Topology of CNS Myelin Proteolipid Protein: Evidence for the Nonenzymatic Glycosylation of Extracytoplasmic Domains in Normal and Diabetic Animals

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ABSTRACT: Myelin proteolipid protein (PLP), the main integral membrane protein in the central nervous system myelin, was labeled at the extracytoplasmic domains with the membrane impermeant reagents pyridoxal 5′-phosphate and tritiated borohydride. Lysine-217, located in the fourth hydrophilic domain of PLP, was found to be the major labeled residue, which defined this domain to be extracytoplasmic in agreement with our previously proposed topological model. The remarkably high reactivity *in vitro* of this residue as compared to all other lysines in PLP led us to investigate the possible modification of PLP *in vivo* by other carbonyl compounds. We demonstrate that PLP is the most highly nonenzymatically glycosylated membrane protein in murine and bovine brain. The degree of modification increases significantly under hyperglycemic conditions, as studied in diabetic mice. The majority of the glycosylation sites are also located at extracytoplasmic domains. The degree of nonenzymatic glycosylation of PLP may be related to late diabetic complications affecting the central nervous system.

Myelin, the multilamellar membrane system which ensheaths the axons in the nervous systems of vertebrates, serves mainly as an electrical insulator which enhances the nerve conduction velocity and allows information transmission with reduced energy consumption. In the central nervous system of mammals, the myelin internodes originate from plasma membrane processes of oligodendrocytes. Two major protein classes account for 60–80% of the myelin proteins, namely the myelin basic proteins (MBPs[1]), which are peripherally associated with the cytoplasmic face of the myelin membrane, and the proteolipid proteins (PLPs), which span the bilayer and occur in two forms, the 30-kDa PLP and the smaller (26 kDa) and less abundant DM-20, a variant which originates from alternative RNA splicing (Nave et al., 1987). The primary sequence of the very hydrophobic PLP is extremely conserved among mammalian species, as well as in birds and amphibia (Schliess & Stoffel, 1991). Several point mutations causing single amino acid substitutions in PLPs have been detected in the human Pelizaeus–Merzbacher disease (e.g. Hudson et al., 1989a; Weimbs et al., 1990; Otterbach et al., 1993) and in animal models like the md-rat (Boison & Stoffel, 1989) and the shaking pub (Nadon et al., 1990). They mostly lead to severe impairment of myelinization in the central nervous system. These observations assigned to PLP an important structural function for building up and maintaining the compact multilayer structure of myelin, although additional functions as an ion channel (Lees & Bizzozero, 1992) or oligodendrocyte differentiation factor (Schneider et al., 1992) have been proposed. Dysmyelinosis in the PLP mutants might be mainly due to the often observed oligodendrocyte death rather than to a direct impairment of a structural function of this protein. Very recently, however, transgenic knock-out mice for PLP have been generated, which demonstrate unequivocally that PLP is responsible for the correct myelin structure, since viable oligodendrocytes do enwrap axons, but only with a very loose and noncompacted multilayered myelin. This results in a largely reduced nerve conduction velocity (Boison & Stoffel, 1994). The observation that only the apposition of the extracytoplasmic membrane faces is impaired, whereas the stacking of the cytoplasmic faces remains normal, proves the pivotal function of PLP to establish the contact between the extracytoplasmic faces, possibly by homophilic or heterophilic protein–protein interaction or by PLP–lipid interaction.

We have recently derived a four-helix model by the determination of the functional states of all 14 cysteine residues, which are present as free, disulfide-bonded, and acylated with long-chain fatty acids (Weimbs & Stoffel, 1992), which is in agreement with a model based on theoretical considerations (Popot et al., 1991). In this model, PLP consists of four membrane-spanning α-helical domains and two extracytoplasmic and three cytoplasmic domains, including the N- and C-termini.

The studies described here further the examination of the correctness of the topological model of PLP by identifying extracytoplasmically located domains and by labeling the lysine residues within these domains with pyridoxal 5′-phosphate and tritiated borohydride. By this approach, lysine-217 within the fourth extracytoplasmic hydrophilic domain of PLP was the predominantly labeled amino acid residue. The facile labeling of amino groups of accessible lysines of PLP with carbonyl compounds led us to study the reactivity of PLP with glucose under hyperglycemic conditions. We demonstrate that PLP is the most highly nonenzymatically glycosylated membrane protein in the brain, most likely also at lysine residues at extracytoplasmic domains. The degree of glycosylation is increased in diabetic mice. Whether this observation might be of importance for the development of late diabetic complications affecting the central nervous system remains to be further studied.

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[1] Abbreviations: HPLC, high performance liquid chromatography; MBP, myelin basic protein; PLP, myelin proteolipid protein; DM-20, smaller isoform of PLP, originating from alternative RNA splicing; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; AGE, advanced glycosylation end products.

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EXPERIMENTAL PROCEDURES

Hyposmotic Shock of Bovine White Matter. White matter of fresh bovine brain was dissected into slices of approximately 2-mm length. Eight tenths of a gram of this material was incubated at 4 °C for 3 h in 50 mL of "hyposmotic buffer" (10 mM sodium borate, pH 8, 0.2 mM EDTA) under constant agitation. After centrifugation (2000*g*, 10 min) the pellet was resuspended in 50 mL of fresh buffer and incubated for another 3 h. The membranes were collected by centrifugation, resuspended in 50 mL of buffer, and directly used for the subsequent labeling experiments.

Permeabilized bovine white matter membranes for the determination of nonenzymatic glycosylation were prepared by homogenization of bovine white matter in hyposmotic buffer with a tissue homogenizer (Ultra-Turrax) at high speed. The membranes were collected by centrifugation (8000*g*, 10 min), washed three times with hyposmotic buffer, subjected to two rapid freezing–thawing cycles, and stored frozen as an aqueous suspension.

