Identification of Membrane-Embedded Domains of Lipophilin from Human Myelin†

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ABSTRACT: The organization of lipophilin in the intact human myelin membrane has been studied by labeling with the carbene photogenerated from 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID). This hydrophobic probe labels mostly lipophilin (the main intrinsic protein of myelin) and the lipids within the bilayer. The domains of lipophilin which are embedded within the membrane have been identified by proteolytic fragmentation of the [^{125}I]TID-labeled myelin, extraction with organic solvents, and separation by chromatography. Four labeled peptides were purified in this way. Polyacrylamide gel electrophoresis, amino acid compositions, automated sequencing, and carboxy-terminal analyses identified a 15K molecular weight peptide, T1 (residues 1–143), as representing the amino-terminal fragment, a 10K peptide, T2 (residues 1–97), representing a smaller amino-terminal fragment, a 5K peptide, T4 (residues 53–97), which represented the COOH-terminal half of peptide T2, and a 7K peptide, T3 (residues 205–268), which represented a sequence near the COOH terminus of lipophilin. The specific radioactivities of the peptides were determined; peptides T1 and T2 had similar specific activities, which were twice the specific activities of peptides T3 and T4. The data provide direct chemical evidence that human lipophilin has membrane-embedded domains between residues 1–97, 53–97, and 205–268, in agreement with some of the predictions of other investigators based on the sequence of bovine myelin lipophilin (proteolipid apoprotein) and a hydrophobicity diagram.

Myelin is a rich source of plasma membrane with a relatively simple biochemical composition (30% protein and 70% lipid). The two major proteins of myelin are lipophilin, an integral membrane protein of the proteolipid type, and basic protein, a peripheral protein (Boggs et al., 1982). The basic protein is believed to be located on the cytoplasmic surface of myelin (Poduslo & Braun, 1975; Golds & Braun, 1976; Omlin et al., 1982) while lipophilin is embedded in the membrane (Boggs et al., 1982) and spans the bilayer (Wood et al., 1980) but also has domains exposed at the surface of the membrane (Poduslo & Braun, 1975; Wood et al., 1977). As such, myelin constitutes an ideal system for the study of protein–lipid interactions in the natural membrane environment.

Since many of the interactions between lipophilin and lipid must be in the nonpolar region of the bilayer, identifying the protein sites involved will contribute to our understanding of this interaction and, therefore, the organization of the myelin membrane. In addition, the nature of the lipid–protein interaction could be particularly important in myelin since disruption or an alteration in this interaction could be responsible for abnormalities of myelin structure as suggested in multiple sclerosis (Chia et al., 1984).

Lipophilin belongs to the class of proteolipids (Schlesinger, 1981) and constitutes ~50% of the total myelin protein. It has a molecular weight of 30000 and a high content of cysteine residues which contribute to the intractability of its hydrophobic core and 2 mol of fatty acids covalently linked by ester bonds to seryl and threonyl residues. All these characteristics make lipophilin one of the most hydrophobic proteins known so far. From the hydrophobicity diagram, computed from the sequence (Stoffel et al., 1982, 1983; Lees et al., 1983) of the bovine protein, these authors suggested that the protein formed at least four hydrophobic regions in the bilayer linked by hydrophilic sequences at the surface of the membrane (Stoffel et al., 1982, 1983; Laursen et al., 1983).

In this paper, we identified several domains of lipophilin present within the lipid bilayer of the human myelin membrane by the use of the photoactivatable precursor 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID),[1] a lipophilic reagent that partitions into the bilayer (Brunner et al., 1980; Brunner & Semenza, 1981). Upon irradiation with light, the diazirine gave rise to the reactive carbene which labeled nearby sequences embedded in the bilayer. This technique of hydrophobic photochemical labeling was combined with proteolytic cleavage of the hydrophilic surface of the membrane and permitted the isolation of those peptides located inside the bilayer. In this way, we examined the orientation of lipophilin in myelin without disrupting the membrane.

Experimental Procedures

Materials. [^{125}I]TID in an ethanolic solution of specific activity 10 Ci/mmol was a gift from Drs. Brunner and Semenza, E.T.H., Switzerland. Penicillocarboxypeptidase S-1 was kindly supplied by Dr. T. Hofmann, Department of Biochemistry, University of Toronto. TPCK–trypsin was purchased from Worthington. Soybean trypsin inhibitor, iodoacetamide, and dithiothreitol were all from Sigma. Sephadex LH60 was obtained from Pharmacia, 88% formic acid from Aldrich, and chloroform from Fisher.

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[1] Abbreviations: [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; LP, lipophilin; BP, basic protein; W, Wolfgram protein; V_e, elution volume; V_t, total volume of this column; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; HPLC, high-pressure liquid chromatography.

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Preparation of Myelin. Myelin was prepared from normal human central nervous system white matter by the method of Lowden et al. (1966). Myelin vesicles were prepared by the method of Steck et al. (1978).

