Acylation of Myelin Basic Protein Peptide 1–21 with Alkyl Carboxylates 2–10 Carbons Long Affects Secondary Structure and Posttranslational Modification†

M. Costentino, L. Pritzker, C. Boulias, and M. A. Moscarello*

Biochemistry Research, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada

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ABSTRACT: A peptide consisting of the first 21 residues of human myelin basic protein (MBP) was synthesized. The N-terminal alanine of portions was blocked in separate experiments with alkyl carboxylates varying in size from 2 to 10 carbon atoms. The effects of these different alkyl carboxylates at the N-terminus on the secondary structure was studied by circular dichroism (250–190 nm). In water, the spectra of the unblocked peptide suggested unordered structure with large negative ellipticities at 198 nm. Addition of an acetyl group altered the magnitude of $[\theta]_{198}$ from -21856 ± 2319 to -11095 ± 1000 deg cm^2 dmol^{-1}, suggesting a significant increase in ordered structure. When peptides with longer alkyl carboxylates, acylated at the N-termini, were studied, the magnitude of θ_{198} approached that of the unblocked peptide but greater negative ellipticities were observed for the C_8 and C_{10} alkyl carboxylates. The θ_{222} values were generally low (-1803 ± 463) but increased with increasing length of the alkyl carboxylate to about -3200 deg cm^2 dmol^{-1}, suggesting that little α-helical structure was present. The spectra were also taken in lipid-mimetic solvents, including 2-propanol, methanol, and lysophosphatidylglycerol (lysoPG). In general the θ_{198} and θ_{222} values were suggestive of increased structure in these environments compared to water. In 90% 2-propanol the θ_{198} of the unblocked peptide did not change when an acetyl group was added to the N-terminus (9088 compared to 8477 deg cm^2 dmol^{-1}). Addition of longer alkyl carboxylates correlated with larger, negative ellipticities. The θ_{222} values became more negative as the length of the alkyl carboxylates increased from 2 to 10 carbon atoms. Large changes in both spectral shape and molar ellipticities were found in 95% 2-propanol. Most notable were large negative ellipticities at θ_{222} suggestive of the presence of segments of α-helical structure, especially with the 4-carbon alkyl carboxylate, which showed the largest negative ellipticities at θ_{222} with prominent minima at 208 and 222 nm. The spectra in lysoPG showed less negative ellipticities at 198 nm and more negative ellipticities at 222 nm as the concentration of lysoPG was increased from 0 to 1000 μM. Since this peptide (1–21) contains the inducing site for the disease experimental allergic encephalomyelitis in the mouse, modulation of the immunological response by secondary structure is suggested either directly as a result of changes in structure or indirectly via effects of posttranslational modifications.

The N-terminus of many membrane proteins has been shown to be blocked, rendering the protein refractory to N-terminal sequencing. In many cases the blocking group is acetate, although a number of proteins are known which carry myristate on an N-terminal glycine residue. These myristoylated proteins include p60^{src}, cyclic AMP dependent protein kinase, the B subunit of the phosphatase calcineurin, NADH cytochrome b_5 reductase, and several mammalian retroviral proteins (Aitken et al., 1982; Carr et al., 1982; Henderson et al., 1983; Schulz & Orozlan, 1983; Ozols et al., 1984). Myristoylation of lyozyme (a soluble basic protein) converted it into a substrate for protein kinase C (PKC) because the presence of the fatty acyl group on the N-terminus allowed the substrate to associate with the phospholipid vesicles (Edashige et al., 1992).

The important role of the N-terminus in protein function has been suggested by four publications in recent months. In the first publication from our laboratory, the N-terminus of myelin basic protein (MBP) from human brain was found to be heterogeneous (Moscarello et al., 1992). Whereas the N-terminal alanine of MBP was considered to be acetylated originally (Hashim & Eylar 1969; Carnegie, 1969), a reinvestigation (Moscarello et al., 1992) has demonstrated that the N-terminal alanine was acylated with C_2, C_4, C_6, C_8, and C_{10} alkyl carboxylic acids. These studies represent the first demonstration that the N-terminus of a protein was heterogeneously acylated. Subsequently, three papers appeared in which proteins of the rod outer segment (ROS) membrane were shown to be heterogeneously acylated. In the first of these the amino terminus of retinal recoverin was shown to be acylated by a small family of fatty acids including myristoleate [14:1], [14:0], [14:2], and [12:0] (Dizhoor et al., 1992). In the second, rod transducin was also shown to be heterogeneously fatty acylated with lauroyl [C:12], myristoyl [C14:0], *cis*-C^5-tetradecanoyl [C14:1], or *cis,cis*-C^5,C^8-tetradecadienoyl [C14:2] fatty acids (Neubert et al., 1992). These authors suggested that the role of the N-terminal fatty acids was in protein–protein, rather than in protein–lipid, interactions (Neubert et al., 1992). Subsequently, a role for the acylated (myristoylated) N-terminus in the Ca^{2+}-dependent binding of recoverin to the membrane has been demonstrated (Dizhoor et al., 1993). Because the α-subunit of rod transducin was eluted from the membrane in the absence of detergent, they concluded that it behaved like a peripheral membrane protein. MBP is also eluted without detergent, so it is unlikely that the acylated N-terminus anchors this protein in the myelin membrane.

