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Analysis of the membrane-interacting domains of myelin basic protein by hydrophobic photolabeling

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Abstract

Myelin basic protein is a water soluble membrane protein which interacts with acidic lipids through some type of hydrophobic interaction in addition to electrostatic interactions. Here we show that it can be labeled from within the lipid bilayer when bound to acidic lipids with the hydrophobic photolabel 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine (TID) and by two lipid photolabels. The latter included one with the reactive group near the apolar/polar interface and one with the reactive group linked to an acyl chain to position it deeper in the bilayer. The regions of the protein which interact hydrophobically with lipid to the greatest extent were determined by cleaving the TID-labeled myelin basic protein (MBP) with cathepsin D into peptides 1–43, 44–89, and 90–170. All three peptides from lipid-bound protein were labeled much more than peptides from the protein labeled in solution. However, the peptide labeling pattern was similar for both environments. The two peptides in the N-terminal half were labeled similarly and about twice as much as the C-terminal peptide indicating that the N-terminal half interacts hydrophobically with lipid more than the C-terminal half. MBP can be modified post-translationally in vivo, including by deamidation, which may alter its interactions with lipid. However, deamidation had no effect on the TID labeling of MBP or on the labeling pattern of the cathepsin D peptides. The site of deamidation has been reported to be in the C-terminal half, and its lack of effect on hydrophobic interactions of MBP with lipid are consistent with the conclusion that the N-terminal half interacts hydrophobically more than the C-terminal half. Since other studies of the interaction of isolated N-terminal and C-terminal peptides with lipid also indicate that the N-terminal half interacts hydrophobically with lipid more than the C-terminal half, these results from photolabeling of the intact protein suggest that the N-terminal half of the intact protein interacts with lipid in a similar way as the isolated peptide. The similar behavior of the intact protein to that of its isolated peptides suggests that when the purified protein binds to acidic lipids, it is in a conformation which allows both halves of the protein to interact independently with the lipid bilayer. That is, it does not form a hydrophobic domain made up from different parts of the protein.

Keywords: Myelin basic protein; Lipid bilayer; 3-(Trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; Photolabel; Cathepsin D; Deamidation

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; GalCer-C8-PL, [^{3}H]*N*-(ω-4-azidobenzoyl)capryloyl galactosylceramide; GalCer-PL, [^{3}H]*N*-(4-azidobenzoyl) galactosylceramide; MBP, myelin basic protein; PC, phosphatidylcholine; PG, phosphatidylglycerol; TFA, trifluoroacetic acid; TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine

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1. Introduction

About 30% of the protein of central nervous system myelin consists of a single protein, myelin basic protein (MBP). MBP is thought to be responsible for adhesion of the intracellular surfaces of the compact multilayered myelin sheath in the central nervous system. It is located at the major dense line of myelin [1,2] and it causes adhesion of lipid vesicles containing negatively charged lipids [3–5]. Although MBP is a water soluble protein which binds to acidic lipids mainly by electrostatic interactions, it also appears to interact with the lipid bilayer through some type of hydrophobic interactions. MBP has a pronounced perturbing effect on the lipid acyl chains. It increases vesicle permeability [6], decreases the temperature and enthalpy of the gel to liquid crystalline phase transition [7–10], increases the surface pressure of lipid monolayers [11,12], and affects the motion of fatty acid spin labels with the nitroxide label close to the terminal methyl group [8–10,13]. In addition, it can be labeled from within the bilayer by the hydrophobic photolabel 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine (TID) [5,14]. On interaction of the protein with lipid, it is partially protected from tryptic hydrolysis [15] and the NMR resonances of some residues are broadened or show chemical shifts [16–20]. All of these effects could be caused by MBP lying on the surface of the bilayer, as indicated by X-ray diffraction [21], with hydrophobic side chains of some amino acids penetrating partway into the bilayer, or the protein may disorganize the bilayer so that the lipid chains interact with the protein on the surface. Alternatively, the protein may fold into a tertiary structure with a hydrophobic domain. In aqueous solution, the protein has a hydrophobic site which can bind heme and other hydrophobic molecules [22–24].

A number of studies have attempted to delineate which part of the protein participates in this hydrophobic interaction the most. Many of these studies have made use of peptides, particularly those obtained by cleavage with BNPS-skatole, which gives an N-terminal two thirds portion, 1–115, and a C-terminal one third portion, 116–169, of MBP (bovine MBP residue numbers) [9,11,25–28]. However, if MBP takes up a tertiary structure in which residues from several regions of the protein are involved in forming a hydrophobic domain, this would be disrupted by cleaving the protein into peptides. Indeed, some studies suggest that the interaction with lipid of intact MBP is different from that of the large peptides 1–115 and 116–169 of bovine MBP [26] and 1–98 and 99–179 of porcine MBP [20]. Therefore a technique by which domains of the intact protein interacting with lipid can be determined may provide more relevant information. In this study we have labeled the protein bound to acidic lipid bilayers with the hydrophobic photolabel TID [29] and with two lipid photolabels which should position the photosensitive group at two locations in the bilayer, a shallow one relatively close to the apolar/polar interface, and a deeper one closer to the center of the bilayer [30]. We then determined which regions of the protein are labeled by TID by peptide mapping using cathepsin D to digest the protein.

MBP isolated from myelin exhibits charge microheterogeneity due to post-translational modifications which increase its negative charge, such as deamidation and phosphorylation [31–33]. These may alter the interactions of MBP with lipid by decreasing the degree of hydrophobic interaction. Different charge isomers of MBP resulting from these modifications can be purified by cation-exchange chromatography [34,32]. In this study we have compared the regions labeled by TID of the two most cationic charge isomers, C1 and C2, of human MBP. C2 has one more negative charge than C1 due primarily to deamidation [33,31,35,36].