Pyridoxal Phosphate/[^{3}H]NaBH$_4$ Labeling. Saponin was pretreated before use with borohydride: 10 mL of a 5% aqueous solution of saponin (from Saponaria species; Sigma) adjusted to pH 8 with 1 M NaOH. Sodium borohydride was added (50 mM) and the solution kept at room temperature for 30 min. After addition of 150 μL of acetic acid, the solution was lyophylized, redissolved in 10 mL of water, and stored frozen at −20 °C until use.

For permeabilization, pretreated saponin was added to 1-mL aliquots of the membrane suspension to final concentrations of 0.04–0.4% and incubated for 30 min at room temperature. The membranes were washed once with hyposmotic buffer and resuspended in 1 mL of this buffer. Interfering aldehydes or ketones were reduced by the addition of sodium borohydride (0.7 mM) and incubation for 30 min at room temperature. A neutralized solution of pyridoxal phosphate was added to a final concentration of 6 mM. After 60 min, membranes were washed, collected by centrifugation, and resuspended in 0.5 mL of hyposmotic buffer containing 2 mCi of [^{3}H]NaBH$_4$ (specific radioactivity: 13.4 Ci/mmol; NEN), incubated for 1 h at room temperature, washed twice with buffer, dissolved in electrophoresis sample buffer, and subjected to SDS-PAGE.

Alternatively, PLP was isolated from the labeled membranes by extraction with 20 volumes of chloroform/methanol (2:1 v/v). The extracted labeled proteins were "diluted" with approximately 5 mg of unlabeled PLP, precipitated by addition of five volumes of diethyl ether, and delipidated by gel filtration chromatography on a column of Sephadex LH-60 (Pharmacia) in chloroform/methanol/0.1 M HCl (10:10:1). Protein was precipitated with five volumes of diethyl ether, collected by centrifugation, washed once in diethyl ether, and dissolved in a solution of 0.5% Triton X-100 (reduced form; Fluka), 100 mM sodium borate, pH 8, 0.5 mM EDTA to a final protein concentration of 1–2 mg/mL. Pyridoxal phosphate was added (20 mM) and the solution incubated for 20 min at 37 °C. After the mixture was cooled on ice, nonradioactive sodium borohydride was added (20 mM) and incubated for 1 h. Ten microliters of acetic acid and 100 μL of 25% SDS per milliliter were added. The protein solution was desalted by gel filtration (Biogel P-30; Biorad) in 50 mM Tris-HCl, pH 8.5, 0.1% SDS, 0.5 mM EDTA. After the addition of urea (6 M), the disulfide bonds were reduced with 20 mM DTT (2 h, 37 °C) and the thiols alkylated with iodoacetamide (50 mM, 2 h, 37 °C). The protein was again desalted (Biogel P-30, 50 mM Tris-HCl, pH 7.4, 0.1% SDS) and subjected to complete proteolytical digestion by addition of $CaCl_2$ (2 mM) and thermolysin (17 mg/mL; Boehringer-Mannheim) and incubation for 48 h at 37 °C. SDS was removed, and the peptides were separated by reversed-phase HPLC (Weimbs & Stoffel, 1992).

HPLC. Peptides were dissolved in water and separated by reversed-phase HPLC on Nucleosil 300-5 C18 (Macherey-Nagel; column size 25 × 0.4 cm^2) by gradient elution: (A) 0.1% TFA, 10 mM triethylamine in water; (B) 0.1% TFA, 10 mM triethylamine in 70% acetonitrile. Peptides were monitored with two detectors connected in series; a photodiode array UV–vis detector (Shimadzu SPD-M6A) and a fluorescence detector (Kratos Spectroflow 980) set to an excitation wavelength of 325 nm. Peptide fractions were collected and rechromatographed to homogeneity but without triethylamine in the eluents.

Peptide Sequencing. Purified peptides were covalently coupled to aminoacyl-derivatized membranes (Sequelon AA; Millipore) according to the suggestions of the manufacturer. The complete amino acid sequences were determined by automated Edman degradation using an Applied Biosystems 477 A gas-phase sequencer.

Electrophoresis. Proteins were separated by SDS-PAGE on 15% gels according to Laemmli (1970), omitting prior mercaptoethanol reduction and boiling of the samples, to avoid aggregation of PLP. Gels were stained with Coomassie blue R-250 and radioactive bands detected by fluorography according to Bonner and Laskey (1974). Quantitation of the autoradiographs was performed by laser densitometry using preflashed X-ray film according to Laskey and Mills (1975). Coomassie-stained gels were dried and scanned directly with the video densitometer (Eagle-eye II, Stratagene).

Myelin Preparation. Diabetic C57 Bl/ksJ (*db/db*) and normal C57 Bl 6 mice were killed by decapitation, and brains were dissected and stored at −80 °C until further processing. The whole brains were homogenized in 15 mL of ice-cold 0.32 M sucrose with a tissue homogenizer (Ultra-Turrax). Myelin membranes were isolated according to Norton and Poduslo (1973). After extensive washing with water, the myelin membranes were stored frozen as an aqueous suspension. Protein concentrations were determined by the BCA protein assay (Pierce) after solubilization in 1% SDS according to the instructions of the manufacturer.

[^{3}H]NaBH$_4$ labeling was performed as described above for the pyridoxal phosphate/[^{3}H]NaBH$_4$ labeling, omitting the borohydride pretreatment and the pyridoxal phosphate incubation step.

In Vitro Glycosylation. Permeabilized bovine white matter membranes were incubated for 24 h at 37 °C in 10 mM sodium phosphate buffer, pH 7.5, with varying concentrations of glucose. Membranes were collected by centrifugation, washed once with 10 mM sodium borate, pH 8.0, resuspended in borate buffer containing 2 mM [^{3}H]NaBH$_4$, and incubated for 1 h at room temperature. Samples for SDS-PAGE analysis were prepared as above.