Photolabeling Reaction. The photolabeling of the myelin membrane was done as described by Brunner and Semenza for the erythrocyte membrane (Brunner & Semenza, 1981) with minor modifications. From 300 to 500 μg of myelin membranes at a concentration of 1 mg/mL on a dry weight basis was suspended in 5 mM sodium phosphate buffer, pH 8.4. An appropriate volume of [^{125}I]TID corresponding to (5–6) $\times$ 10^7 cpm was added to the membrane solution. To conserve [^{125}I]TID, lower amounts of radioactivity were used for some experiments. After 30-min equilibration of the membrane suspension with the labeling reagent, the mixture was exposed to a beam of light from a 100-W high-pressure mercury lamp (PRA Photochemical Research Associates, Inc., London, Ontario). The light beam was cooled by passage through a reservoir of circulating cold water and was directed through a saturated solution of $CuSO_4$ in a round quartz cell down the tube at the membrane suspension. The membranes were kept in suspension by stirring. After photolysis, the labeled myelin membranes were washed 4 times in sodium phosphate buffer, pH 8.5 (10 mM), containing 1% bovine serum albumin (BSA) and 3 times in the buffer without BSA in order to remove the nonbound reagent. The labeled myelin samples were dissolved in a sample buffer containing 2% (w/v) SDS and 5% (v/v) mercaptoethanol and analyzed as such by SDS–polyacrylamide gel electrophoresis containing 8 M urea (Laemmli, 1970). For autoradiography, dried slab gels were exposed to Kodak films at −70 °C.

Isolation of Proteins. Basic protein and lipophilin were isolated from 300 mg of unlabeled myelin containing a known amount (1.7 $\times$ 10^6 cpm) of [^{125}I]TID-labeled myelin by the methods of Lowden et al. (1966) and Gagnon et al. (1971), respectively, with a few modifications. After acid extraction of basic protein from myelin, the pellet was dissolved in a small volume of chloroform/methanol (2:1) and centrifuged to remove the Wolfgram proteins (another proteolipid fraction of myelin which constitutes 10–20% of the total protein). This chloroform/methanol solution was either directly chromatographed on a Sephadex LH60 column (2.5 $\times$ 75 cm), for a quantitative recovery of lipid, or reextracted with water by dialysis in order to remove small amounts of basic protein still attached to lipophilin. This step was then followed by chromatography on the Sephadex LH60 column in order to separate lipophilin from the lipids. In all the experiments described in this paper, the Sephadex LH60 column was eluted with chloroform/methanol (1:1) containing 5% 0.1 N HCl. Aliquots of the chloroform/methanol solution containing lipophilin and aliquots of basic protein precipitated from the acid extract by cold ethanol were counted and hydrolyzed for amino acid analysis (Cockle et al., 1978).

Preparation and Purification of Tryptic Peptides from Lipophilin. For digestion with trypsin, 300 mg of myelin containing a small amount of [^{125}I]TID-labeled myelin (1.44 $\times$ 10^6 cpm) was suspended in 10 mM sodium phosphate buffer, pH 8.4. Tryptic cleavage was performed for 24 h at room temperature with gentle stirring. An enzyme to substrate ratio of 1:30 was used, and the enzyme was added in two equal portions during the 24-h interval. The digest was extracted with 4 volumes of acidified chloroform/methanol (1:1) containing 5% (v/v) 0.1 N HCl. The material present at the interface, insoluble in either the upper aqueous phase or the lower chloroform phase, was found to be soluble in 90% formic acid and was further separated into a protein and a lipid peak on a Bio-Gel P150 column eluted with the same solvent. The chloroform phase was collected and pooled, concentrated on a rotary evaporator, and chromatographed on a Sephadex LH60 column (2.5 $\times$ 75 cm) equilibrated in the same solvent in order to separate the lipids from the tryptic fragments.

In another series of experiments, the enzymic digest (300 mg of myelin) was stopped after 24 h by the addition of soybean trypsin inhibitor, and the myelin membrane suspended in the sodium phosphate buffer, pH 8.4, was subjected to reduction with dithiothreitol (120 mg) overnight under N_2 at 43 °C, followed by alkylation with 240 mg of iodoacetamide for 2 h at 43 °C (Stoffel et al., 1982). The membrane suspension was then extensively dialyzed (3 days) in Spectrapor dialysis tubing of molecular weight cutoff 3500 against the phosphate buffer to remove dithiothreitol and iodoacetamide. The reduced and alkylated tryptic digest of the myelin membrane was extracted with 4 volumes of chloroform/methanol (1:1) containing 5% (v/v) 0.1 N HCl, and the chloroform phase was collected, pooled, and concentrated. Ice-cold ether was then added (4 volumes) to the chloroform phase to precipitate the peptide material and in this way separate it from most of the ether-soluble lipids. After centrifugation at 10000 rpm, the pelleted tryptic peptides were redissolved in a small volume of chloroform/methanol (1:1) containing 5% (v/v) 0.1 N HCl, centrifuged briefly to remove any undissolved material, and chromatographed on the Sephadex LH60 column. The column was eluted at a flow rate of 5.6 mL/h, and fractions of 2.8 mL were collected. Elution of the protein was monitored by measuring the absorbance at 280 nm and the emission of γ radiation. Phosphorus analysis was done on each fraction according to the method of Bartlett (1959). Homogeneous peptide fractions were isolated by rechromatography on the same column. The pooled fractions were dried to remove the chloroform by rotary evaporation and the peptides redissolved in a small amount of formic acid (80%) and lyophilized.