2. Materials and methods

2.1. Chemicals

Dipalmitoylphosphatidylglycerol (DPPG), egg phosphatidylcholine (PC), and phosphatidylglycerol (PG) prepared from egg PC were purchased from Avanti (Birmingham, AL). Dipalmitoylphosphatidylcholine (DPPC) and psychosine were purchased from Sigma (St. Louis, MO). All were chromatographically pure and were stored at −20°C. Spleen cathepsin D was purchased from Sigma. An ethanolic solution of TID (specific activity 10 mCi/μmol) was purchased from Amersham (Canada). ω-Aminocaprylic acid, 9-flurenylmethylchloroformate, *N*,*N*′-dis-

uccinimidylcarbonate, and *N*-hydroxysuccinimide were purchased from Aldrich (Milwaukee, WI). 4-Azidobenzoic acid was purchased from Chemical Dynamics (South Plainfield, NJ) and succinimidyl-4-azidobenzoate-3,5-[^{3}H]) (specific activity, 41.6 Ci/mmol) was purchased from NEN-Dupont (Boston, MA).

2.2. Purification of MBP and fractionation into charge isomers

MBP consisting predominantly of the 18.5 kDa isomer was prepared from bovine and from human brain white matter as described by Cheifetz and Moscarello [4]. The human tissue was obtained postmortem from patients who had had Alzheimer's disease and was supplied by the Canadian brain tissue bank (Toronto, ON). The MBP purification method is the extensively used procedure of chloroform/methanol delipidation followed by extraction with acid. The possibility that the protein is denatured cannot be excluded. However, there is no procedure available for extraction of MBP which is known to prevent any putative denaturation since there is no way to determine whether this protein is in its native conformation. However, MBP purified by similar procedures as used here was found by Gow and Smith [37] using CD spectroscopy to have a similar conformation as MBP extracted with $CaCl_2$ in the absence of denaturants. These authors have argued that this single chain protein which lacks cysteine should be able to refold spontaneously to its thermodynamically stable state in different environments.

The human MBP was fractionated into its charge isomers by ion-exchange chromatography at alkaline pH on CM52 as described [4,31]. The charge isomers were further purified by chromatography on Sephadex G-75 in 0.1% trifluoroacetic acid (TFA) as described [38]. C1 and C2 gave single bands at the position expected for the 18.5 kDa isomer of MBP by polyacrylamide gel electrophoresis (PAGE) performed according to Laemmli [39]. Amino acid analyses, performed at the Hospital for Sick Children/Pharmacia Biotechnology Centre on a Waters PICO-TAG system, gave the amino acid composition expected for MBP; the expected number of arginines (19/mole protein) were detected for both C1 and C2 (not shown) indicating that C-terminal Arg loss from C2 was not responsible for its lower net positive charge. We found that C1 had similar CD spectra in solution and in the presence of detergent as found by Gow and Smith [37] for MBP extracted with $CaCl_2$ (K.M. Koshy, J.M. Boggs, unpublished). Addition of detergent induced significantly more secondary structure for C1 as found for MBP extracted with $CaCl_2$.

2.3. Synthesis of GalCer photolabels

Cold succinimidyl azidobenzoate was synthesized from 4-azidobenzoic acid and *N*-hydroxysuccinimide as described [40] and used for trial syntheses of the GalCer photolabels. It was also used to lower the specific activity of purchased [^{3}H]succinimidylazidobenzoate to a specific activity of 0.216 mCi/μmole for synthesis of [^{3}H]*N*-(4-azidobenzoyl)galactosylceramide (GalCer-PL). [^{3}H]Succinimidylazidobenzoate (250 μCi) was reacted with 0.5 mg of psychosine in 250 μl of a mixture of THF, water, and triethylamine (90:10:5) for 18 h. The solvent was removed under a stream of nitrogen and the residue dried under vacuum. GalCer-PL was purified by TLC on silica gel plates using chloroform/methanol (80:20) as the solvent. The specific activity was estimated at 0.09 mCi/μmole based on the radiochemical yield.

For synthesis of [^{3}H]*N*-(ω-4-azidobenzoylamido)-capryloyl galactosylceramide, *N*-(ω-aminocapryloyl)-psychosine was synthesized by an adaptation of the method of Sonnino et al. [41]. The Fmoc derivative of ω-aminocaprylic acid obtained by the reaction of 9-flurenylmethylchloroformate with ω-aminocaprylic acid was converted to the *N*-hydroxy succinimide ester by reaction with *N*,*N*′-disuccinimidyl carbonate. The product was reacted with psychosine in the presence of triethylamine in DMF. The protecting group was removed using 20% piperidine in THF to give *N*-(ω-aminocapryloyl)psychosine. The latter was purified by column chromatography on silica gel using chloroform/methanol/ammonium hydroxide (80:20:1) as the eluting solvent.

375 μCi of [^{3}H]succinimidylazidobenzoate adjusted to 1.2 mCi/μmole with cold succinimidylazidobenzoate was reacted with 0.2 mg of *N*-(ω-aminocapryloyl)psychosine dissolved in 150 μl of THF/water/triethylamine (90:10:5) for 18 h. The product, [^{3}H]*N*-(ω-4-azidobenzoylamido)capryloyl galactosyl-

ceramide (GalCer-C8-PL) was purified as above. The specific activity was estimated at 0.43 mCi/μmole.

Each step of these synthetic procedures was carefully monitored by micro-thin layer chromatography using appropriate visualizing reagents such as orcinol for the sugar head group, ninhydrin for the free amino group, iodine vapor, and radioactivity where feasible. The products at each stage had the characteristics expected, e.g. presence or loss of a free amino group, TLC mobility, etc.