RESULTS AND DISCUSSION

Lysine-Specific Vectorial Labeling of Myelin Proteins. Vectorial labeling experiments require that the membrane protein under investigation is present in its unperturbed, native structure and orientation in a sealed membrane preparation. Membrane impermeant probes should then label only those protein domains which are located at the accessible membrane surface (Jennings, 1989). We chose pyridoxal 5′-phosphate as a membrane impermeable labeling reagent which reacts specifically with lysine residues of proteins to form a Schiff base which can subsequently be reduced with sodium boro-

hydride to the stable and fluorescent amino compound. Phosphopyridoxyl-lysine residues can be radioactively labeled with tritiated borohydride. Twelve lysine residues are equally distributed over all of the hydrophilic domains of PLP.

Conventional methods for the preparation of myelin membranes involve techniques potentially disrupting the integrity of these membranes. We applied a gentle preparation method which is based on the observation of McIntosh and Robertson (1976) that hyposmotical treatment of whole rat optic nerves dissociates the myelin sheaths and separates and exposes apposed extracytoplasmic faces to membrane impermeant labeling reagents in the aqueous medium. Cytoplasmic membrane surfaces remain tightly packed. A valuable control for membrane integrity is therefore the degree to which the abundant MBP rich in lysine residues is labeled. On a molar basis MBP equals approximately PLP in the myelin membrane (Braun, 1984).

Initial experiments proved that membranes prepared according to this procedure from rat optical nerve are very suitable for vectorial labeling. PLP was highly labeled whereas MBP remained almost unlabeled (data not shown).

We adapted this preparation method to freshly dissected slices of bovine brain white matter in order to obtain more starting material. After hyposmotical treatment, the membranes were labeled with pyridoxal phosphate and (^{3}H)NaBH$_4$, protein size fractionated by SDS-PAGE, and analyzed by fluorography (Figure 1B). PLP and its smaller isoform DM-20, as well as some proteins of higher molecular weight, are highly labeled, whereas MBP shows only a very weak radioactive signal despite its high abundance. In a control experiment the membranes were permeabilized by increasing amounts of saponin prior to the labeling step. Saponin permeabilizes biological membranes by complex formation with cholesterol without perturbing the bilayer, native structure, and orientation of membrane proteins (St. John et al., 1982; Kyte et al., 1987). This procedure resulted in a strong labeling of almost all myelin proteins visible on the Coomassie-stained gel, including MBP (Figure 1A, lanes 3–5). Saponin treatment renders the membranes freely permeable to pyridoxal phosphate and (^{3}H)NaBH$_4$. Figure 1C shows the quantitation of the radioactive labeling of densitometry. After permeabilization with 0.4% saponin, the specific radioactivity of MBP increases by a factor of 12 over MBP in untreated membranes. The increase for PLP by a factor of 1.5 could be either due to an additional labeling of cytoplasmic domains or to a higher labeling of extracytoplasmic lysine residues due to improved accessibility. The specific radioactivity of PLP in nonpermeabilized membranes is approximately 7 times higher than that of MBP, whereas in the saponin-treated control both proteins are labeled to a comparable degree. This demonstrates that the membranes prepared from bovine white matter are suitable for the vectorial protein labeling by pyridoxal phosphate and (^{3}H)NaBH$_4$, contrary to myelin membranes isolated by tissue homogenization and density gradient centrifugations (lane 6).

Localization of the Pyridoxal Phosphate Labeling Sites. For the identification of the labeled phosphopyridoxyl lysyl residues in the PLP sequence, a vectorial labeling experiment was performed as described above and PLP was isolated. As estimated from the amount of radioactivity incorporated into the PLP preparation, the molar ratio of PLP protein to phosphopyridoxyl lysyl groups of PLP was approximately 1000:1. We therefore performed a "bulk" derivatization of the free lysine residues of PLP with pyridoxal phosphate and (nonradioactive) borohydride in detergent solution for dilution of

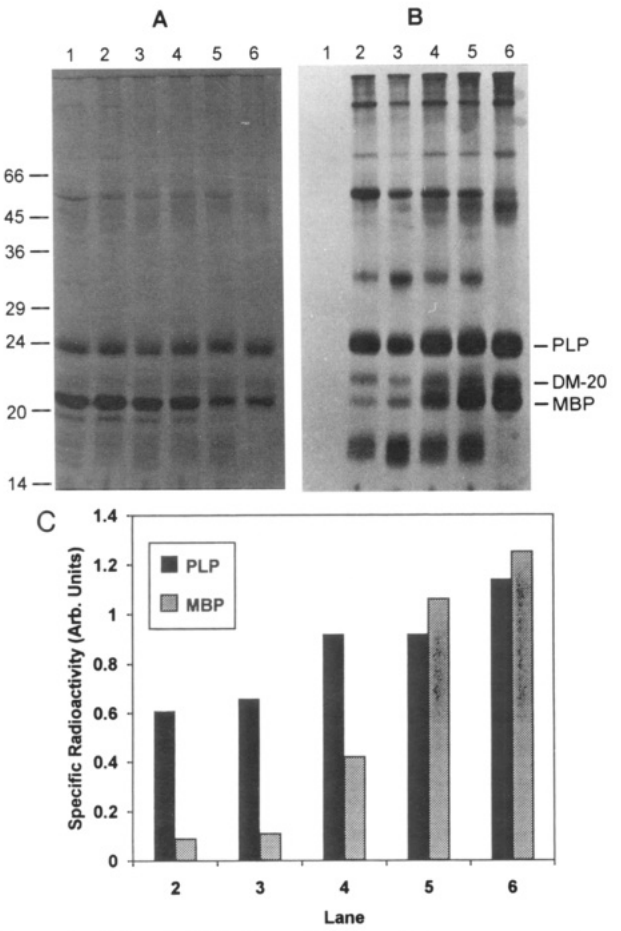

FIGURE 1: Vectorial labeling of bovine white matter membrane proteins with pyridoxal phosphate/(^{3}H)NaBH$_4$. Hyposmotically shocked bovine white matter membranes (lanes 1–5) or isolated myelin membranes (lane 6) were either permeabilized by treatment with 0.04% (lane 3), 0.15% (lane 4), or 0.4% (lane 5) saponin or untreated (lanes 1, 2, and 6). Membrane proteins were labeled as described. Control (lane 1): pyridoxal phosphate was added. SDS-PAGE (15%): panel A, Coomassie blue stain, the positions of the PLP and MBP bands and of molecular weight markers are indicated; panel B, fluorography of an identical gel (9-h exposure time); panel C (bottom), quantitation of the proteins and radioactivity by densitometry; values for the specific radioactivity in lanes 2–6 are expressed in arbitrary units for PLP and MBP.