Characterization of Tryptic Peptides. The lyophilized peptide material was dissolved in the sample buffer containing 2% (w/v) SDS and 5% (v/v) mercaptoethanol and analyzed on SDS–polyacrylamide gels containing 8 M urea (Laemmli, 1970). The dried slab gel was autoradiographed. Small amounts of the peptides were used for amino acid analyses in a Durrum 500 amino acid analyzer after hydrolysis in 5.7 N HCl under vacuum in a sealed tube, at 110 °C for 24 h (Cockle et al., 1978). The peptides were also subjected to automated sequence studies in a Beckman 890 C sequencer (Edman, 1967). Carboxy-terminal amino acid analysis was performed on the isolated fragments using penicillocarboxypeptidase S-1 in a pyridine/formate buffer at pH 3.0 for 4 h (Hui et al., 1974) at an enzyme to substrate ratio of 1:40). The digestion was stopped by the addition of 10% trichloroacetic acid, which precipitated the undigested protein. After a brief centrifugation, the supernatant containing the C-terminal amino acids was lyophilized and subjected to amino acid analysis.

Results

Photolabeling of Myelin Membranes. Myelin membranes were labeled as described under Experimental Procedures. After exposure to light for 1 min, 51% of the total radioactivity originally present in the reaction mixture was covalently bound to myelin. Further photolysis for 2 min did not change the amount of label bound. To determine the distribution of radiolabel among myelin components, the labeled membranes were dissolved in the sample buffer and run on 15% SDS–polyacrylamide slab gels. The Coomassie blue staining pattern

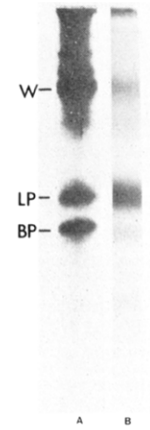

FIGURE 1: Distribution of [^{125}I]TID in myelin proteins analyzed by SDS–polyacrylamide gel electrophoresis. Myelin (500 μg) was suspended in 10 mM sodium phosphate buffer, pH 8.4, and after equilibration with 10^7 cpm of [^{125}I]TID (sp act. 10 Ci/mmol) was irradiated. The noncovalently bound label was removed as described under Experimental Procedures. [^{125}I]TID-labeled myelin was dissolved in the sample buffer and subjected to SDS–PAGE electrophoresis on a slab gel (15%). (A) Coomassie blue stain of myelin (80 μg applied). (B) Autoradiogram of the dried slab gel containing 20 μg of myelin.

Table I: Distribution of Radiolabel in the Components of the Myelin Membrane[a]

fraction	% photolysis	
	with glutathione[b]	without glutathione
myelin	100	100
lipophilin[c]	9.0	9.3
basic protein[d]	0.27	0.25
Wolfgram protein	0.67	0.60
lipid	78	80

[a] Proteins were isolated from myelin containing 1.7×10^6 cpm after all the noncovalently bound radioactivity was removed, as described under Experimental Procedures. [b] Glutathione concentration 30 mM. [c] The specific activity of lipophilin was obtained by counting an aliquot for radioactivity and by determining the protein concentration on a second aliquot after amino acid analysis. It was found to be 1.2×10^{11} cpm/mol of protein. [d] The specific activity of basic protein was determined in the same way as for lipophilin. It was found to be 7.22×10^9 cpm/mol.

is shown in Figure 1A and the autoradiograph in Figure 1B. Although several Coomassie blue staining proteins were observed in Figure 1A as expected, the autoradiography showed a major radioactive band which coincided with lipophilin (LP), a barely visible radioactive band corresponding to basic protein (BP), and a faint band corresponding to the Wolfgram protein fraction (W). When cylindrical SDS–polyacrylamide gels were cut into 2-mm slices and counted, lipophilin was found to have incorporated about 10 times more radioactivity than basic protein and 2 times more than the Wolfgram proteins.

The isolation of each protein as described under Experimental Procedures allowed us to determine the quantitative distribution of radioactivity in the two major proteins (Table I). About 80% of the myelin-incorporated label became associated with the lipid, and about 10% bound covalently to the proteins. This indicated that most of the reagent reacted with the lipids, which represent the main chemical components of the membrane. The specific activity of lipophilin (1.2×10^{11} cpm/mol) was ~16 times higher than the specific activity of basic protein (7.22×10^9 cpm/mol). Since the Wolfgram fraction consists of several proteins, no specific activity was calculated. This is in good agreement with the SDS–PAGE analysis (Figure 1) and suggested that a much larger portion of lipophilin than of basic protein was embedded in the non-

Table II: Distribution of Radiolabel among the Peptides of the Tryptic Digest[a]

phases	% of total radioactivity	% protein	% lipid
total radioactivity[b] in membrane (1.44×10^6 cpm)	100	10	80
water/methanol	0.8		
interface	6	3.2	2.4
chloroform/methanol/acid[d]	85	6.2	78