The fourth communication confirmed the heterogeneous N-terminus of the α-subunit of transducin, Tα, and showed that it had functional significance (Kokame et al., 1992). These authors demonstrated that the nature of the N-terminal

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blocking group influenced both the GTPase activity of transducin and its ability to be ADP-ribosylated. The tryptophan fluorescence emission spectrum of recombinant recoverin was shown to be insensitive to Ca^{2+} (Ray et al., 1992). However, the myristoylated recombinant protein was red shifted in the presence of Ca^{2+}, similar to the emission spectrum obtained with retinal recoverin. Since the three tryptophans were located at positions 31, 104, and 156, myristoylation of the N-terminus probably induced a conformational change affecting the environment(s) of one or more of the tryptophans (Ray et al., 1992). Substitution of the N-terminal Gly by Ala of Pr 55^{gag} polyprotein precursor of human immunodeficiency virus (HIV-1) prevents *N*-myristoylation and inhibits viral replication (Gottlinger et al., 1989; Bryant & Ratner, 1990). An important role for N-terminal myristoylation is suggested by these studies.

The importance of the N-terminal blocking group in the induction of experimental allergic encephalomyelitis (EAE), the animal model of multiple sclerosis in the human, has been studied in the mouse. EAE was induced in these animals with the N-terminal peptide consisting of residues 1–9 (the least number of amino acids required for activity) of MBP only if the N-terminus was acetylated. The unblocked peptide was inactive (Zamvil et al., 1986). Since EAE is an autoimmune disease, these studies suggested that blocking the N-terminus modulated the immune response. With our report (Moscarello et al., 1992) that the N-terminus of MBP consists of alkyl carboxylates 2–10 carbons long, of which the 4- and 6-carbon acyl groups form the major species, it is tempting to consider that they play an important role in modulating the immune response in EAE, possibly by affecting the secondary structure of active peptides.

In order to study the effect on secondary structure of the N-terminus of MBP, we synthesized the amino-terminal peptide 1–21 because this peptide had been obtained by cyanogen bromide cleavage of MBP at methionine 21 in the original studies (Moscarello et al., 1992) and it contained an ADP-ribosylation site and several sites of phosphorylation. The amino terminus was left unblocked or was blocked with a C_2, C_4, C_6, C_8, or C_{10} alkyl carboxylate. Each synthetic peptide was purified by HPLC, and the structures were confirmed by FABMS. We studied the effect of the various N-terminal blocking groups on the secondary structure as determined by circular dichroism. Since Ser^7 represents a principal site of phosphorylation in MBP and Ser^{12} a second more labile site when a Ca^{2+}- and lipid-dependent protein kinase C from brain is used, we were able to correlate conformational factors with phosphorylation at these sites (Ramwani & Moscarello, 1990). Peptide 1–21 also contains a site for ADP-ribosylation by cholera toxin at Arg^9 (Boulias & Moscarello 1993). Conformational factors were also correlated with this enzymatic reaction.

MATERIALS AND METHODS

Peptide 1–21 (sequence: ASQKRPSQRHGSKYLAT-ASTM) was synthesized by the solid-phase method in a Biolynx 4170 Crystal peptide synthesizer (Nova Biochem, Cambridge, U.K.) using Fmoc chemistry with counterion distribution monitoring (CDM). The synthesis was carried out on 1 g of Novasyn KA-100 (Nova Biochem) (1.0 g equiv per 0.1 mmole of peptide). The Fmoc group on the N-terminal alanine was removed in a Biolynx. The peptide–Novasyn was washed with dimethylformamide twice followed by three ether washes, dried under vacuum in a dessicator, and divided into 5 equal parts. Each portion was treated separately as follows:

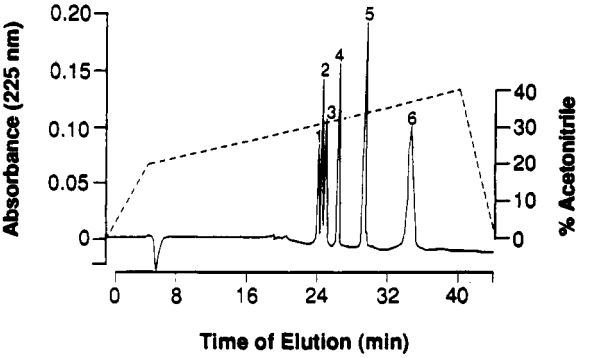

FIGURE 1: HPLC profile of a mixture of synthetic peptides resolved on a C:18 reversed-phase column. From left to right the peptides are as follows: 1, unblocked 1–21; 2, Ac 1–21; 3, Bu 1–21; 4, Hx 1–21; 5, Oc 1–21; 6, Dc 1–21.

(*a*) *The Peptide with a Free N-Terminus.* Cleavage and simultaneous deprotection of the peptide was carried out in trifluoroacetic acid (TFA) (12 mL), containing thioanisole (2.0 mL), *m*-cresol (0.2 mL), ethanedithiol (1.0 mL), ethyl methyl sulfide (1.5 mL), and trimethylbromosilane (2.0 mL) at 0 °C for 20 min. The resin support was removed by suction through a sintered glass filter, and the filtrate was dried by rotary evaporation. Equal volumes of diethyl ether and 0.1% TFA were added to the residue and shaken. The aqueous layer containing the peptide was washed with diethyl ether three times. The peptide solution was lyophilized and desalted on Sephadex G10 in 0.1% TFA. An aliquot was used to check purity on a reversed-phase HPLC (Waters) with a μ Bondapak C:18 column in 0.1% TFA/acetonitrile solvent. The HPLC profile of all the peptides is shown in Figure 1.

(*b*) *Acylation with Acetic Anhydride.* The resin-bound peptide was soaked with a minimum volume of DMF for 60 min and then treated with acetic anhydride (20 equiv) and 4-(*N*,*N*-dimethylamino)pyridine (DMAP; 5 equiv) at room temperature for 60 min. The resin-bound peptide was washed twice sequencially in DMF, *tert*-amyl alcohol, glacial acetic acid, *tert*-amyl alcohol, and diethyl ether. The solids were vacuum dried in a dessicator. The cleavage, deprotection, and isolation of the peptide were carried out as for the unblocked peptide (a, above). Acylation of other aliquots of the peptide was carried out essentially as described for acetic anhydride, using butyric, hexanoic, or decanoic anhydride. Acylation with octanoic acid was carried out with diisopropylcarbodiimide (DIC; Pierce Chemicals) as follows: octanoic acid (40 equiv) and DIC (20 equiv) were mixed in DMF and shaken for 30 min at room temperature to produce the anhydride *in situ*, which was then used immediately for acylation, as described above.