the labeled protein and subsequent protein analysis. Under these conditions 5–6 mol of lysine residues was derivatized per mole of PLP, as determined by UV spectroscopy (not shown). Protein was denaturated by reductive carboxyamidomethylation in the presence of SDS and completely digested with thermolysin. The derived peptides were separated by reversed-phase HPLC; those bearing the phosphopyridoxyl group were detected by their specific fluorescence (Figure 2A) and UV spectroscopy (not shown). Twelve major peptides were collected and rechromatographed to homogeneity, the radioactivity was determined by scintillation counting, and the amino acid sequences were determined by Edman degradation. Table 1 summarizes the analytical results. Only peptide 10 with the sequence AFPGK is the most abundantly labeled. It contains more than 80% of the total radioactivity of all collected peptides. All other peptides are neglibly labeled. Radiosequencing of peptide 10 showed that that radioactivity is associated with lysine 217 (Figure 3).

Lys-217 must be located at the extracellular face of the myelin membrane in support of our previously proposed topological model (Figure 4). Lys-191 and -228 as well as two lysines in the second hydrophilic domain (Lys-44 and

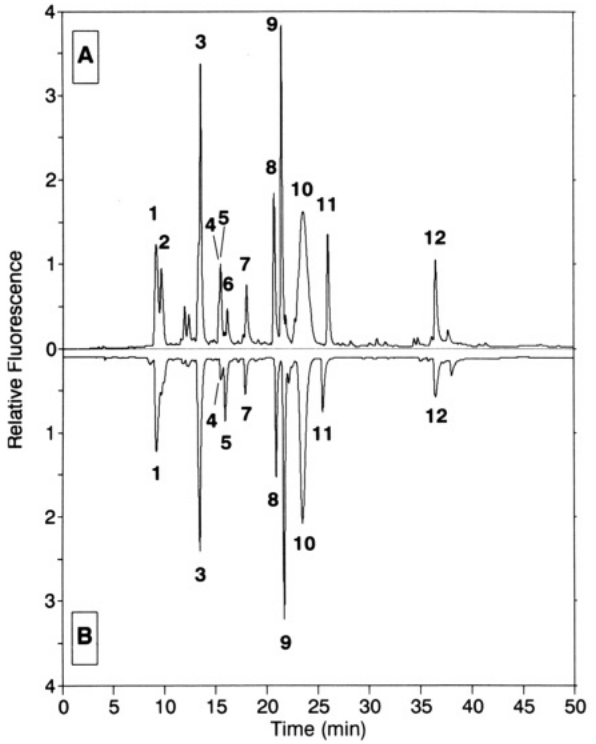

FIGURE 2: Reversed-phase HPLC separation of proteolytic peptides from PLP labeled with pyridoxal phosphate/(^{3}H)NaBH$_4$. Proteins in hyposmotically shocked bovine white matter membranes were labeled with pyridoxal phosphate/(^{3}H)NaBH$_4$ directly (A) or after permeabilization with 0.4% saponin (B) as described in Figure 1. PLP was isolated, and all remaining accessible lysine residues were derivatized by pyridoxal phosphate and nonradioactive borohydride in detergent solution (Triton X-100). After complete cleavage with thermolysin, the derived peptides were separated by reversed-phase HPLC as described in Experimental Procedures. The major peptides (1–12) were collected and rechromatographed to homogeneity, the radioactivity was determined by liquid scintillation counting, and the amino acid sequences were determined by Edman degradation (see Table 1). The elution positions of some peptides are slightly shifted in B as compared to A, due to the frequent use of the column.

Table 1: Amino Acid Sequences and ^{3}H-Radioactivity of the Eluted Peptides of Figure 2[a]

peptide no.	amino acid sequence	sequence position	% of total radioactivity	
			nonpermeabilized	permeabilized
1	LK	288	1.9	8.1
2	VTGGQK	121	0.9	
3	ICGKG	110	0.8	10.4
4	LTGTEK	44	2.8	0.3
5	VLK	268	1.6	1.8
6	FSK	52	0.8	
7	LGHPDK	150	1.5	4.3
8	IAAPSK	191	1.5	4.3
9	IAAPSKTS	191	1.1	9.0
10	AFPGK	217	84.0	59.1
11	FSKNY	52	2.3	3.4
12	IFGDYK	104	0.7	3.3

[a] Aliquots of the rechromatographed peptides of Figure 2 were used for liquid scintillation counting and for automated Edman degradation. The amino acid sequences are represented in the single-letter code. The pyridoxylamine-derivatized lysine residues are underlined, and the position in the amino acid squence of PLP is indicated (compare to Figure 4). The total radioactivity of all collected peptides in the labeling experiments with intact (A in Figure 2) and saponin-permeabilized membranes (B in Figure 2) was set to 100%, and the percentage for each individual peptide is indicated.