[a] The tryptic digest of myelin was extracted with chloroform/methanol (1:1) containing 5% 0.1 N HCl. [b] The values for the radioactivity associated with protein and lipid were taken from Table I. [c] The interphase material was dissolved in 90% formic acid and chromatographed on Bio-Gel P150 in order to separate the protein from the lipid. [d] The chloroform phase was concentrated by rotary evaporation and then chromatographed on the Sephadex LH60 column as described under Experimental Procedures.

polar environment of the membrane, in agreement with the known properties of these proteins (Boggs et al., 1982) and with the study of Harris & Findlay (1983) on the organization of bovine myelin. Some of the labeling experiments were performed in the presence of glutathione (30 mM), which acted as a scavenger for the carbene in the aqueous phase of the membrane suspension and, therefore, indicated if any labeling occurred at the aqueous surface of the membrane (Bayley & Knowles, 1978a,b). The amount and distribution of label incorporated into the lipids and proteins were not affected by the presence of the thiol (Table I). Therefore, we concluded that the [^{125}I]TID labeled only the hydrophobic portion of the bilayer.

Isolation of Membrane-Embedded Peptides. Myelin vesicles made according to the procedure described by Steck et al. (1978) gave a population which was 70% multilamellar vesicles and the rest single-layered vesicles when studied in thin-section electron microscopy. Labeled myelin vesicles were extensively digested with trypsin to remove exposed protein segments from the surface of the membrane. After extensive digestion with trypsin more than 90% of the vesicle population was unilamellar. Thus, trypsin penetrated into the intraperiod as well as major dense-line regions and digested the protein material at these sites. After removal of peptides from the surface of the bilayer by trypsin, the hydrophobic peptides were extracted from their membrane environments by acidified chloroform/methanol as described under Experimental Procedures.

Only a small amount of radioactive material was extracted into the upper aqueous phase (0.8%), demonstrating that very little label was attached to the exposed protein segments (Table II). This phase was not analyzed further. The insoluble material at the interface contained ~6% of the total radioactivity. Most of the radioactivity, 85%, was recovered in the lower, chloroform phase which contained some of the tryptic peptides and most of the lipids. The peptides from this chloroform phase were separated from the lipids by chromatography on Sephadex LH60 in acidified chloroform/methanol and contained ~6.2% of the radioactivity associated with this phase. About 78% of the radioactivity was found with the lipid. Since lipophilin has 16 Cys residues out of which at least 6 are disulfide bonds (Cockle et al., 1980), reductive carboxamidomethylation of the digested myelin membrane was performed before extraction with chloroform/methanol. The separation of the reduced and alkylated peptides on Sephadex LH60 is shown in Figure 2. The peptide material was recovered in fractions 20–53. Fractions 60–80 contained phospholipids and a small amount of material absorbing at 280 nm. Four peaks of radioactivity and the absorbance at

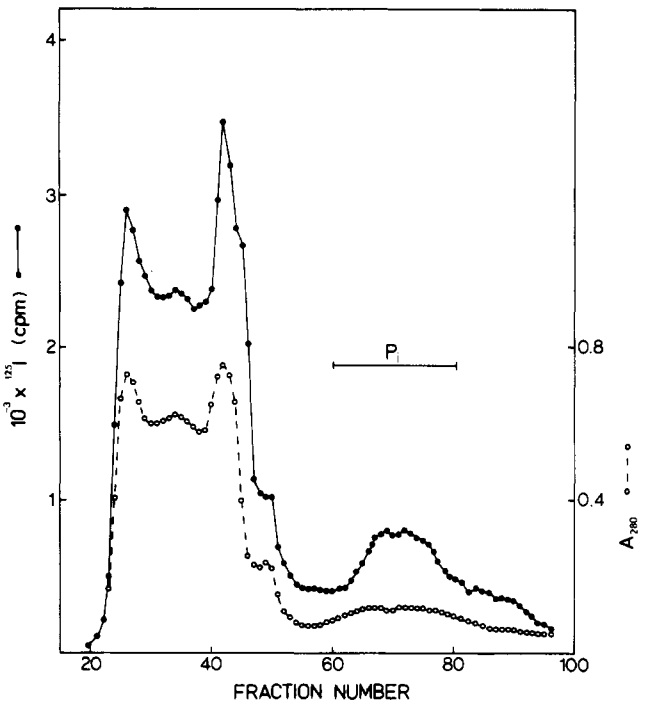

FIGURE 2: Gel filtration of [^{125}I]TID peptides on Sephadex LH60. [^{125}I]TID-labeled myelin was digested with trypsin as described under Experimental Procedures. The digest was reduced and alkylated, extracted with acidified chloroform/methanol, concentrated, and chromatographed on Sephadex LH60 (2.5 × 75 cm). The column was eluted with a flow rate of 5.6 mL/h. Fractions of 2.8 mL were collected and counted for ^{125}I, and their absorbancies at 280 nm were recorded. Fractions 20–28 for peak 1, 31–37 for peak 2, 39–45 for peak 3, and 48–53 for peak 4 were pooled and concentrated by rotary evaporation. P_i indicates the phosphorus-containing fractions.