2.4. Preparation of vesicles

The dry lipid was dispersed in buffer at a concentration of 4 mg/0.75 ml by vortex mixing at a temperature above the lipid phase transition temperature to give large multilayered vesicles. The buffer contained 10 mM NaCl and 10 mM HEPES and was adjusted to pH 7.4. 30 mM glutathione was included in early experiments but was found to have no effect on the labeling of MBP and was later omitted. C1 or C2 was dissolved in the buffer at a concentration of 4 mg/ml and the pH was checked. 0.25 ml was added to the lipid suspension so that the protein to lipid weight ratio was 1:4. The sample was vortexed again and incubated at a temperature above the lipid phase transition temperature for at least 3 min. In order to label protein in the absence of lipid, 0.25 ml of protein solution was added to 0.75 ml of buffer.

2.5. Photolabeling reaction with TID

TID was diluted with 0.4 ml ethanol. The samples to be labeled were flushed with nitrogen and 4–8 μl of TID solution containing approx. $1–2\times10^7$ cpm were added to 1 ml of vesicle suspension and the sample was mixed in the dark. It was equilibrated at room temperature in the dark for 20–30 min and then irradiated for 2 min using a 100 W high pressure Hg lamp (Photochemical Research Associates, London, ON) with the sample tube immersed in ice/water as described [14,5]. The light beam was cooled by passage through a reservoir of circulating cold water and directed through a filter consisting of a saturated solution of $CuSO_4$.

The labeled protein was delipidated as described previously [14] with some modifications. The sample was acidified to pH 1 with 2 N HCl and extracted with 4 ml of acidified chloroform/methanol 1:1 (v/v) containing 5% 0.1 N HCl. The extraction was repeated three times with fresh organic phase. The aqueous phase was then lyophilized, redissolved in 2 ml 0.05% TFA and chromatographed on C18 Sep-Pak columns (1 ml per column) (Waters, Milford, MA) in order to remove small molecular weight labeled compounds. The MBP was eluted with 40% acetonitrile containing 0.05% TFA. 40–80% of the labeled protein was recovered in the 40% acetonitrile eluate. Eluates from the same sample were pooled and lyophilized. The protein was redissolved in 1 ml of distilled water and aliquots were counted in a γ counter and assayed for protein content by the method of Peterson [42]. 5–10 μg/lane was loaded on precast 16% acrylamide tricine gels (Novex, San Diego, CA) [43]. SDS-PAGE was performed at 100 V (constant voltage) until the tracking dye reached the bottom of the gel. After staining with 0.05% Coomassie brilliant blue R and destaining as described [44], the gel was sliced into ten strips of approximately equal width, keeping stained bands intact, and the strips were counted in a γ counter. 1 ml of a color-eluting solution (3% SDS in 50% isopropanol) was added to the gel strips and they were incubated at 37°C for 24 h [44]. Absorbance of the dye at 595 nm (A_{595nm}) was read in a spectrophotometer. Background values of both cpm and absorbance were obtained from regions of the gel far removed from any bands and were subtracted from the values due to the bands. Specific activities were then obtained as cpm/A_{595nm}. In some cases, autoradiograms were prepared and the autoradiograms and the Coomassie blue stained gel were scanned on a densitometer. Specific activities of each band were determined as the ratio of densities of the autoradiogram to that of the stained gel. Similar results were obtained by both procedures.

TID labels lipid in addition to protein. Therefore the purification procedures must be able to remove all labeled lipid from the protein. Control experiments were performed to determine if this was the case. Lipid vesicles in the absence of MBP were labeled similarly with TID and MBP was added after irradiation. The samples were treated similarly to those in which MBP was added before irradiation. When MBP was added after irradiation, the specific activity of the MBP band was not above background

levels indicating that the extraction and purification procedures and gel electrophoresis resulted in removal of all TID-labeled lipid and other compounds from the protein.

2.6. Photolabeling with lipid photolabels

Lipid-C1 vesicles containing the GalCer photolabels (2.8×10^6 cmp/mg lipid) were prepared as described above except that the final lipid concentration of the vesicle suspension was 0.53 mg/ml. The lipid photolabels were incorporated by combining chloroform solutions of the lipid and photolabel, evaporating the solvent under nitrogen, followed by evacuation in a lyophilizer for 2 h, before hydration with buffer and addition of C1 at a protein to lipid weight ratio of 1:4. The sample was equilibrated in the dark at room temperature for 20 min and irradiated for 30 s, using the same lamp described above with the copper sulfate filter replaced by a short wavelength cut-off filter (Oriel, Stratford, CT) to cut off wavelengths below 235 nm. The short irradiation time was found to cause no degradation of MBP. In order to determine the amount of labeled lipid which was non-covalently bound to the peptide after gel electrophoresis, a control sample of vesicles without C1 was also photosensitized. Unlabeled C1 was added to the photosensitized vesicles, using the same procedures as when adding it to lipid before photosensitization. The control samples as well as the other samples were run on gels as described below.