Each peptide was purified on a C:18 Polymer Lab Reverse Phase-S (300 Å) column using a Waters 600 multisolvent delivery HPLC system. The sequence of the unblocked peptide was determined on a Porton sequencer with an on-line microbore HPLC system. All peptides were subjected to FAB mass spectrometry as described earlier for the natural peptides (Moscarello et al., 1992).

Circular dichroic (CD) spectra were measured on a Jasco-720 spectropolarimeter over the range of 250–190 nm. Four spectra were accumulated over 2500 data points using the following conditions: sensitivity, 20 mdeg; response, 1 s; scanning speed, 50 nm/min; path length, 0.1 cm at 25 °C. Mean residue ellipticities, θ, in deg cm^2 dmol^{-1} were calculated using the equation $\theta = \theta_{obs}(MRW)d^{-1}c^{-1} \times 10^{-1}$, where θ_{obs} is the ellipticity measured in degrees, MRW is the mean residue weight taken as 108.9, c is the concentration of peptide, and d is the optical path length (0.1 cm). Calibration was done

in 0.06% ammonium-*d* 10-camphorsulfonate at 29.5 nm. In all cases the concentration of peptide was determined by amino acid analyses on a Waters Pico Tag system after hydrolysis in vacuo for 24 h in 5.7 N HCl. All lysoPG spectra were obtained in 5 mM HEPES buffer, pH 7.4.

ADP-Ribosylation. ADP-Ribosylation of each peptide was carried out with cholera toxin as follows: Cholera toxin was activated with 20 mM dithiothreitol (DTT) in 2.5 mM potassium phosphate buffer, pH 7.5, at 30 °C for 30 min. The incubation mixture contained 25 μg of peptide, 10 mM thymidine, 1 mM ATP, 100 μM GTP, 400 μM ADP-ribose, 2.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.5 mM [^{32}P]-NAD^+ (1 μCi/assay), and 5 μg of preactivated cholera toxin in 100 mM potassium phosphate buffer, pH 7.5, in a total volume of 100 μL and was incubated at 37 °C for 90 min. After incubation, the samples were lyophilized and dissolved in 100 μL of deionized water, and peptides were isolated from the incubation mixture by reversed-phase HPLC using a Super Pak C:8 column (LKB/Pharmacia) in 0.05% TFA and a linear acetonitrile gradient (0–45%) over 45 min. The radioactive peptides were collected; an aliquot of the peptides was used to measure radioactivity, and a second aliquot was used for amino acid analysis after hydrolysis in 5.7 N HCl in the gas phase for 24 h. The amino acid mixture was resolved on a Waters Pico Tag system from which the concentration of the peptide was obtained. The specific activity was expressed as moles of ADP-ribose/mole of peptide.

To determine rates of ADP-ribosylation, aliquots (7 μL) were removed from the incubation mixture at different times and added to 7 μL of cold 10% trichloracetic acid (TCA). The entire 14 μL was spotted on Immobilon membranes (7 × 7 mm), which were then air dried, wetted with methanol, dropped into a beaker containing 500 mL of 5% TCA, and stirred for 20 min. This wash procedure was repeated two more times. The radioactivity of the peptide was determined on a portion of the membrane. A second portion was used for amino acid analysis as described above.

Phosphorylation. Phosphorylation of peptides was done with a protein kinase C (PKC) isolated from human white matter, purified 2000-fold. The incubation mixture contained 30 μg of peptide in 20 mM Tris buffer, pH 7.5, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 50 μM ATP (1 × 10^6 cpm [γ-^{32}P]ATP); vesicles containing 25 μg of phosphatidylserine; and 2 μg of diolein in a total volume of 250 μl at 30° for 60 min. The reaction was stopped by freezing in a dry ice/acetone mixture, and the reaction mixture was delipidated by extraction with ether. The phosphorylated peptides were separated from the reagents of the incubation by HPLC as described above for the ADP-ribosylated peptides. A portion of each radioactive peak was used to determine radioactivity, and a second portion was used for amino acid analysis from which the concentration of peptide was determined. The specific activity was expressed as moles of PO_4/mole of peptide.

Isolation of Phosphorylated Peptides. Each phosphorylated peptide was digested with trypsin at an enzyme/peptide ratio of 1:50 (w/w) in 0.1 M ammonium carbonate and 2 mM $CaCl_2$ at 37 °C for 48 h. Each digest was fractionated on a Pharmacia Super Pak Pep S C:2/C:18 reversed-phase column. The column was equilibrated with 0.05% TFA and eluted with a 0–60% acetonitrile gradient. The radioactive peaks were collected for counting and for amino acid analyses to identify the peptides.

RESULTS

HPLC Profile of Peptides. Aliquots of the purified peptides were mixed together and injected onto the C:18 column (Figure 1). All peptides were resolved in order according to the nature of the N-terminal blocking groups as follows from left to right: unblocked, C_2, C_4, C_6, C_8, and C_{10} alkyl carboxylates.