280 nm were obtained and were pooled as described in the legend of Figure 2.

Characterization of [^{125}I]TID-Labeled Peptides. Each of the peptide peaks was rechromatographed on the same column for further purification, as shown in Figure 3. Rechromatography of peak 1 (fractions 20–28, Figure 2) is not shown because it rechromatographed as a single peak (peptide T1) eluting with $V_e/V_t = 0.2$.

Peak 2 gave a major peak with ascending and descending shoulders, representing small amounts of contaminants from peaks 1 and 3 (Figure 3A). Purified peptide T2 was recovered in the major peak of $V_e/V_t = 0.24$. Peak 3 separated into two peaks, one at $V_e/V_t = 0.24$ containing mainly peptide T2 and one at $V_e/V_t = 0.32$ containing purified peptide T3 (Figure 3B). Peptide T4 was recovered in the main peak of $V_e/V_t = 0.38$ obtained from the rechromatography of peak 4 (Figure 3C). The shoulder represented a small amount of contaminant from peak 3.

To estimate the molecular weight of the hydrophobic peptides, the column of Sephadex LH60 was calibrated with molecular weight standards. From the calibration curve (Figure 3 inset), it appeared that there was a good correlation between the elution volume of the peptides and their molecular weights, similar to the results of Gerber et al. (1979), who eluted their Sephadex LH60 column with formic acid/ethanol. The molecular weight deduced for each peptide was as follows: 15000 for T1; 10000 for T2; 7000 for T3; 5000 for T4. SDS–PAGE of T2 and T3 is shown in Figure 4. They migrated as single bands, demonstrating that they were pure. In each case, the radioactive peptide migrated at the same position as the peptide stained with Coomassie blue, confirming the elution profile on the LH60 column (Figure 2). Peptide T3 migrated as a single band of molecular weight ~5700 as

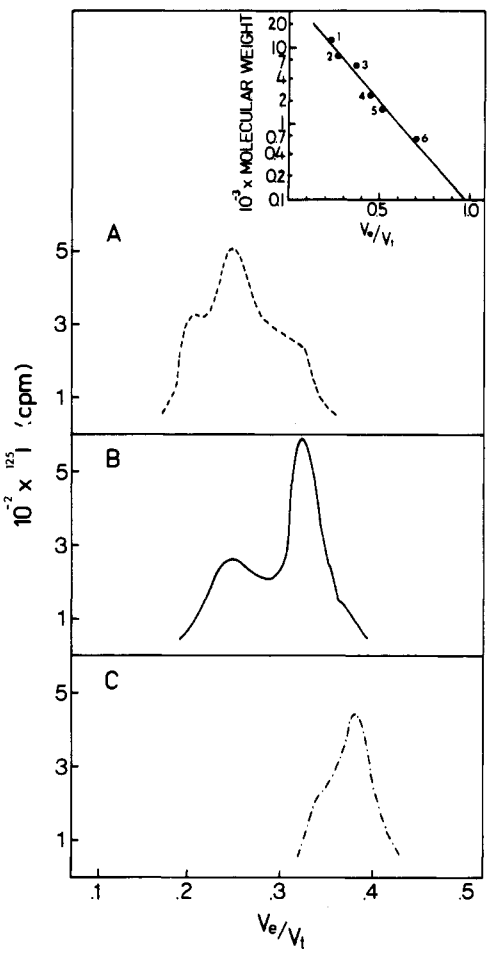

FIGURE 3: Rechromatography of peptide peaks on the Sephadex LH60 column. Fractions of 2.8 mL were collected and counted, and their radioactivity was plotted as a function of the ratio of V_e (elution volume) to V_t (total volume). (A) Rechromatography of peak 2; (B) rechromatography of peak 3; (C) rechromatography of peak 4. The inset shows the calibration of the Sephadex LH60 column (2.5 × 75 cm) developed at a flow rate of 5.6 mL/h with the following molecular weight standards: (1) cytochrome *c* (horse) of M_r ~13000; (2) CNBr I of M_r ~8250 obtained by CNBr digestion of cytochrome *c*; (3) insulin of M_r ~5700; (4) CNBr II of M_r ~2420; (5) CNBr III, M_r ~1650; (6) heme, M_r ~594.

shown in the Coomassie blue staining pattern (Figure 4A) and the autoradiograph (Figure 4B). Peptide T2 also migrated as a single component after performic acid oxidation, which was necessary because the original reduction and alkylation of this peptide were not complete (only two out of six Cys residues were recovered as carboxymethylcysteine by amino acid analysis). On the SDS gel, one band identified by Coomassie blue staining (Figure 4C) and by autoradiography (Figure 4D) was obtained corresponding to a molecular weight of 6900. Peptide T4 is not shown since its electrophoretic mobility was too high for this particular gel system. A Coomassie blue stained gel of T1 showed a single component. These molecular weights deduced from the gel are lower than those determined from column chromatography (Figure 3) and sequence analysis. This is in agreement with similar studies on other membrane proteins in which SDS–PAGE yielded anomalous molecular weights (Ohnoki & Martonosi, 1980; Hartshorne & Catterall, 1984).