2.7. Quantitation of lipid photolabel bound to C1

Since C1 did not run as far as labeled lipid on the gels, we were able to separate the protein from labeled lipid by SDS-PAGE without prior extraction of the lipid. 5 μg MBP/lane was loaded onto each of three adjacent lanes on 16% tricine gels. After electrophoresis, staining, and destaining as described above, the gel was scanned in a gel scanner (White/UV Transilluminator, DiaMed Lab Supplies, ON). The band intensity was integrated using UVP Grab-It software. For elution of the radioactive protein from the gel, each lane was sliced into 14 strips keeping the three adjacent lanes loaded with the same sample together. Each strip of three lanes was placed into scintillation vials in small pieces. Gel oxidizing solution, freshly made from 19 parts of 30% H_2O_2 and 1 part of 14.8 M NH_4OH [45], was added (900 μl/vial), the vials were incubated at 37°C overnight, and the solution was neutralized by addition of 100 μl of glacial acetic acid per vial. Finally, 6 ml of scintillation cocktail was added and ^3H radioactivity was counted in a β counter. Labeled lipid appeared in strips well below the C1 band. Control samples, in which the protein was added to the labeled vesicles after irradiation, had low counts (12% or less of the values for protein added before irradiation for both PC and PG) in the gel slice containing the protein indicating that most of the non-covalently bound lipids were removed by SDS-PAGE. Specific activities were obtained (cpm/μg) for controls and samples. After subtraction of control values, the sample values were divided by the original radioactivity available per μg protein and multiplied by 100 to give % labeling values (percent of added photolabel bound to protein).

2.8. Cathepsin D digestion

TID-labeled bovine MBP (used to work out the conditions and conserve purified human C1 and C2) and human C1 and C2 were digested with cathepsin D using a modification of the procedure of Vacher et al. [22]. Photolabeling has been found to alter fragmentation patterns by preventing cleavage at modified residues [46]. We also found that labeled MBP was often cleaved less readily than unlabeled MBP and that the relative specific activities of the different peptides depended on the degree of cleavage. Therefore, only cathepsin D digests in which complete digestion of the protein was obtained, were processed further to determine the specific activity of the peptides from C1 and C2. The enzyme to protein ratio and incubation time were adjusted in order to obtain complete digestion of TID-labeled MBP. To samples of MBP (50 μg/30 μl) in 0.01 M sodium citrate buffer, pH 3.5, were added various amounts of a cathepsin D solution in the same buffer (2.5 μg/10 μl) to achieve weight ratios of cathepsin D to MBP of 1/200 to 1/25. The samples were incubated at 37°C for 4 h, frozen, and lyophilized. In order to identify the peptides, the digest was dissolved in 0.05% TFA and the peptides were sepa-

rated by HPLC on a Waters reversed phase C18 μBondapak column using an acetonitrile gradient. Fractions were lyophilized, dissolved in sample buffer, and run on tricine gels. The peptides were identified from the amino acid compositions determined by amino acid analysis as described above (not shown). Cathepsin D digests of TID-labeled protein were run on tricine gels and the specific activity of each peptide was determined either by autoradiography or by counting gel slices and eluting the dye as described above. For counting, duplicate or triplicate lanes were run and counted together. In each experiment using a new sample of vesicles, the digestion, gel electrophoresis, and determination of specific activity were repeated several times. The specific activities of peptides 44–89 and 90–170 were normalized to that of 1–43 and normalized values from all such determinations were averaged.

Cleavage of labeled C1 and C2 with cyanogen bromide to give peptides 1–21 and 22–170 and with BNPS-skatole to give peptides 1–116 and 117–170 [9,11,47], was also carried out but neither reagent gave complete cleavage, precluding use of the TID labeling data on these peptides.

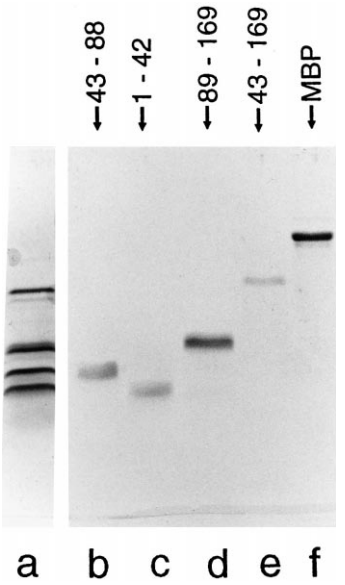

Fig. 1. Coomassie blue stained tricine gel of (a) cathepsin D total digest of bovine MBP; (b–e) HPLC fractions of cathepsin D digest; (f) undigested bovine MBP. (b) peak 1, (c) peak 2, (d) peak 3, (e) peak 4 of HPLC chromatogram shown in Fig. 2. Lane a is from a different gel than b–f.

3. Results

The ability of TID to label the purified human MBP charge isomers C1 and C2 from the lipid bilayer was determined similarly to our earlier studies with unfractionated bovine MBP containing a mixture of charge isomers [14,5]. Although MBP is a water soluble protein, it is significantly labeled by TID when bound to acidic lipid vesicles. At the lipid to protein ratios used in this study and in the gel state of the synthetic lipid DPPG, 2–6% of the total TID added is bound to MBP (Table 1). Although MBP decreases the phase transition temperature of acidic lipids, the MBP-DPPG complex is still in the gel phase at the temperature used for photolabeling [10]. In the liquid crystalline phase of natural lipids such as egg PG, the amount of TID bound to MBP

Table 1
Comparison of labeling of C1 by TID and lipid photolabels in solution and bound to acidic lipid[a]

Lipid	TID		GalCer-PL		GalCer-C8-PL	
	% photolabeling	PG/PC	% photolabeling	PG/PC	% photolabeling	PG/PC
DPPC	0.42 ± 0.22					
DPPG	3.7 ± 2.0	9.4 ± 4.6				
	$n = 5$					
Egg PC	0.15		0.07 ± 0.01		0.11	
Egg PG	0.5	3.3	1.4 ± 0.3	20.7 ± 6.6	1.8 ± 0.5	15.7
			$n = 3$		$n = 3$	

[a]Percentage of photolabel added which reacted with protein and ratio of PG to PC values. Mean ± standard deviation is shown for % photolabeling except in those cases where only one experiment was carried out. Specific activity was determined as cpm/μg protein by cutting out protein bands from gels. The difference in TID labeling between natural and synthetic lipids is similar to that which we reported earlier [14].