-52) also considered to be extracytoplasmic are not significantly labeled. The reasons might be either inaccessibility of these residues to the labeling reagent or a low reactivity to form a

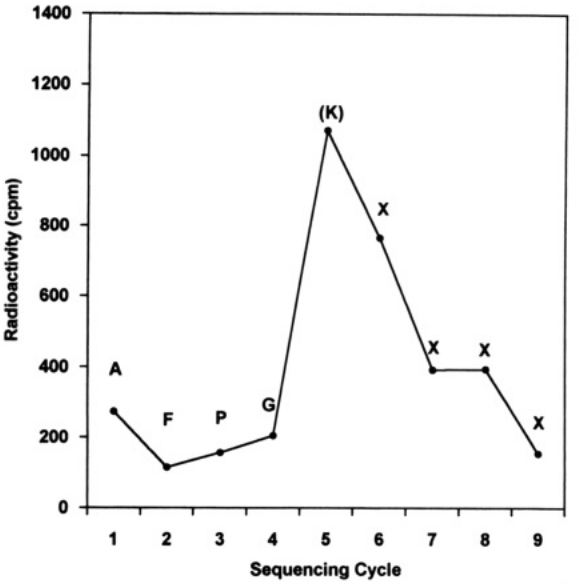

FIGURE 3: Radiosequencing of peptide 10 from Figure 2. An aliquot of the rechromatographed peptide was C-terminally coupled to an aminoaryl-derivatized membrane (Sequelon AA) and subjected to automated Edman degradation. Half of the cleaved phenylthiohydantoin (PTH) amino acids were used for the determination by on-line HPLC separation and the other half collected for liquid scintillation counting. The identified amino acids are indicated in the single-letter code. "X" represents a cycle without any detectable amino acid. The majority of the radioactivity appeared in the 5th cycle, and no corresponding PTH-lysine could be detected in the HPLC.

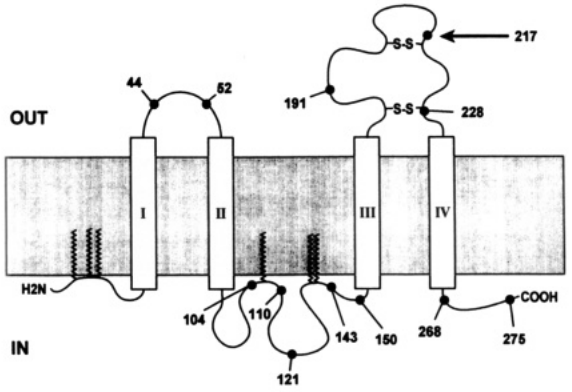

FIGURE 4: Topological model of the proteolipid protein. Four hydrophobic domains (I–IV) span the myelin membrane bilayer. The N- and C-termini, as well as the six fatty acid-acylated cysteine residues (indicated as zigzag lines), are located at the cytosolic membrane face, whereas the two disulfide bridges in the hydrophilic domain between the transmembrane domains III and IV are placed at the extracellular face (Weimbs & Stoffel, 1992). The 12 lysine residues are indicated with their sequence positions. Lysine residue 217 (arrow) can be highly labeled by pyridoxal phosphate/(^{3}H)-NaBH$_4$ in intact membranes and must therefore be located at the extracellular membrane face.

Schiff base due to stabilizing electrostatic interactions with acidic groups. The latter is supported by the observation that no incorporation of pyridoxal phosphate could be detected when SDS, instead of the nonionic Triton X-100, was used as detergent in the "bulk" labeling step (data not shown). The anionic headgroup of SDS probably interacts with the lysine residues of PLP in the micelles, thereby inhibiting the reactivity toward pyridoxal phosphate.

In the control experiment the radioactive labeling was performed with saponin-permeabilized membranes, PLP being processed identically as described above. As expected, the peptide pattern in the HPLC separation was almost the same as above (Figure 2B). It was, however, surprising that peptide

10 remained by far the most highly labeled peptide (59% of total radioactivity). The radioactivity of most other peptides increased only modestly.

Lysine residues located at active sites, substrate binding sites, or allosteric sites of enzymes often show a considerably higher reactivity toward pyridoxal phosphate than all other lysines in these proteins—an effect frequently used for specific enzyme inhibition (e.g. Paech & Tolbert, 1978; Kemp et al., 1987; Mahrenholz et al., 1988; Basu et al., 1989; Green et al., 1992). Lys-217 could be important for its structural function.

The fourth hydrophilic domain, containing Lys-217, is the largest of the two extracytoplasmic domains in PLP (Figure 4). It is very likely that this domain is involved in the protein–protein or protein–lipid interaction responsible for the apposition of the extracytoplasmic faces of the myelin membrane. Lys-217 could be particularly important for this interaction. Furthermore this residue is evolutionary, being conserved in the PLP sequences of all vertebrate species investigated so far (Schliess & Stoffel, 1991) and in the recently discovered family of PLP/DM-20-related proteins in rodents and elasmobranchs (Yan et al., 1993; Kitagawa et al., 1993).

The peak shape of peptide 10 in the chromatography and rechromatography, unlike that of the other peptides is broader, but yet symmetrical. In order to investigate if this behavior could be due to a previously undetected modification, we subjected the peptide to fast atom bombardment mass spectroscopy. However, the correct value for the protonated molecular ion of the phosphopyridoxyl-derivatized peptide was observed (data not shown), which makes additional modification in this peptide unlikely. Possibly, the peak broadening could be the result of a particular conformation of this peptide.

Presence of Borohydride-Reducible Groups in PLP. Vlassara et al. (1983) and Epand et al. (1985) found evidence for the presence of aldehyde-reacted proteins in the central nervous system myelin. In both studies the incorporation of radioactivity into myelin proteins after treatment with (^{3}H)$NaBH_4$ was observed. On the basis of the reactivity of PLP toward pyridoxal phosphate *in vitro*, membranes from bovine white matter were incubated directly with (^{3}H)$NaBH_4$ and the Schiff base formation *in vitro* with other carbonyl compounds both in nonpermeabilized (Figure 5, lane 1) and saponin-permeabilized membranes (lane 2) analyzed by SDS-PAGE and fluorography. PLP is the protein which incorporates the highest amount of tritium. In a control experiment the membranes were completely dissolved in SDS buffer prior to the labeling (lane 3). Saponin treatment completely permeabilizes the membranes, as indicated by the ratio of ^{3}H-labeled PLP and MBP, which remained constant as compared to lane 2. Furthermore, SDS-dissolved membranes pretreated with nonradioactive borohydride prior to the addition of (^{3}H)$NaBH_4$ (lane 4) showed no tritium incorporation, demonstrating that the labeling is a specific reduction.