The amino acid compositions and partial N-terminal sequences for the four peptides are given in Table III. The amino acid compositions of these peptides are very hydrophobic. Peptide T3 contained most of the Met residues in lipophilin, suggesting that this fragment originated from the

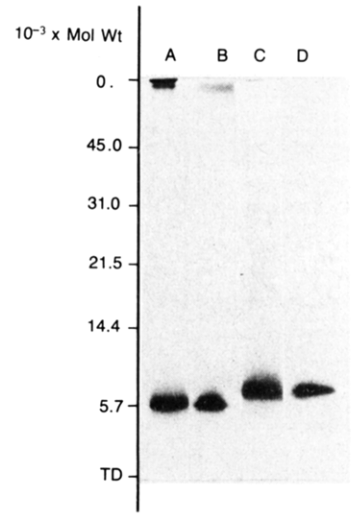

FIGURE 4: SDS–PAGE of [^{125}I]TID-labeled intramembranous peptides. After rechromatography on Sephadex LH60, the [^{125}I]TID-labeled peptides were concentrated by rotary evaporation and lyophilized, dissolved in the sample buffer, and subjected to SDS–PAGE electrophoresis on a slab gel (15%). (A) Coomassie blue stain of peptide T3 (80 μg); (B) autoradiograph of the dried slab gel of peptide T3 (40 μg); (C) Coomassie blue stain of performic acid oxidized peptide T2 (80 μg); (D) autoradiography of gel of performic acid oxidized peptide T2 (40 μg).

Table III: Amino Acid Composition[a] and Partial Sequence Analysis[b] of the Purified Peptides

amino acid	T1[c]	T2[d]	T3[e]	T4[f]
Asp	4.4 (4)	2.4 (2)	2.5 (3)	2.7 (3)
Thr	8.5 (9)	5.5 (6)	5.0 (5)	2.2 (2)
Ser	4.5 (5)	2.7 (3)	3.1 (3)	0.7 (1)
Glu	11.2 (11)	7.0 (7)	2.5 (3)	3.8 (4)
Pro	1.5 (2)	1.4 (1)	2.2 (2)	0.3 (0)
Gly	21.3 (21)	11.5 (12)	5.0 (5)	5.8 (6)
Ala	14.2 (14)	11.0 (11)	8.5 (9)	5.6 (6)
Val	7.5 (8)	5.5 (6)	6.0 (6)	3.2 (3)
CM-Cys	3.8 (4)	1.8 (2)	1.5 (2)	
Met	0.4 (0)	0.4 (0)	2.5 (3)	0.2 (0)
Ile	6.0 (6)	4.4 (4)	3.2 (3)	3.0 (3)
Leu	17.4 (17)	12.4 (12)	8.4 (8)	5.5 (6)
Tyr	8.8 (9)	9.3 (9)	2.4 (2)	7.3 (7)
Phe	10.3 (10)	9.5 (10)	6.5 (7)	5.5 (6)
Lys	6.2 (6)	2.5 (3)	2.5 (3)	0.5 (1)
His	5.5 (6)	2.5 (3)	1.3 (1)	1.0 (1)
Arg	4.9 (5)	2.3 (2)	1.2 (1)	1.3 (1)

[a] All values have been obtained from hydrolysis in 5.7 N HCl at 110 °C for 24 h. Values represent moles of amino acid per mole of peptide rounded off to the nearest whole residue (in parentheses). The molecular weights of the peptides have been estimated from their elution volume of Sephadex LH60 (Figure 4). [b] N-Terminal sequences for each peptide were obtained by automated Edman degradation in a Beckman 890 C sequencer. [c] T1: Gly-Leu-Leu-Glu-Cys-X-Ala-Arg-X-Leu-Val-Gly-Ala-Pro-Phe. [d] T2: Gly-Leu-Leu-Gly-Cys-X-Ala-Arg-X-Leu-Val-Gly-Ala-Pro-Phe. [e] T3: Met-Tyr-Gly-Val-Leu-Pro-Trp-Asn-Ala-Phe-Pro-Gly-Lys-Val. [f] T4: Asn-Tyr-Gln-Asp-Tyr-Glu-Tyr-Leu-Ile-Asn-Val-Ile.

sequence near the COOH terminus of the molecule[2] (Stoffel et al., 1982). Peptide T4 had a composition relatively high in Tyr residues and contained no carboxymethylated Cys residues. The N-terminal sequences of the first 15 residues for T1 and T2 were identical. Their respective amino acid compositions also suggested that they were related peptides.

When the amino acid composition and Edman degradation results were compared with the known sequences of bovine myelin lipophilin (Stoffel et al., 1982, 1983) and proteolipid apoprotein (Laursen et al., 1983), the sequences for peptide

[2] I. Kahan and M. A. Moscarello, unpublished results.