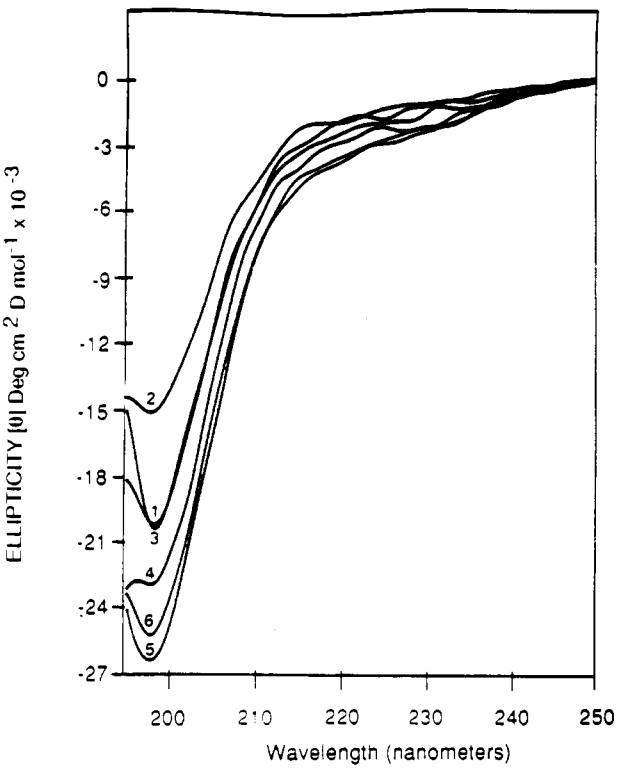

FIGURE 2: Circular dichroism of peptides in water from 250 to 190 nm: 1, unblocked 1–21; 2, Ac 1–21; 3, Bu 1–21; 4, Hx 1–21; 5, Oc 1–21; 6, Dc 1–21.

Table 1: Mean Residue Ellipticities of All Peptides in Water[a]

peptide	mean residue ellipticity (deg dm^2 dmol^{-1}) θ_{198}	θ_{222}
1–21	21 856 ± 2319	1803 ± 463
Ac 1–21	11 095 ± 1000	1854 ± 436
Bu 1–21	19 913 ± 481	2342 ± 514
Hx 1–21	22 893 ± 3160	2586 ± 312
Oc 1–21	26 150 ± 2873	3253 ± 158
Dc 1–21	24 463 ± 1153	3195 ± 107

[a] The unblocked peptide 1–21 represents the mean ± SD of five independent spectra. All others represent the mean ± SD of four independent spectra. [b] Ac = acetyl; Bu = butyl; Hx = hexyl; Oc = octyl; Dc = decyl.

Circular Dichroism. The spectra of all the peptides in water are collected in Figure 2, which represents a single set of spectra only so that the absolute values obtained from the figure may not correspond to those in Table 1. The values in Table 1 represent the means and standard deviations of four or five sets of spectra. When compared to the unblocked peptide, the acetyl-blocked peptide showed the greatest amount of secondary structure as determined by the change in the θ_{198} values from −21 856 ± 2319 to −11 095 ± 1000 deg cm^2 dmol^{-1} (Table 1). With the C_4-acylated peptide the θ_{198} was similar to that of the unblocked peptide. The C_6-, C_8-, and C_{10}-acylated peptides showed increased negative ellipticities with the C_8 showing the greatest negative ellipticity, suggesting that this peptide contained the least amount of ordered structure. Although the θ_{222} values were small, they increased from −1803 ± 463 to −3195 ± 107 deg cm^2 dmol^{-1}, no significance is attached to the $[\theta]_{222}$ values.

The mean residue ellipticities of the spectra (spectra not shown since the shapes of the spectra were similar to those

Table 2: Mean Residue Ellipticities of Peptides in 90% 2-Propanol

peptide	mean residue ellipticity (deg cm^2 dmol^{-1}) θ_{198}	θ_{222}
1–21	9088	4100
Ac 1–21	8477	3246
Bu 1–21	10 190	4111
Hx 1–21	7598	5771
Oc 1–21	19 000	5450
Dc 1–21	14 940	5252

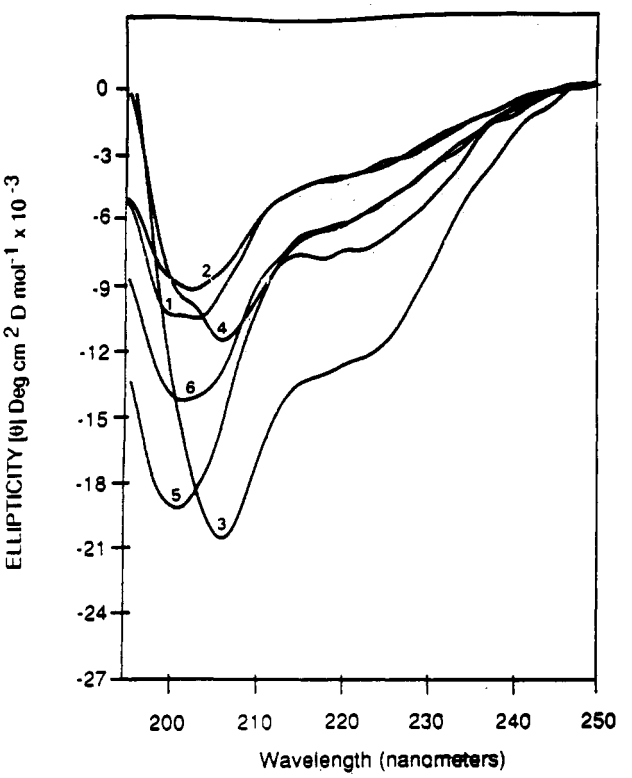

FIGURE 3: Circular dichroism from 250 to 190 nm of peptides in 95% 2-propanol: 1, unblocked 1–21; 2, Ac 1–21; 3, Bu 1–21; 4, Hx 1–21; 5, Oc 1–21; 6, Dc 1–21.

in water) obtained in 90% 2-propanol are collected in Table 2. All ellipticities at 198 nm were less negative than those obtained in water, suggesting an increase in secondary structure in this solvent. The values of the C_8- and C_{10}-blocked peptides were changed less than the others (*i.e.*, showed less ordered structure). The ellipticities at 222 nm, although still small, were more negative than the values obtained in water for all peptides.