Only the extracytoplasmic protein domains of nonpermeabilized membranes are accessible to the nonpenetrating borohydride. The increase of the specific radioactivity of PLP after saponin-permeabilization is by a factor of 1.7, which is comparable to the value of 1.5 observed in the pyridoxal phosphate-labeling experiment (Figure 1). This suggests that the majority of the borohydride-reducible groups in PLP are also located extracytoplasmically. As expected, the specific radioactivity of MBP increases to a factor of 3.4 after saponin permeabilization.

The result that PLP is the major borohydride-reducible protein in myelin is at variance with the results of Vlassara et al. and Epand et al., who found MBP to be the major reducible myelin protein whereas PLP was labeled only to a minor extent. However, both groups reported about an unidentified high molecular weight protein which was highly labeled and did not enter the SDS-PAGE gel. The discrepancy can be explained by the well-known irreversible aggregation of PLP during the delipidation steps applied in those studies.

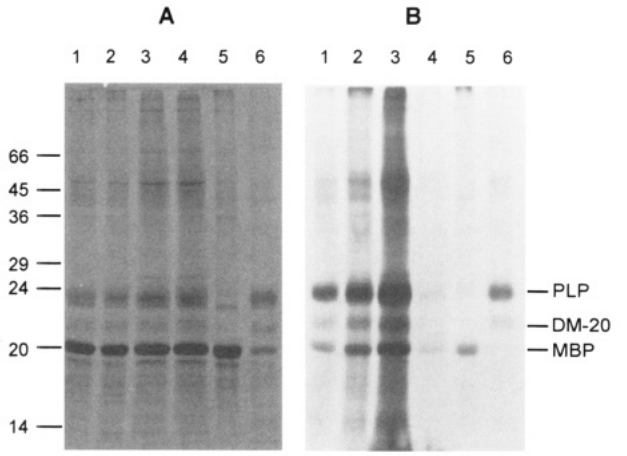

FIGURE 5: Direct labeling of bovine white matter membrane proteins with (^{3}H)$NaBH_4$. Hyposmotically shocked bovine white matter membranes were incubated directly with 2 mCi of (^{3}H)$NaBH_4$ for 1 h, washed twice, and subjected to SDS-PAGE (lane 1): lane 2, prior to the addition of (^{3}H)NBH_4, the membranes were permeabilized by treatment with 0.4% saponin; lane 3, prior to the addition of (^{3}H)$NaBH_4$, the membranes were dissolved in 4% SDS; lane 4, as a control, the SDS-dissolved membranes as in lane 3 were pretreated with 20 mM nonradioactive borohydride for 45 min prior to the addition of (^{3}H)$NaBH_4$. PLP was extracted from the membranes, exactly labeled as in lane 1, by the addition of 20 volumes of chloroform/methanol (2:1). Undissolved proteins were collected by centrifugation and subjected to SDS-PAGE (lane 5), whereas the chloroform/methanol-soluble proteins were precipitated by the addition of five volumes of diethyl ether, collected by centrifugation, washed once with ether, and analyzed by SDS-PAGE (lane 6): panel A, Coomassie blue stain; panel B, fluorography of an identical gel (exposure time 11 days).

Normally, proteins do not contain borohydride-reducible chemical bonds except disulfide bridges and, in some cases, thioester linkages to fatty acids (Torchinsky, 1981). Although PLP contains both of these latter bonds (Weimbs & Stoffel, 1993), their reduction with (^{3}H)$NaBH_4$ yields thiol groups, which rapidly exchange their tritium. Hence no incorporation of radioactivity can be expected. Vlassara et al. (1983) demonstrated that glycosylated lysine residues and their rearrangement products are the major borohydride-reducible adducts present in total myelin proteins and that their amount increases in diabetic rats.

Epand et al. (1985) speculated that unsaturated fatty acids which are linked to PLP could also be reduced by (^{3}H)$NaBH_4$. To discriminate between these possibilities, we incubated myelin proteins, radioactively labeled with (^{3}H)$NaBH_4$ as above, under denaturating conditions (2% SDS, 6 M urea) with 0.4 M hydroxylamine, pH 7.4, and 50 mM dithiothreitol for 2 h at 37 °C to cleave the thioester-linked fatty acids completely (Weimbs & Stoffel, 1993). Upon subsequent SDS-PAGE separation and fluorography, no loss of radioactivity as compared to the untreated sample was observed (data not shown), and hence the PLP-bound unsaturated fatty acids do not contribute significantly to the borohydride labeling. Therefore the majority of the borohydride-reducible groups in PLP may originate from nonenzymatic glycosylation.

[2] A column filled with Matrex Gel PBA-30 (Amicon) was used according to the instructions of the manufacturer.

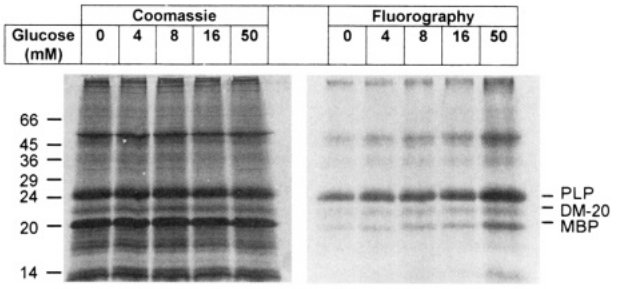

FIGURE 6: *In vitro* glycosylation of bovine white matter membrane proteins. Permeabilized bovine white matter membranes were incubated for 24 h at 37 °C in sodium phosphate buffer, pH 7.5, without glucose (lane 1) and with 4 mM (lane 2), 8 mM (lane 3), 16 mM (lane 4), or 50 mM glucose (lane 5), respectively. The membranes were washed, and nonenzymatically glycosylated proteins were labeled by treatment with $(^3H)NaBH_4$. The proteins were separated by SDS-PAGE in duplicate and the gels stained with Coomassie blue (panel A, left) or processed for fluorography (panel B, right; exposure time 43 h). The positions of PLP, DM-20, MBP, and molecular weight standards are indicated.