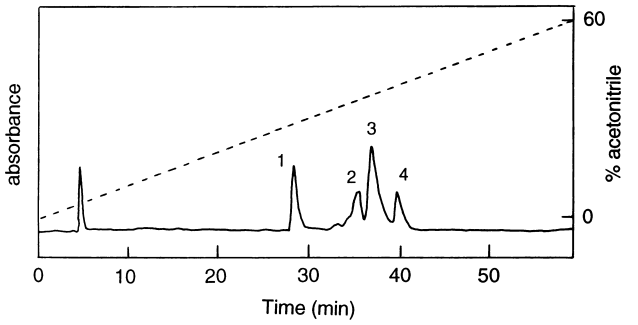

Fig. 2. HPLC chromatogram of cathepsin D digest of bovine MBP eluted with an acetonitrile gradient. Peaks 1–4 were run on the tricine gel shown in Fig. 1b–e.

is about 10% of that in gel phase lipids (Table 1 and [14]]. We have attributed this to a deeper location of TID in the bilayer in the liquid crystalline phase farther away from MBP near the surface of the bilayer [14]. If a higher ratio of lipid to protein is used, a lower percentage of TID is transferred to MBP due to greater competition by the lipid for TID. However, labeling of MBP is still significant and greater than that reported for other water soluble proteins bound to lipid bilayers [46,48].

MBP is also labeled by TID in aqueous solution in the absence of lipid (not shown). However, due to the competition by lipid for TID, the degree of labeling of MBP in the absence of lipid cannot be compared to that in the presence of lipid. In order to compare the labeling of lipid-bound MBP to that of unbound MBP in solution under similar conditions, it is compared to the labeling of MBP in the presence of DPPC or egg PC [14]. MBP does not bind to large vesicles of DPPC or egg PC [8] although it has been reported to cause fragmentation of PC bilayers [49] and to bind to monomers of PC [28] and micelles of lyso PC [16]. This could contribute to the labeling of MBP in the presence of PC. In addition, MBP may dimerize in solution [71] forming hydrophobic sites from which water is excluded, which are then labeled by TID, as found for several other proteins in solution [61,62].

MBP was labeled significantly more when bound to DPPG than when in solution in the presence of DPPC as shown for C1 in Table 1. On average, both charge isomers, C1 and C2, were labeled 5–6 times more when bound to DPPG than when in the presence of DPPC (Table 2). In natural lipids, the labeling of C1 bound to egg PG was 3 times higher than that in the presence of egg PC (Table 1). These results are consistent with our previous study [14]. This greater labeling of MBP when bound to acidic lipid must occur from within the lipid bilayer. Since any carbene generated from TID in the aqueous phase would be scavenged efficiently by water, it is considered very unlikely that lipid-bound TID labels lipid-bound protein at any site exposed to water molecules [59].

The ability of photolabels to label MBP from within the lipid bilayer was confirmed using two lipid photolabels, GalCer-PL and GalCer-C8-PL. Only liquid crystalline phase egg PC and egg PG were used because of the probability of phase separation of the lipid photolabels in gel phase lipids. 1.4–1.8% of the lipid photolabels reacted with C1 bound to egg PG vesicles, while only 0.07–0.11% reacted with the protein in solution in the presence of egg PC vesicles (Table 1). C1 bound to PG was consistently labeled about 33% more by the deeper GalCer-C8-PL than by the more shallow GalCer-PL. The much greater labeling of the protein in PG relative to that in solution by these lipid photolabels indicates that the protein is readily labeled by photolabels from within the lipid bilayer. This supports the conclusion that the labeling of the protein bound to PG by the more water soluble TID is also due to labeling from the lipid bilayer. These results indicate a close association of MBP with the bilayer. We showed earlier that MBP was labeled significantly more by TID than

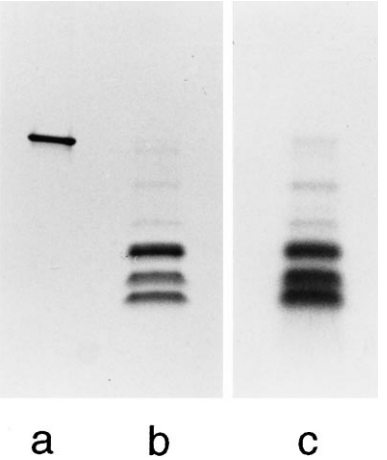

Fig. 3. Coomassie blue stained tricine gel (a,b) and autoradiogram (c) of human C1: (a) undigested unlabeled C1; (b,c) cathepsin D digest of C1 labeled with TID.