Since membrane proteins and peptides reside in lipid–hydrocarbon environments, CD spectra were obtained with each peptide dissolved in 95% 2-propanol, a lipid-mimetic solvent. These are shown in Figure 3. Increasing the 2-propanol concentration from 90 to 95% resulted in large changes in the shape of the spectra. The most notable changes were represented by the large negative ellipticities at 222 nm especially for the C_4- and C_6-blocked peptides. The largest spectral change was observed when the N-terminus was blocked with a 4-carbon alkyl carboxylate showing prominent minima at 208 and 222 nm suggestive of the presence of α-helical structures (curve 3, Figure 3).

The spectra of the different peptides were obtained in 14 concentrations of 2-propanol from 0 to 95% (data not shown). In the case of the unblocked and the acetylated peptides, the θ_{198} values changed gradually from about –20 000 to –10 000 deg cm^2 dmol^{-1} in an almost linear fashion. The curves for

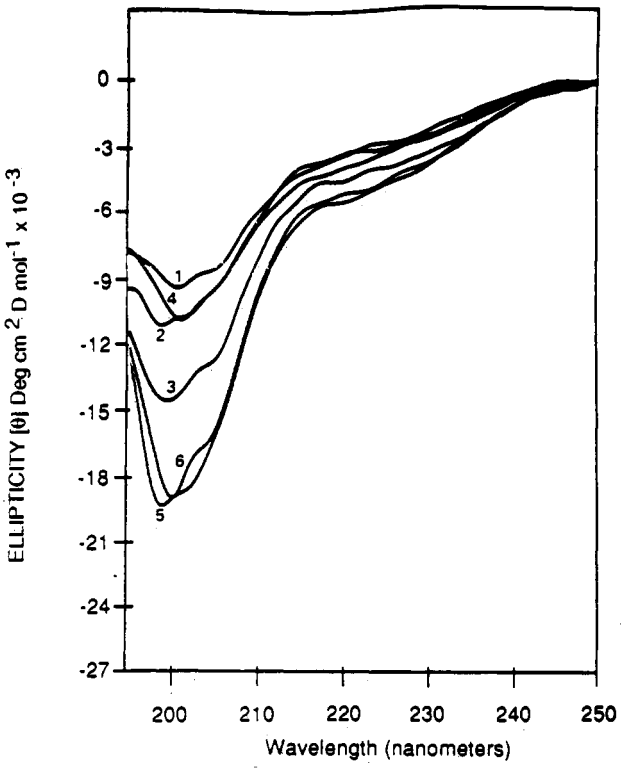

FIGURE 4: Circular dichroism of peptides in 1 mM lysophosphatidylglycerol from 250 to 190 nm: 1, unblocked peptide; 2, Ac 1–21; 3, Bu 1–21; 4, Hx 1–21; 5, Oc 1–21; 6, Dc 1–21.

the 6- and 8-carbon alkyl carboxylates also changed gradually from 0 to 95% 2-propanol, but the ellipticities were generally more negative, *i.e.*, the θ_{198} value in 0% 2-propanol was about –25 000 deg cm^2 dmol^{-1} for both, which decreased slowly to about –20 000 deg cm^{2}dmol^{-1}.

The θ_{222} values for the peptides remained constant up to about 70% 2-propanol, increased gradually up to 90%, and then increased sharply from 90 to 95% 2-propanol, suggesting a cooperative change in conformation of all peptides in high 2-propanol concentrations. Similar curves were obtained in various concentrations of methanol; since they were qualitatively similar to the curves in 90% 2-propanol, they are not reproduced here. Therefore, in the lipid-mimetic solvents used, the largest spectral changes were observed at the highest concentrations used.

The spectra in 1000 μM lysophosphatidylglycerol (lysoPG) are shown in Figure 4, and the mean residue ellipticities, θ_{198} and θ_{222}, are shown in Figure 5 at various concentrations of lysoPG. The θ_{198} values for the unblocked peptide and the C_2- and C_6-blocked peptides change in a linear fashion from –11 000 to –15 000 deg cm^2 dmol^{-1} at 0% lysoPG to –8000 to –11 000 deg cm^2 dmol^{-1} at 1000 μM lysoPG. Therefore, with increasing concentration of lysoPG the ellipticities at 198 nm became less negative for each peptide. At all concentrations, the θ_{198} values of the C_4- C_8-, and C_{10}-blocked peptides were more negative than those of the unblocked or the C_2- or C_6-blocked peptides. The ellipticities at 222 nm became more negative (Figure 5B) for all peptides with increasing concentration of lysoPG.

LysoPG is an anionic lipid which binds to basic proteins and peptides primarily by electrostatic interactions. The data suggest that the unblocked peptide, which had the least negative θ_{198} value (Figure 4) and therefore the most structure, binds more lysoPG than the octyl- and decyl-blocked peptides, which had θ_{198} values about 2-fold greater than that of the unblocked peptide. These large negative ellipticities at 198