Table IV: Distribution of Radioactivity among Peptides Fractionated from the Chloroform Phase[a]

residues	% radioactivity associated with peptide material	μmol of peptide[b]	sp act.[c] (cpm/mol)
total	6		
T1, 1–143	1.65	0.32	5.6×10^{10}
T2, 1–97	1.5	0.24	5.8×10^{10}
T3, 205–268	2.4	0.80	3.1×10^{10}
T4, 53–97	0.45	0.16	3.0×10^{10}

[a] Peptides were isolated on Sephadex LH60 as described under Experimental Procedures. [b] The amount of each peptide was determined after rechromatography on Sephadex LH60 by amino acid analysis, as described under Experimental Procedures. [c] The total cpm associated with each peptide was determined by counting an aliquot of each pooled peptide after rechromatography.

T1 and peptide T2 for the human protein were deduced to represent two N-terminal fragments. Peptide T1 corresponded to residues 1–143, giving a theoretical molecular weight of ~16000, and peptide T2 corresponded to residues 1–97, giving a theoretical molecular weight of ~11000. T3 represented the C-terminal fragment of lipophilin (residues 205–268) of theoretical molecular weight ~7700, and T4 represented the fragment consisting of residues 53–97 of theoretical molecular weight ~5000. This interpretation was confirmed by end-group analysis with penicillocarboxypeptidase S-1. This analysis identified Arg and Val as the terminal and subterminal residues of T2, respectively, Lys-Gly at the C-terminus of T1, and Lys-Leu at the C-terminus of T3. Peptide T4 was insoluble in this buffer, and no C-terminus could be established for it.

Distribution of Radioactivity among Peptides. The radioactivity associated with each peptide fraction in the chloroform phase is shown in Table IV. The recovery of the lipophilin fragments in the chloroform phase was 60–65% on the basis of initial amounts of lipophilin in the myelin membrane. Peptides T1 (residues 1–143) and T2 (residues 1–97) have similar specific activities which are about twice the specific activities of peptides T3 and T4.

DISCUSSION

In this study, we used [^{125}I]TID to label the human myelin membrane. Lipophilin, the predominant intrinsic membrane protein, was labeled with about 16 times greater specific activity than basic protein, the predominant extrinsic protein. Moreover, only 0.8% of the total label associated with the membrane or ~7% of the label associated with the protein was extracted into the aqueous phase after extensive tryptic proteolysis. This phase contained mainly peptides originating from basic protein as well as surface peptides derived from lipophilin. The presence of the hydrophilic scavenger glutathione had no effect on the distribution of label among the myelin proteins. These data demonstrate that labeling occurred mainly within the bilayer. Analysis of other proteins like sucrose isomaltase, an integral protein of the brush-border membrane (Spiess et al., 1982), and acetylcholinesterase from *Torpedo marmorata* (Stieger et al., 1984) has shown that the sites on these proteins labeled by TID are localized within fragments known to be embedded in the membrane, consistent with our conclusion that the labeling occurred from within the bilayer.

For the purpose of localizing the membrane-embedded regions of lipophilin, extensive tryptic proteolysis of myelin, followed by extraction of the membrane-embedded peptides into acidified chloroform/methanol and gel chromatography, was used to obtain four pure radioactive peptides: T1 (residues

1–143), T2 (residues 1–97), and T4 (residues 53–97), situated near the N-terminus of the protein; and T3 (residues 205–268), situated at its C-terminus. The peptide corresponding to the N-terminal 52 residues was not isolated in the chloroform phase as would have been expected since tryptic cleavage occurred at Lys-52. This fragment probably remained at the interface, since 3.2% of the radioactivity in the interface was associated with peptide material (Table II). Our attempts to separate the peptides from the interface either on Bio-Gel P columns or by HPLC on silica supports eluted with 90% formic acid have not been successful so far.

Peptides T1 and T2 have similar activities and share the N-terminal 97 residues. This implies that no [^{125}I]TID has been incorporated in the C-terminal 45 residues of T1.

The specific activity of T2 was approximately twice that of T4, implying that there was at least one other site of TID incorporation in residues 1–52. If it is assumed that the specific activity is proportional to the number of transmembrane domains, then it can be inferred that residues 1–52 of T2 contain one transmembrane domain joined, by a sequence accessible to trypsin at Lys-52, to a second transmembrane domain formed by residues 53–97.

A third region embedded in the bilayer was recovered in peptide T3 (residues 205–268). Since the specific activity of T3 was similar to that of T4, T3 may also span the myelin membrane only once. Thus, the four fragments isolated by us may contain three membrane-embedded domains of lipophilin. However, the possibility that some intramembranous domains are partially shielded from contact with the carbene or lack residues for which the carbene may exhibit chemical selectivity cannot be ruled out at the present time.

