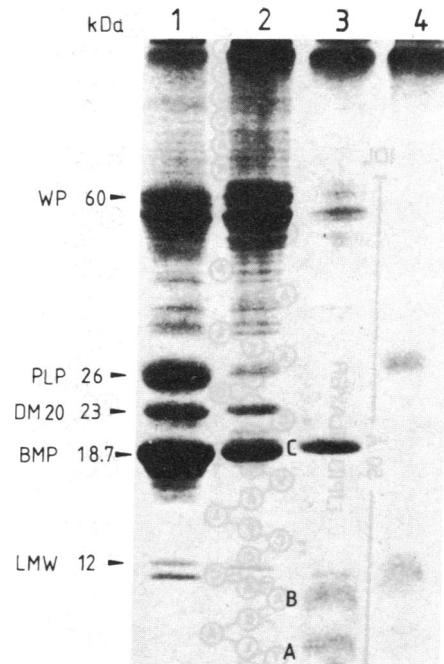


FIG. 3. NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis of proteins of isolated myelin (lane 1), myelin after acid treatment (lane 2), myelin proteins and their fragments after hypotonic shock and trypsin treatment (lane 3), and products after trypsinization of acid-treated myelin (lane 4). WP, Wolfgram protein; DM20, myelin protein of 23 kDa; LMW, low molecular weight proteins of about 12 kDa.