Nonenzymatic glycosylation of protein amino groups occurs during aging and under hyperglycemic conditions (Cohen, 1986; Cerami et al., 1988) via a Schiff-base formation between the monosaccharide and the amino group followed by an Amadori rearrangement leading to a ketoamine adduct and subsequently further rearrangements and dehydratization reactions to form the so-called advanced glycosylation end products (AGEs). The intermediates and part of the AGEs can be reduced by borohydride to a chemically stable linkage to the protein amino group. Nonenzymatic glycosylation is preferentially detected in long-lived proteins, a fact that is believed to play an important role in the development of some of the chronic complications in diabetes (Kennedy & Baynes, 1984; Cerami et al., 1988; Sensi et al., 1991; Brownlee, 1992).

In order to further examine the presence of *in vivo* nonenzymatically glycosylated lysine residues in PLP, we digested $(^3H)NaBH_4$-treated PLP completely with thermolysin. The glycosylated peptides were specifically bound to a column of phenylboronate beads via their 1,2-cis diol groups. Approximately half of the radioactivity bound to the column and was eluted with a buffer containing 200 mM sorbitol (data not shown),[2] indicating that the majority of the borohydride-reducible groups originate from nonenzymatic glycosylation. The observation that not all of the radioactive peptides bound to the column may be explained by the presence of advanced glycosylation end products which might have lost their 1,2-cis diol groups. Attempts to localize the glycosylation site(s) by analysis of the derived proteolytical peptides failed due to the very low concentration of labeled groups. By analogy the abundant pyridoxal phosphate labeling of extracytoplasmic Lys-217, we conclude that it has a similarly high affinity toward glucose being a major site of nonenzymatic glycosylation of PLP.

In Vitro Glycosylation of PLP. We investigated if PLP can be nonenzymatically glycosylated *in vitro* by incubation of permeabilized bovine white matter membranes with varying concentrations of glucose (Figure 6). After 24 h of incubation with 4–16 mM glucose (72–288 mg/dL), which corresponds to blood glucose concentrations in normoglycemic to moderate hyperglycemic individuals, a slight increase (30–50%) in nonenzymatical glycosylation can be detected for PLP, as compared to the control. Incubation with 50 mM (900 mg/dL) glucose, corresponding to hyperglycemic coma in humans, increased the borohydride reducibility by 90%. Although other proteins are similarly affected, PLP remains by far the protein with the highest absolute amount of nonenzymatic glycosylation.

Nonenzymatic Glycosylation of PLP in Diabetic Mice. The effect of high glucose concentrations on the nonenzymatic glycosylation of PLP *in vivo* was examined in the diabetic mutant mouse strain C57Bl/ksJ (*db/db*), which suffers from insulin resistance and severe hyperglycemia (Coleman & Hummel, 1974). Myelin membranes were prepared from the brains of seven diabetic mice 2.5 to 4.5 months of age (blood glucose concentrations 265 and 690 mg/dL (mean 510 mg/dL) and of seven normal, age-matched controls (approximately 100 mg/dL). They were incubated with $(^3H)NaBH_4$, and the proteins were separated by SDS-PAGE (Figure 7A). The quantitation is shown in Figure 7B. In diabetic as well as control mice, the pattern of radiolabeled proteins was similar, but PLP was the prominently labeled protein. The radioactivity of PLP of diabetic mice was significantly higher (factor of 2.1) than that of the controls. The pattern of radioactive myelin proteins was similar in diabetic and control mice. However, a considerable amount of the radioactivity is associated with very high molecular weight proteins in diabetic mice which are aggregates of PLP. PLP was also the major radioactive protein when total brain membranes of diabetic and control mice were labeled by $(^3H)NaBH_4$ (Figure 8). Radioactive MBPs are hardly detectable in contrast to MBP

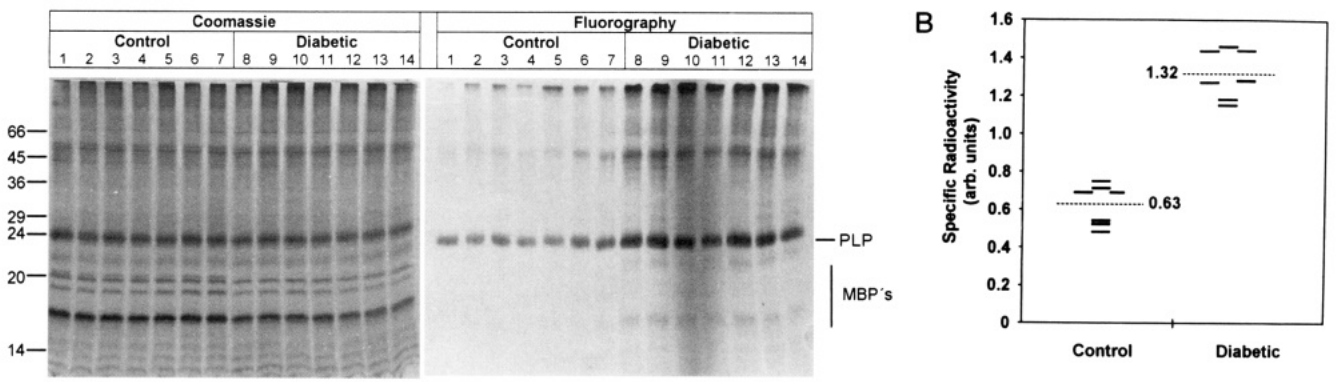

FIGURE 7: $(^3H)NaBH_4$ labeling of myelin proteins of diabetic and normal mice. Myelin membranes were prepared from seven diabetic mutant C57 Bl/ksJ (*db/db*) mice (lanes 8–14) and from seven age-matching normal controls (lanes 1–7). Borohydride-reducible bonds were labeled by treatment with 2 mCi of $(^3H)NaBH_4$ in 10 mM sodium borate, pH 8.0, for 1 h at room temperature. Membranes were washed twice with buffer and dissolved in electrophoresis sample buffer, and equal amounts were subjected to SDS-PAGE on the same gels in duplicate (A, left). One gel was stained with Coomassie blue, and the other was processed for fluorography (exposure time 80 h). The positions of molecular weight markers, PLP, and the region of the MBP isoproteins are indicated. (B, right) Protein amounts and radioactivity were determined by densitometry. The specific radioactivity (in arbitrary units) of the PLP bands is represented in the diagram for diabetic and normal mice. The mean values are indicated by dotted lines.