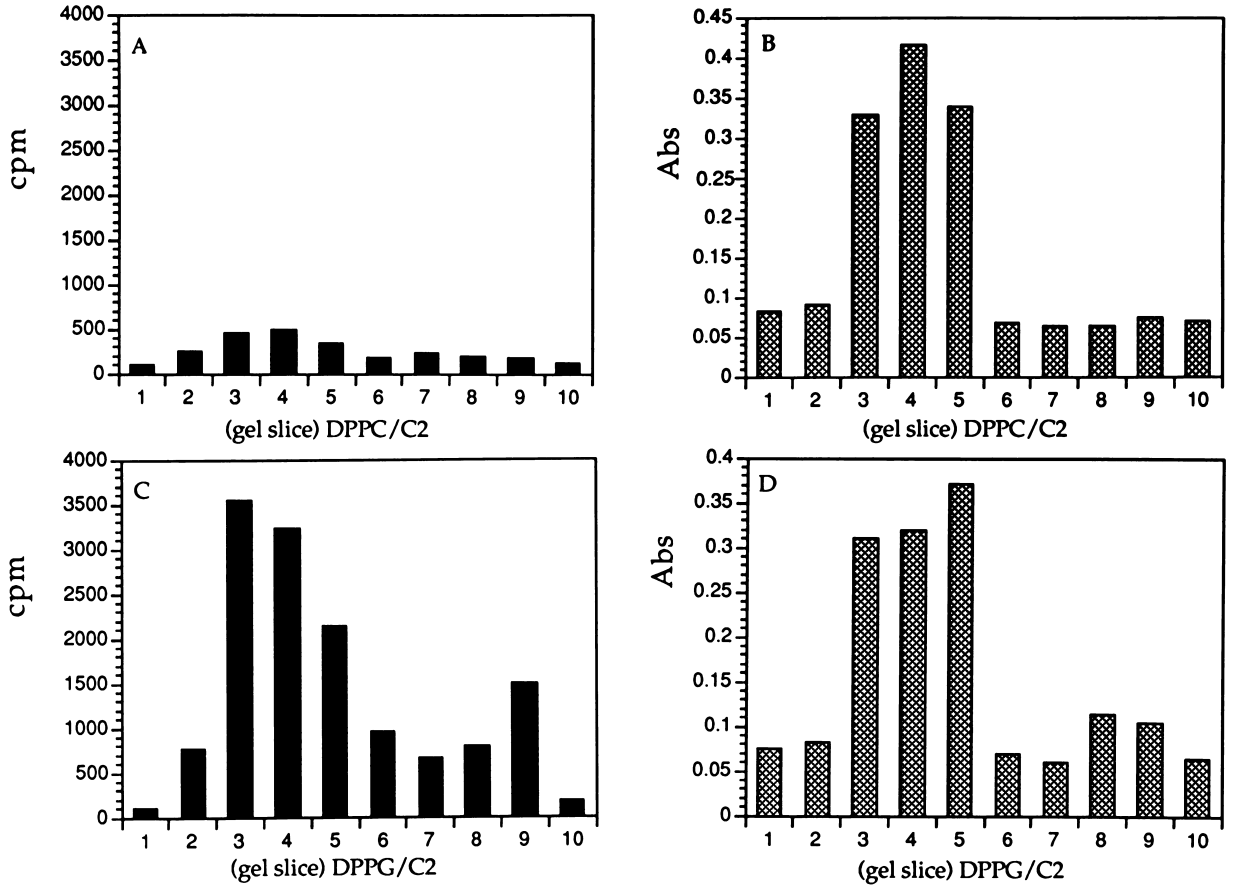

Fig. 4. TID labeling profile of cathepsin D digests of C2 (A,B) labeled in the presence of DPPC and (C,D) labeled in DPPG and run on a tricine gel. Cpm due to bound TID (filled bars, A,C) determined by counting gel slices. Absorbance (Abs) due to Coomassie blue dye eluted from gel slices (shaded bars, B,D). Gel slices are labeled 1–10 starting from the bottom of the gel and end one slice above the location of intact C2 (at band 9) since no other Coomassie blue stained bands were apparent in gels of digests or in gels of undigested MBP.

polylysine bound to DPPG indicating that this close association of MBP with the bilayer is not due simply to electrostatic binding to the bilayer, but to some degree of hydrophobic interaction of MBP with the bilayer.

The greater labeling of the protein bound to egg PG by the lipid photolabels than by TID is probably due to the lower reactivity with lipid C-H bonds of the nitrenes generated from the aryl azide lipid photolabels compared to the carbenes generated from TID [50,51]. Thus lipid does not compete with the protein for the lipid photolabels as effectively as it does for TID. The greater labeling of C1 in solution by TID in the presence of DPPC compared to egg PC is probably due to the three times greater solubility of TID in egg PC compared to gel phase DPPC [14].

Although the mean relative labeling of C2 by TID was somewhat greater than that for C1 (Table 2) the differences were not statistically significant. Furthermore, in three experiments where both C1 and C2

Table 2
Comparison of labeling of C1 and C2 by TID from lipid bilayer

Relative labeling[a]	
C1	C2
4.7 ± 1.3	6.1 ± 1.1
$n = 9$	$n = 5$

[a]Ratio of specific activity of labeled protein bound to DPPG to that of protein in presence of DPPC. Specific activities were determined from cutting out bands from gels in some cases as in Table 1, and in other cases from autoradiograms of gels. The mean ± standard deviation is shown.

were used at the same time, C2 was labeled more than C1 in only one of them. Thus there was no significant difference in labeling of C1 and C2 by TID from the lipid bilayer.

After TID labeling of bovine MBP or human C1 and C2 either bound to DPPG, in aqueous solution in the presence of DPPC, or in aqueous solution in the absence of lipid, the extracted proteins were then digested with cathepsin D in order to determine the relative labeling of different domains of the two charge isomers in different environments. Cathepsin D has been reported to cleave bovine and guinea pig MBP at two sets of Phe-Phe linkages (for bovine MBP at Phe-Phe 42–43 and 88–89 giving peptides 1–42, 43–88, and 89–169) [47,52]. Cleavage at Phe-Phe 42–43 is preferential and incomplete cleavage at Phe-Phe 88–89 results in peptide 43–169 also. Fig. 1 shows a Coomassie blue stained tricine gel of digested bovine MBP. Four bands of smaller molecular weight than MBP were obtained. In order to identify the peptides, they were separated by HPLC giving 4 peaks (Fig. 2). Each peak was run on tricine gels (Fig. 1b–e) and identified from its amino acid composition (not shown). Peak 1 was 43–88, peak 2 was 1–42, peak 3 was 89–169, and peak 4 was 43–169. It was thus possible to separate peptides 1–42 and 43–88 on the gel despite the similarity in their molecular weights. Thus SDS-polyacrylamide gel electrophoresis rather than HPLC was used to separate the TID-labeled peptides and determine their specific activities.