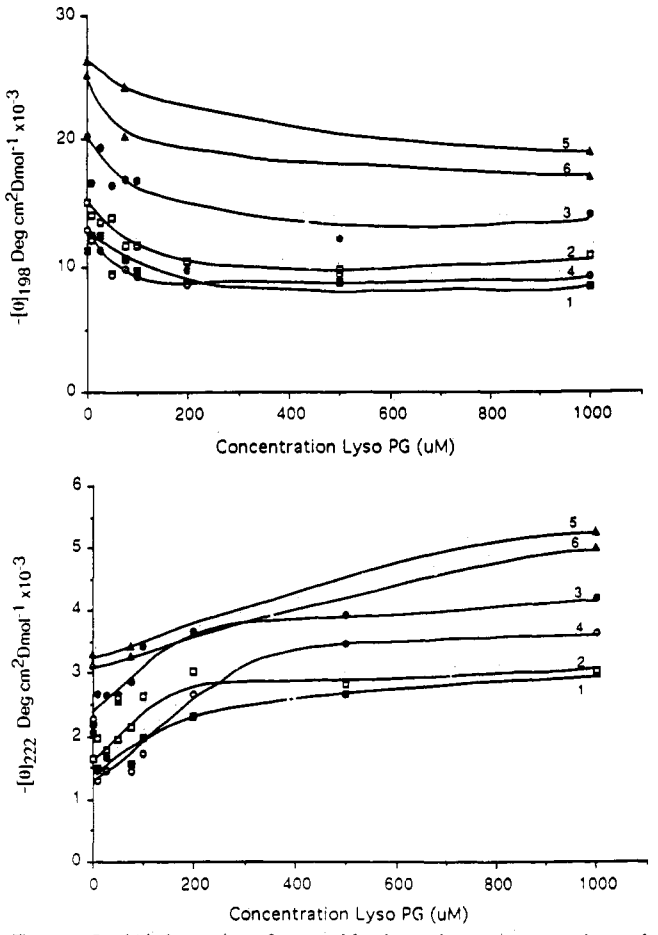

FIGURE 5: (A) θ_{198} values for peptides in various concentrations of lysoPG: 1, unblocked 1–21; 2, Ac 1–21; 3, Bu 1–21; 4, Hx 1–21; 5, Oc 1–21; 6, Dc 1–21. (B) θ_{222} values for peptides in various concentrations of lysoPG: 1, unblocked 1–21; 2, Ac 1–21; 3, Bu 1–21; 4, Hx 1–21; 5, Oc 1–21; 6, Dc 1–21.

nm are similar to the ellipticities obtained in water for these peptides (Figure 2). We concluded that the peptides with the long alkyl chains, such as 8 and 10 carbons, may interfere with lipid binding. From an examination of the sequence of the peptide (Table 4), Lys at position 4 and Arg at postion 5 in addition to loss of the N-terminal amino group could represent lipid-binding sites affected by the N-terminal alkyl carboxylate. Since Pro at position 6 may induce a bend in the molecule, Arg at position 9 could also be affected. Lys at position 13 would remain as the major binding site for the lipid.

ADP-Ribosylation. Each peptide was ADP-ribosylated with [^{32}P]NAD in the presence of cholera toxin as described in Materials and Methods (Table 3). The lowest specific activity (0.47 mol of ADP ribose/mol of peptide) was obtained when the alkyl carboxylate was 10 carbons long, while the highest specific activity (0.97 ± 0.06 mole of ADP-ribose/mol of peptide) was obtained when the alkyl carboxylate was 6 carbons long. The rate of ADP-ribosylation generally paralleled the specific activity except that the rate when the alkyl carboxylate was 6 carbons long was almost 3-fold greater than when it was 10 carbons long.

Since ADP-ribosylation of myelin basic protein with cholera toxin was found to occur on arginyl residues (Boulias & Moscarello, 1993), it was necessary to determine which of the two arginyl residues (Arg^5 or Arg^9) was ADP-ribosylated. To do this, the peptide with the highest specific activity was digested with endoproteinase Lys C (1:50, w/w) in 1% ammonium bicarbonate, pH 8.0, at 37 °C for 42 h. The digest was lyophilized and dissolved in water, and the peptide was isolated from the reagents by HPLC on a C:18 Super Pak (LKB/Pharmacia) reversed-phase column. A single radioactive peptide was obtained, which was identified as residues 5–13 by amino acid analysis, with a specific activity of 0.86 mole of ADP-ribose/mol of peptide. Automated sequence analysis yielded R^{5}PSQ in good yield, and we concluded that ADP-ribosylation occurred at Arg^9, the same site ADP-ribosylated in the intact protein. To confirm this assignment, a second aliquot of the same radioactive peptide was bound to the polybrene membrane and divided into two equal portions after the first cycle in the sequencer, *i.e.*, after removal of Arg^5. One half was used to continue the sequence, which confirmed RPSQ, the first four amino acids of peptide 5–13. The other half of the membrane was used to measure the radioactivity, which was 3483 cpm. Since a total of 7961 cpm was bound to the membrane originally, recovery of 3483 from half the membrane represented an 88% recovery of radioactivity associated with peptide 6–13. ADP-ribosylation must have occurred at Arg^9, the same site ADP-ribosylated in the first 21 residues of the intact protein.

Table 3: ADP-Ribosylation and Phosphorylation of MBP Peptide 1–21 N-Terminally Blocked with Various Alkyl Carboxylates

peptide	no. of carbon atoms at N-terminus	ADP-ribosylation sp act.[a]	ADP-ribosylation rate[b]	phosphorylation sp act.[c]
1–21	0	0.56[d]	0.059	0.73 ± 0.022
$CH_3-\overset{O}{\overset{|}{C}}-N-$1–21	2	0.76	0.054	0.55 ± 0.013
$CH_3(CH_2)_2-\overset{O}{\overset{|}{C}}-N-$1–21	4	0.72	0.075	0.95
$CH_3(CH_2)_4-\overset{O}{\overset{|}{C}}-N-$1–21	6	0.97 ± 0.06	0.075	0.94
$CH_3(CH_2)_6-\overset{O}{\overset{|}{C}}-N-$1–21	8	0.77	0.042	0.94
$CH_3(CH_2)_8-\overset{O}{\overset{|}{C}}-N-$1–21	10	0.47	0.028	1.40

[a] Specific activity = (mol of ADP-ribose)/(mol of peptide). [b] Rate = (nmol of ADP-ribose transferred)/(μg of peptide)/min. [c] Specific activity = (mol of PO_4)/(mol of peptide). Five independent determinations were obtained for peptide 1–21 and acetylated 1–21. For all other peptides, the values represent the means of two independent determinations, which varied by less than 3%. [d] Mean of two determinations for 0, 2, 4, 8, and 10 carbon alkyl carboxylates, which varied by less than 10%. For the 6-carbon alkyl carboxylate the mean and standard deviation of four independent determinations is reported.