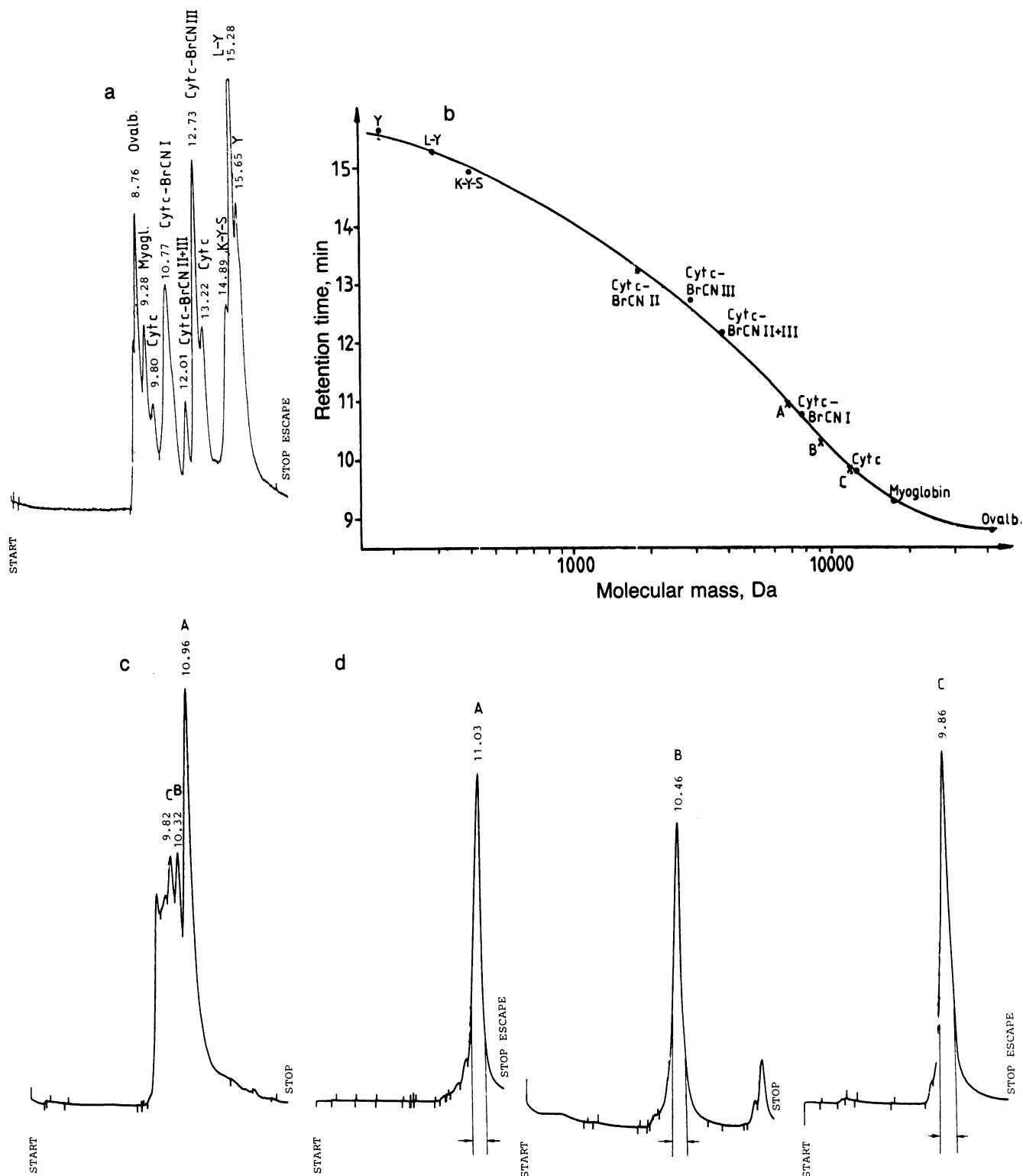


FIG. 4. Preparative gel permeation HPLC. (a) Elution pattern of calibration peptides and protein from preparative gel permeation HPLC column. (b) Semilogarithmic plot of retention times versus molecular masses of calibration proteins and of myelin polypeptides A, B, and C. (c) HPLC elution pattern of the polypeptide mixture after trypsin treatment. (d) HPLC elution pattern of the purified fractions A, B, and C (20-mg aliquots were separated). The column was 30 × 2 cm with 5- $\mu$ m 1:1:1 Si 100/Si 60/Si 50; the solvent was 90% formic acid; flow rate was at 4.5 ml/min; and UV detection was at 280 nm. Ovalb., ovalbumin; Myogl., myoglobin; Cyt c, Cytochrome c; Cyt c-BrCN I, II, and III, cyanogen bromide fragments I, II, and III of cytochrome c; Y, tyrosine; L-Y, leucine-tyrosine dipeptide; K-Y-S, lysine-tyrosine-serine tripeptide.

tion (Fig. 4d). The band with a retention time of 9.82 min corresponds to the 17- to 18-kDa protein, identical with basic myelin protein and traces of the NH<sub>2</sub>-terminal tryptic fragment of proteolipid protein due to incomplete separation. The NH<sub>2</sub> terminus of BMP is *N*-acetylated and, therefore, escapes automatic Edman degradation but was identified by

NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis. The band, which was eluted at a retention time of 10.46 min, represents the 11-kDa fragment. Automated Edman degradation over 15 cycles revealed the NH<sub>2</sub> terminus to be from Gly-1 through Arg-97. Identification of this cleavage site is supported by the molecular mass of the fragment and the amino

acid analysis, which yielded two lysine residues. Two polypeptides coeluted at retention time of 10.96 min in a 1:1 ratio. They were sequenced in parallel and proved to correspond to the sequence from Gly-127 through Lys-191 (7.3 kDa) and from Thr-192 through Phe-276 (9.0 kDa).

### DISCUSSION

Our understanding of the molecular structure of CNS myelin requires substantial chemical and physical information about the complex lipid and protein components of this membrane. Considerable compositional data have accumulated, which have been reviewed (13, 14). The two main proteins BMP and PLP are considered myelin specific (15). They are present in myelin in approximately equimolar amounts (14, 15). This study describes a topochemical study of the orientation and integration of one of these main protein components of CNS myelin, PLP, into the lipid bilayer. This approach became feasible after we succeeded in determining the complete amino acid sequence of this most hydrophobic integral protein and in having available rapid and efficient procedures for the separation and identification of hydrophobic polypeptides.

The model proposed in Fig. 1 concerns the folding of the five strongly hydrophobic sequences that are bordered by charged amino acid residues. Three of them span the lipid bilayer and two are harbored in the hydrocarbon layer of the membrane. The hydrophobicity index (16, 17) of the hydrophobic sequence from Cys-9 to Cys-34 is 2.31, from Tyr-59 to Val-96 is 2.72, from Phe-151 to Cys-190 is 2.36, and from Phe-232 to Leu-267 is 2.35. They are in the range of the hydrophobicity index of the 23-amino-acid residue sequence of glycoporphin (hydrophobicity index, 2.62) spanning the lipid bilayer (17, 18). Assuming an  $\alpha$ -helical conformation of the hydrophobic sequences, about 30 amino acid residues are sufficient to span the 4.6- to 5-nm-thick myelin lipid bilayer (19). The tightly packed membrane layers were dissociated by hyposmotic shock (11) to make their surfaces accessible for the proteolytic attack. Electron microscopy demonstrated that this cleavage occurs in the intraperiod dense line, which corresponds to opposing outer myelin membrane surfaces. The result of the tryptic cleavage at sites of protein segments protruding out of the extracytosolic side of the myelin membrane are in full agreement with the proposal that four of the six hydrophilic sequences, namely, the NH<sub>2</sub> terminus and loops 2, 3, and 4 (Fig. 1), are oriented toward the extracytosolic side. We were unable to detect fragments arising from cleavage at Lys-44 or -48 (residues of hydrophilic loop 2); which should be oriented toward the cytosolic side. Our experimental results are incompatible with the integration of PLP in the myelin membrane proposed recently largely on our sequence data (20). Further support of this model comes from our observation that, in isolated PLP, disulfide bonds link the hydrophilic NH<sub>2</sub>-terminal cysteine-rich sequence to the hydrophilic loops 4 and 2 and also link loops 2 and 4 (Fig. 1). This is only possible if the correspond-

ing cysteine residues are in appropriate distances at the same side of the bilayer. In our myelin preparations, BMP was protected against the tryptic attack. This gives further evidence for the localization of this basic protein in the cytoplasmic cleft between the myelin bilayers. Our observation is consistent with the inability of nonpermeant chemical labels to bind to BMP in intact myelin preparations (21, 22). PLP on the other hand can be <sup>125</sup>I-labeled with lactoperoxidase (21). The hydrophilic sequences of PLP on the external side of the myelin membrane may represent the sites of the proteolytic attack under pathological condition, at which the disintegration of the membrane starts, which finally leads to demyelination. They also can form the antigenic sites of the myelin membrane.

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