Human C1 and C2 were cleaved similarly to bovine MBP, giving peptides 1–43, 44–89, and 90–170 (and 44–170 for incomplete cleavage). A Coomassie blue stained tricine gel and an autoradiogram of a cathepsin D digest of TID-labeled C1 are shown in Fig. 3 and show that every peptide band is labeled. No other regions of the gel contained radioactivity. Labeling profiles for a similar gel of the cathepsin D digest of C2 labeled in DPPG and in the presence of DPPC are shown in Fig. 4. For Fig. 4, the bands were cut out and cpm determined by counting and the absorbance of the stained band was determined by dye elution. Mean values averaged from a number of experiments for the relative specific activities of the three peptides, normalized to that of 1–43, for C1 and C2 labeled in DPPG and in aqueous solution are shown in Table 3. Both charge isomers were labeled about twice as much on peptides 1–43 and 44–89 as on 90–170. Peptides 1–43 and 44–89 were labeled to a similar extent. There was no significant difference between C1 and C2 and no effect of environment on the domains labeled, even though the peptides from protein labeled in DPPG bound much more TID than peptides labeled in solution in the presence of DPPC (e.g. compare Fig. 4A and C). Thus the proteins were labeled more on the N-terminal half than the C-terminal half when bound to DPPG, when in aqueous solution in the presence of DPPC, and when in aqueous solution in the absence of lipid. In the one experiment where C1 was labeled in egg PG and in the presence of egg PC, relatively similar results were obtained for the peptide labeling pattern as in DPPG (Table 3).

Table 3
Distribution of TID in different regions of the protein for human MBP components C1 and C2 labeled from different environments

			Relative labeling[a]	
	n	1–43	44–89	90–170
C1/DPPC	15	1	0.88 ± 0.31	0.51 ± 0.16*
C1/DPPG	16	1	0.82 ± 0.21	0.48 ± 0.21*
C1 only	11	1	0.92 ± 0.20	0.36 ± 0.14*
C2/DPPC	12	1	0.88 ± 0.24	0.67 ± 0.24***
C2/DPPG	12	1	0.72 ± 0.14	0.41 ± 0.12*
C2 only	11	1	0.82 ± 0.27	0.51 ± 0.21**
C1/egg PC	2	1	1.32 ± 0.31	0.72 ± 0.22
C1/egg PG	2	1	1.06 ± 0.06	0.78 ± 0.16

[a]Labeling of different cathepsin D peptides normalized to that of 1–43. Mean ± standard deviation of *n* different determinations from several photolabeling experiments, except for egg PC and egg PG, where the mean ± range of two determinations from one photolabeling experiment is indicated. Values for 1–43 and 44–89 are significantly different from that for 90–170 at $*P < 0.005$, $**P < 0.01$, and $***P \cong 0.05$ by a 2-tailed Student's *t*-test. A similar labeling pattern for a particular type of sample was observed in every experiment.

C1 photolabeled with the lipid photolabels was also cleaved with cathepsin D. Although labeled bands appeared in the positions of peptides 1–42, 89–169, and 43–169 on the gel, there was no band in the position where 43–88 usually runs, and two new bands appeared between the positions of 89–169 and 43–169. Thus labeling by lipid photolabels may have affected migration of peptide 43–88 on the gel. Therefore, the digest of the lipid-labeled protein was not analyzed further.

4. Discussion

These results show that the hydrophobic photolabel TID and lipid photolabels react with MBP from the lipid bilayer despite the fact that it is a water soluble, highly charged membrane protein which binds to acidic lipids mainly by electrostatic interactions. Polylysine, which also binds electrostatically to lipids, is labeled by TID much less than MBP [14]. This indicates that some of the MBP amino acid side chains are accessible to the hydrocarbon region of the lipid bilayer. Although these photolabels are usually used in order to identify the transmembrane regions of intrinsic membrane proteins [29,53], they have also been shown recently to label a number of other water soluble membrane proteins and amphipathic peptides bound to lipid bilayers leading to the conclusion that these proteins interact with the lipid bilayer by some type of hydrophobic interaction [48,54–60].

Although the deeper GalCer-C8-PL labeled C1 even more than the shallow GalCer-PL, this does not necessarily mean that the protein penetrates deeply into the bilayer. Although depth-dependent labeling of proteins has been achieved using similar shallow and deep lipid photolabels [62–65], a protein lying on the surface which perturbs lipid packing, as MBP does [6–13], could cause the longer acyl chain of GalCer-C8-PL to flip up and probe the same region of the bilayer as that probed by the more shallow GalCer-PL. This might be the mechanism by which MBP restricts the motion of fatty acid chains almost their entire length, as indicated by fatty acid spin labels [8–10,13]. This would also allow both photolabels to contact residues dipping into the bilayer from protein lying on the surface of the bilayer. Labeling of MBP by these lipid photolabels was about 10 times less than that of an amphipathic peptide, magainin, [30] which is thought to form pores by inserting in a transmembrane fashion [66]. At higher concentrations, MBP causes DPPG to form an interdigitated bilayer [10]. This could also result in similar labeling of MBP by GalCer-C8-PL as GalCer-PL. However, interdigitation does not occur in the liquid crystalline phase used for measurement of photolabeling of MBP by the lipid photolabels. Thus, despite the greater labeling of MBP by GalCer-C8-PL relative to GalCer-PL, the most reasonable conclusion is that MBP probably lies on the surface of the bilayer with some hydrophobic amino acid side chains dipping into the bilayer, allowing reaction with both TID and lipid photolabels.