Table 4: Identification of Sites Phosphorylated on the Peptides Acylated with Different Alkyl Carboxylates[a]

no. of carbon atoms at N-terminus	mol of PO_4/mol of peptide			
	1–4	5–9	10–13	14–21
0	0.16[b]	0.34	0	0
2	0	0.25	0.11	0
4	0	0.42	0.30	0
6	0	0.27	0.76	0
8	0	0.28	0.89	0
10	0	0.11	0.95	0

[a] Sequence of peptide 1–21 where X = alkyl carboxylate and arrows show tryptic sites:

X—^{1}ASQKRPSQRHGSKTLATASTM^{21}

No digestion occurred at R5 because of the adjacent P. [b] The value 0.16 represents an underestimate of the specific activity. Peptide 1–4 was eluted near the beginning of the chromatogram. The amino acid analyses revealed small amounts of D, G, T, Y, C, and H. When corrected, the specific activity of 1–4 became 0.27 mol of PO_4/mol of peptide.

Phosphorylation. The phosphorylation of peptide 1–21 is also shown in Table 3. The specific activity of the unblocked peptide was 0.73 ± 0.022 mol of PO_4/mol of peptide; the specific activity decreased slightly to 0.55 ± 0.013 mol of PO_4/mol of peptide when the N-terminus was acetylated and then rose to a maximum of 1.4 mol of of PO_4/mol of peptide when the alkyl carboxylate was 10 carbons long. Because peptide 1–21 contains four seryl and three threonyl residues, phosphoamino acid analyses were done, which showed that only seryl residues were phosphorylated.

To obtain the sites phosphorylated, each peptide was digested with trypsin (1:50, w/w) in 0.1 M ammonium carbonate, pH 8.0, at 37 °C for 24 h. The resulting peptides 1–4, 5–9, 10–13, and 14–21 were isolated by HPLC on a Super Pak Prep S C:2/C:18 reversed-phase column. Because of the Pro adjacent to Arg[5], no digestion was obtained at the carboxyl end of this Arg. The specific activity of each of the peptides is shown in Table 4. Peptide 1–4 was only phosphorylated in the unblocked peptide and only to a small extent (0.16 mol of PO_4/mol of peptide). Although peptide 5–9 was phosphorylated in all samples, maximum phosphorylation was obtained with the 4-carbon alkyl carboxylate blocked N-terminus and the least with the 10-carbon blocking group. Peptide 10–13 was not phosphorylated when the N-terminus was unblocked but became a progressively better substrate for protein kinase C as the length of the alkyl carboxylate increased to 10 carbons. The specific activity increased from 0 to 0.95 mol of PO_4/mol of peptide, demonstrating a marked effect of the N-terminal blocking group on phosphorylation at Ser[12].

DISCUSSION

The present investigations arose out of our earlier studies in which we demonstrated that the N-terminal alanine of myelin basic protein (MBP) was not solely acetylated as originally described but was in fact heterogeneously acylated with C_2, C_4, C_6, C_8, and C_{10} alkyl carboxylates (Moscarello et al., 1992) with C_4 and C_6 being the major species. Since this report, heterogeneous acylation at the N-terminus of recoverin and transducin with C_{12} and C_{14} saturated and unsaturated fatty acids has been reported (Dizhoor et al., 1992; Neubert et al., 1992; Kokame et al., 1992). Since the retina is considered part of the central nervous system, heterogeneous N-terminal blocking groups may be unique to this tissue. In fact, mass sepectrometry of bovine heart catalytic subunit of cAMP-dependent protein kinase detected

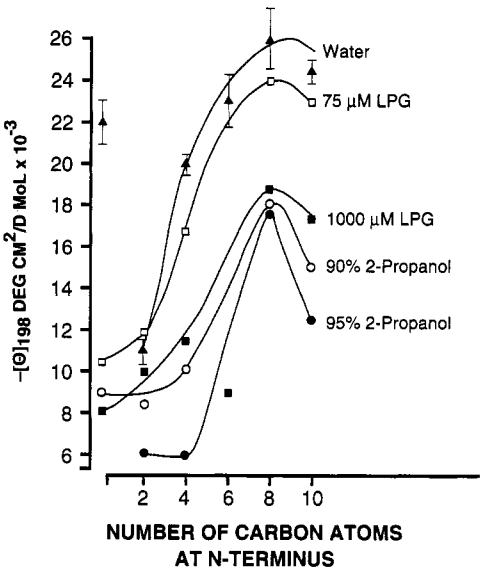

FIGURE 6: θ_{198} as a function of the number of carbon atoms at the N-terminus of peptide 1–21 of human myelin basic protein: Water (▲); lysophosphatidylglycerol (LPG), 75 μM (□); lysophosphatidylglycerol, 1000 μM (■); 2-propanol, 90% (○); 2-propanol, 95% (●).

only myristic acid (Neubert et al., 1992). The same authors argue that the N-terminal acylation is probably not related to anchoring of the protein in the lipid bilayer since the retinal proteins can be extracted readily without detergent. In a more recent report, the amino terminus of recombinant recoverin acylated with myristoylate was shown to bind membranes in a Ca^{2+}-dependent manner (Dizhoor et al., 1993), which was similar to the binding when heterogeneously acylated recoverin was used. In this case, myristoylation bound the protein to the membrane by either protein–lipid or protein–protein interactions. MBP is readily extracted without detergent; therefore, it is unlikely that the short alkyl carboxylates function to anchor this protein in the membrane.