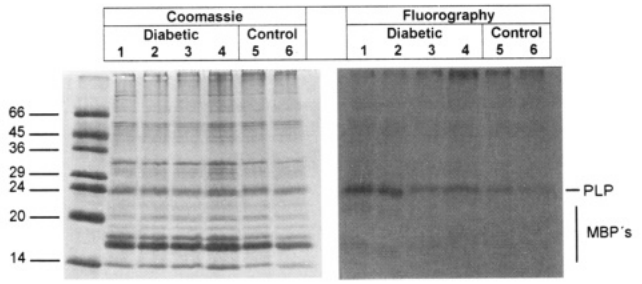

FIGURE 8: (^{3}H)$NaBH_4$ labeling of murine brain total membranes. Total brain membranes of four diabetic (lanes 1–4) and two control mice (lanes 5 and 6) were labeled with (^{3}H)$NaBH_4$ as in Figure 7 and subjected to SDS-PAGE. The gels were stained with Coomassie Blue or processed for fluorography (exposure time 75 h).

in bovine brain membrane preparations from different animals (compare Figures 5 and 6), which may reflect the age dependence of the degree of nonenzymatic glycosylations. It should be mentioned that the glucose concentration in the extracellular fluid (ECF) of the brain has not been determined and might differ from the plasma glucose level due to the glucose transport across the blood–grain barrier. Therefore we cannot give an exact correlation between blood and ECF glucose levels.

In summary, we conclude that PLP is the most highly nonenzymatically glycosylated membrane protein in the brain. The degree of glycosylation increases significantly under hyperglycemic conditions. PLP is an abundant protein in brain with a long half-life in the range of months (Benjamins & Smith, 1984), conditions which will favor the accumulation of glycosylation events at the PLP.

Several diabetic complications in advanced stages of the disease have been correlated with enzymatic glycosylation of certain proteins, e.g. cataracts and eye lens proteins, nephropathy and collagen, atherosclerosis and apolipoproteins (Kennedy & Baynes, 1984; Cerami et al., 1988; Sensi et al., 1991; Brownlee, 1992). Reports about CNS complications are very rare. In a recent review, Mooradian (1988) concluded from the collected data that mild cognitive dysfunction, like reduced memory or increased reaction time, is a common complication of chronic hyperglycemia. Ryan (1988) reviewed reports which correlate the development of mental deficiencies with type I diabetes at higher age. Morphologically the CNSs of diabetic patients revealed severe degeneration and loss of myelin sheaths accompanied by astrocytosis (Reske-Nielsen and Lundbaek (1963) and reports cited in Mooradian (1988)), which might be responsible for the reduced nerve conduction velocity observed in diabetic central nervous system (see references cited in Mooradian (1988)).

Our observation that PLP is the principal nonenzymatically glycosylated myelin protein of the CNS suggests that an excessive accumulation of the glycosylated form of this protein may be the first step in inducing a gradual degradation of myelin sheaths which then could cause the observed neurobehavioral symptoms during chronic hyperglycemia. Three different mechanisms in PLP-mediated myelin degradation seem to be plausible:

(1) Amino acid residues critical for PLP function may be glycosylated. The modification could also change the tertiary structure of the protein with an impairment of the myelin sheath integrity.

(2) The nonenzymatically glycosylated PLP could induce the degradation of myelin by phagocytosis by microglial cells. Microglial cells are present in demyelinating areas of diabetic white matter and contain PAS-positive material within the cytoplasma (Reske-Nielsen & Lundbaek, 1963). Several AGE-binding receptor proteins have been identified on murine macrophages (Vlassara et al., 1985), in rat liver (Yang et al., 1991), and on bovine endothelial cells (Schmidt et al., 1992). Although it has not yet been examined, it can be assumed that similar AGE receptors are present also in brain tissue and could recognize nonenzymatically glycosylated myelin proteins. This phenomenon has been described for peripheral myelin of diabetic rats (Vlassara et al., 1984).

(3) Finally, a recent hypothesis for the development of the long-term complications of diabetes mellitus states that nonenzymatically glycosylated proteins are susceptible to autoxidation by molecular oxygen, which leads to the generation of oxygen-free radicals (Gillery et al., 1989; Baynes, 1991; Ceriello et al., 1992). These reactive compounds could subsequently increase the rate of peroxidation of unsaturated fatty acids of myelin membrane lipids. Chia et al. (1983) demonstrated a dramatic increase of lipid peroxidation in human myelin during aging, accompanied with an increased instability of the lipid bilayer. Elevated levels of lipid peroxidation in diabetes have been detected in blood and blood cells (Baynes, 1991), but we are not aware of studies concerning the rate of lipid peroxidation in the CNS in diabetes.

CONCLUSION

The result that the fourth hydrophilic domain of PLP is located at the extracytoplasmic face of the myelin membrane supports our proposed topological model. Since this domain is the only large extracytoplasmic part of PLP according to this model, it is likely that it is involved in the proposed interaction which leads to the apposition of the extracytoplasmic myelin membrane faces. Lys-217 within this domain shows a comparatively high reactivity toward Schiff-base formation with pyridoxal phosphate *in vitro* and possibly with glucose *in vivo* and may be of particular importance for the function of PLP by analogy to reactive lysine residues within functionally important sites in other proteins. PLP was found to be the most highly nonenzymatically glycosylated membrane protein in the brain of normal and diabetic animals, and the degree of modification increased under hyperglycemic conditions. It is expected that this holds true also for human tissue, and it would be interesting to investigate in future experiments a possible correlation of the degree of nonenzymatic glycosylation of PLP with late diabetic complications affecting the central nervous system, particularly when central demyelination is observed.

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