In this study, we have shown that although MBP bound to lipid is labeled by TID at sites in both the N and C-terminal halves, the N-terminal half is labeled more than the C-terminal half suggesting that the N-terminal half interacts hydrophobically with the lipid bilayer more than the C-terminal half. Several other studies of the intact protein bound to lipid also indicated that sites in the N-terminal half interacted hydrophobically with lipid more than the C-terminal half. MBP was protected from tryptic digestion when bound to lipid at five of 13 possible sites in the N-terminal half but was not protected at any of the ten tryptic sites in the C-terminal half [15]. Amino acids in the intact protein whose NMR resonances were broadened or shifted upon interaction with zwitterionic detergent or lipid micelles occurred more frequently in the N-terminal half [16,17,19].

A similar pattern of TID labeling was obtained for the protein in solution, although the degree of labeling of all peptides was considerably less than when the protein is labeled from the bilayer. The similar labeling pattern in solution and in a lipid environment might be consistent with a conclusion that the protein in solution takes up a tertiary or quaternary conformation which forms a hydrophobic site and that it interacts with the lipid in the same conformation. The greater labeling of the N-terminal half could then be due to its greater contribution to this hydrophobic site compared to the C-terminal half. Detailed conformational models of MBP have been proposed which contain a β-sheet composed of five strands from different regions of the protein. Both surfaces of one of these β-sheet models has hydrophobic domains [67] while the other model has a hydrophobic interior [68]. Residues from the N-terminal half and from the C-terminal half contribute equally to the five strands of β-sheet [67,68], but only the N-terminal half has two sets of hydrophobic Phe-Phe residues.

However, most studies of the interaction of MBP peptides with lipids [9,11,27,28] also indicated greater hydrophobic interaction of the N-terminal BNPS-skatole peptide than the C-terminal peptide. Peptide 38–118 binds heme similarly to MBP suggesting the

hydrophobic heme-binding site is located in the N-terminal half and that it does not depend on the presence of the C-terminal half [22]. A hydrophobic domain formed as a result of intramolecular interactions between different regions of the molecule, as in the structures mentioned above, would be disrupted on cleavage into peptides. The fact that most studies of the intact protein and studies of the isolated N- and C-terminal peptides all indicate greater hydrophobic interaction by the N-terminal half or two thirds suggest that when MBP binds to lipid, its conformation must allow the N- and C-terminal portions of the protein to interact independently with the bilayer.

A predicted model having a tertiary structure consistent with this suggestion has recently been presented by Ridsdale et al. [69] to accommodate the β-strands of the Stoner and Martinson models in addition to the dimensions of a structure of MBP obtained from three-dimensional reconstruction of electron microscopical data of MBP bound to a lipid monolayer [70]. The predicted model differs from the previous models in the strand order and direction and location of the loops containing α-helical domains. It could allow fairly independent interactions of the N-terminal two thirds and C-terminal one third, corresponding to the BNPS-skatole peptides, with lipid, particularly for the N-terminal portion which contains four of the five predicted β-strands and one of the α-helical domains, while the C-terminal portion contains one β-strand and one α-helical domain. The β-strands contain both hydrophobic and basic residues. The highest hydrophobicity of MBP occurs in regions 14–21, 36–46, 85–96, and 148–154 [27], all of which are primarily the residues of four of the predicted β-strands. If the β-sheet structure lies flat on the surface of the bilayer, it could interact with lipid through electrostatic interactions and its hydrophobic side chains could dip into the lipid bilayer. Thus both TID and the lipid photolabels could be reacting with hydrophobic side chains of protein bound to the bilayer surface. The N terminus also is bound to an acyl chain of length varying from two to ten carbons, with four to six predominating [72]. This may also contribute to greater hydrophobic interaction of the N-terminal half. However, we found that TID labeling of peptide 1–43 was similar to that of 44–89.

The pattern and degree of labeling of C2 was similar to that of C1. C2 has been reported to be deamidated at Gln-101 or Gln-146 [31] and possibly also at Asn-90 [33]. This would be expected to make the C-terminal half more hydrophilic but would not affect the N-terminal half. Since the C-terminal half interacts less hydrophobically with the bilayer, an increase in its hydrophilicity might be expected to have little effect on the labeling of MBP by TID. The greater participation of the N-terminal half in hydrophobic interactions may also explain why C2 had a similar ability as C1 to increase the permeability of lipid vesicles [36].

In summary, we have shown that the N-terminal half of MBP interacts hydrophobically with lipid more than the C-terminal half by using a more direct technique than has been used previously and by studying the interaction of the intact protein with lipid rather than peptides. The similar TID labeling pattern of the lipid-bound protein to that of the protein in aqueous solution indicates that any tertiary structure adopted by the protein must be relatively similar in both environments. The consistency of these results on the intact protein with earlier conclusions arrived at by studying the interactions of large MBP peptides with lipid suggests further that the conformation taken up by the protein when bound to lipid must allow each half of the protein to interact independently with the bilayer. Since the C-terminal half interacts hydrophobically with lipid less than the N-terminal half, deamidation of Gln or Asn in the C-terminal half of MBP, as occurs in charge component C2, would therefore not be expected to have much effect on its hydrophobic interaction with lipid, as was found. It is possible that the protein may behave differently in situ in myelin. Interaction of MBP with other proteins in myelin in addition to lipid may affect its conformation and the way it interacts with lipid.

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