Support for the hypothesis that these membrane-embedded sequences are of sufficient length to span the membrane was obtained from circular dichroism studies of lipophilin and of peptides T3 and T4 incorporated into PC vesicles which suggested that both the intact protein (Cockle et al., 1978) and the peptides[3] have a high content of α-helical structure. The relative thickness of the myelin bilayer was reported to be 50 Å (Kirshner & Caspar, 1977). Since the pitch height per residue for an α-helix is 1.5 Å, 33 residues would be needed to span the 50-Å-thick bilayer. Therefore, a 44-, 52-, or 63-residue sequence would probably be sufficiently long to span the myelin bilayer once.

While this work was in progress, the complete amino acid sequence of bovine lipophilin was elucidated, and models of lipophilin organization in the membrane on the basis of its sequence were proposed (Stoffel et al., 1982, 1983). Similar predictions were made for the bovine proteolipid apoprotein (Laursen et al., 1983). These authors deduced from the hydrophobicity indices, and the number and position of hydrophobic amino acids of lipophilin, the presence of four long sequences that were capable of spanning the membrane and one short sequence that may be only slightly embedded in the membrane. The four long hydrophobic stretches were formed by residues 9–34, 59–90, 151–190, and 232–267. The short hydrophobic sequence was included between residues 205 and 218. The sequences in between the hydrophobic residues form hydrophilic loops outside the bilayer.

The short and long hydrophobic sequences from the C-terminus of lipophilin were situated on the TID-labeled tryptic fragment T3 (residues 205–268) isolated by us. The sequence defined by residues 59–90 was almost identical with that of our radioactive fragment T4 (residues 53–97). Therefore, the chemical data described here support the models of lipophilin in myelin for the sequences 59–90 and 207–267.

The N-terminal 52 residues were postulated to be membrane embedded from analysis of the specific activities of peptides T2 (5.8×10^{10} cpm/mol) and T4 (3.0×10^{10} cpm/mol). This supports the hydrophobicity analysis which predicts a hydrophobic segment between residues 9 and 34. A comparison of the specific activities of T1 (5.6×10^{10} cpm/mol) and T2 (5.8×10^{10} cpm/mol) suggests that no carbene was incorporated in the sequence 98–145 and confirms the prediction that this segment, rich in polar residues, is situated outside the bilayer.

The tryptic fragment corresponding to the hydrophobic sequence 151–190 was not present in the chloroform phase analyzed by us. Therefore, we cannot conclude whether this sequence incorporated any label. Since the specific activity of lipophilin (1.2×10^{11} cpm/mol) was greater than those of T1 and T3, the existence of at least one more intramembranous domain could be inferred. However, at present, the identity of this domain is unknown.

The data reported here provide the first direct evidence for the existence of intramembranous domains of lipophilin in the human myelin membrane. A complete characterization of [^{125}I]TID labeling of lipophilin will help to provide an accurate model of this major protein.

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Nuclear Magnetic Resonance Determination of Metal–Proton Distances in a Synthetic Calcium Binding Site of Rabbit Skeletal Troponin C†

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ABSTRACT: The binding of gadolinium to a synthetic peptide of 13 amino acid residues representing the calcium binding loop of site 3 of rabbit skeletal troponin C [AcSTnC(103-115)amide] has been studied by using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. In particular, the proton line broadening and enhanced spin–lattice relaxation have been used to determine proton–metal ion distances for several assigned nuclei in the peptide–metal ion complex. These distances have been used in conjunction with other constraints and a distance algorithm procedure to demonstrate that the structure of the peptide–metal complex as shown by ^{1}H NMR is consistent with the structure of the EF calcium binding loop in the X-ray structure of parvalbumin but that the available ^{1}H NMR distances do not uniquely define the solution structure.

Calcium has been implicated in varous cellular functions [see reviews by Kretsinger (1979, 1980), Wang & Waisman (1979), and Means et al. (1982)] through its binding to calmodulin (Cheung, 1970; Kakiuchi & Yamazaki, 1970) and troponin C (Ebashi et al., 1968). These two proteins and several others including parvalbumin (Pechère et al., 1971; Benzonana et al., 1972; Nockolds et al., 1972; Coffee & Bradshaw, 1973), S-100 (Isobe & Okuyama, 1978, 1981; Mani et al., 1982), the myosin light chains (Frank & Weeds, 1974; Jakes et al., 1976; Collins, 1976; Kendrick-Jones & Jakes, 1977; Chantler & Szent-Gyorgyi, 1978), and the intestinal calcium binding proteins (Hofmann et al., 1979; Fullmer & Wasserman, 1981) possess two to four EF hand[1] domains (Kretsinger & Nockolds, 1973). These regions are about 30–35 amino acids long and are composed of two α-helical segments flanking a calcium binding loop (Gariépy & Hodges, 1983).

We have been involved in the "molecular dissection" of a model EF hand domain using synthetic analogues of site 3 of rabbit skeletal troponin C (Reid et al., 1980, 1981; Gariépy

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[1] Abbreviations: AcSTnC(103-115)amide, synthetic N-terminal acetylated rabbit skeletal troponin C fragment, residues 103-115, with a C-terminal amide; EF hand, second calcium binding domain of carp parvalbumin; TnC, troponin C; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; FID, free induction decay; ^{1}H NMR, proton nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; M_r, molecular weight; 2D, two dimensional.

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