The first question to be answered concerned the possibility that the different N-terminal groups affected the secondary structure of the peptides. By circular dichroism we obtained mean residue ellipticities at 198 and 222 nm from the spectra. We compared the spectra in water and in different membrane-mimetic solvents. When studied in water, the addition of an acetyl group to the N-terminus changed the θ_{198} value from −22 000 to −11 000 deg cm^2 dmol^{-1}, reflecting an increase in ordered structure. As the number of carbons at the N-terminus was increased to 4, 6, 8, and 10, the θ_{198} value changed sharply in the opposite direction from −11 000 to −26 000 deg cm^2 dmol^{-1} for the C_8-blocked peptide. The θ_{198} value for the C_{10}-blocked peptide was slightly less at −23 500 deg cm^2 dmol^{-1}. Since θ_{198} is suggestive of unordered structure, the less negative ellipticities with the addition of an acetyl group suggested an increase in ordered structure. Increasing the length of the alkyl carboxylate up to 10 carbons regenerated the unordered structure (Figure 6). In lipid-mimetic environments such as 90% 2-propanol (and 90% methanol) and 1000 μM lysophosphatidylglycerol, the spectra were of similar shape but the amount of unordered structure varied; *e.g.*, with the C_8-blocked peptide the θ_{198} value was about −18 000 deg cm^2 dmol^{-1} in all solvents, compared to −26 000 deg cm^2 dmol^{-1} in water. In the presence of 95% 2-propanol a dramatic change in shape of the curves occurred (Figure 3). The θ_{198} values were less negative for all peptides when compared to the ellipticities in the various solvents used, although the values when eight carbons were at the N-terminus were similar to

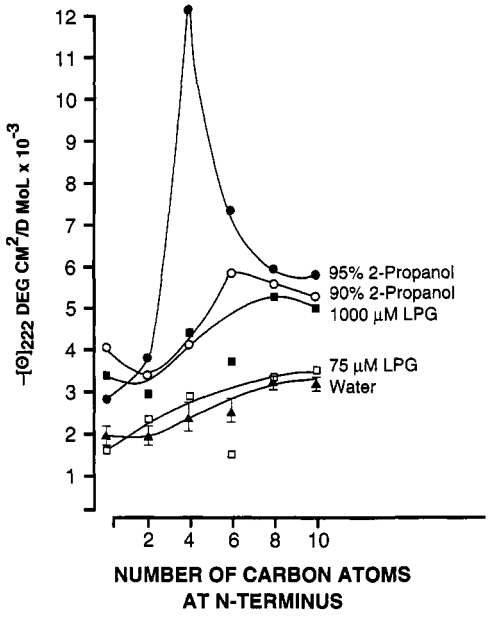

FIGURE 7: θ_{222} as a function of the number of carbon atoms at the N-terminus of peptide 1–21 of human myelin basic protein: Water (▲); lysophosphatidylglycerol (LPG), 75 μM (□); lysophosphatidylglycerol, 1000 μM (■); 2-propanol, 90% (○); 2-propanol, 95% (●).

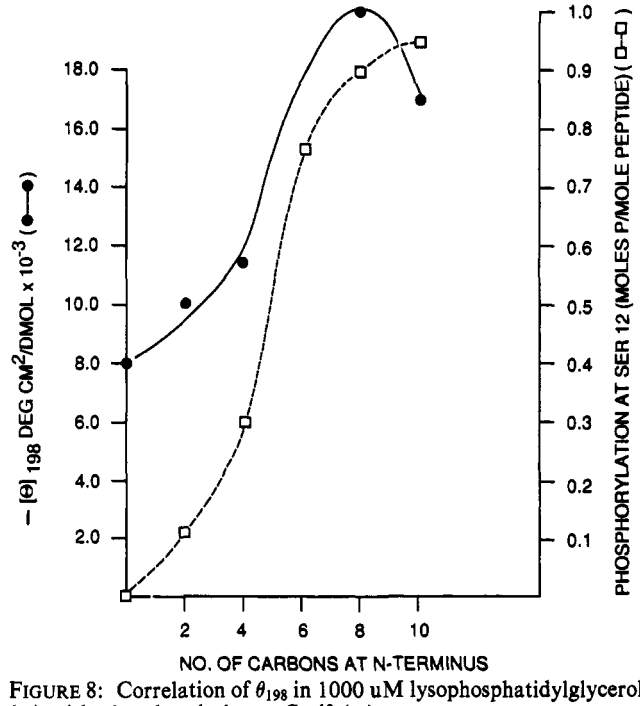

FIGURE 8: Correlation of θ_{198} in 1000 uM lysophosphatidylglycerol (●) with phosphorylation at Ser^{12} (□).

those in 90% 2-propanol and in lysophosphatidylglycerol (1000 μM).

The θ_{222} values were generally small. However, the value increased from −1600 deg cm^2 dmol^{-1} for the unblocked peptide to −3400 deg cm^2 dmol^{-1} when the length of the carbon chain was increased to 8 or 10 carbons in water or in 75 μM lysoPG. In 90% 2-propanol or 1000 μM lysophosphatidylglycerol, higher ellipticities were observed for all peptides; *e.g.*, θ_{222} increased from −3300 deg cm^2 dmol^{-1} for the unblocked peptide to −5700 deg cm^2 dmol^{-1} for the peptide containing the C_6 alkyl carboxylate (Figure 7). The θ_{222} values in 95% 2-propanol were unusual for some of the peptides. Although the values for the unblocked and the acetylated peptides were similar to those in 90% 2-propanol, the value for the butylated peptide was vastly different from all the rest: the θ_{222} value was more than −12 000 deg cm^2 dmol^{-1}. The value for the hexylated peptide in 95% 2-propanol was also considerably higher than those for the hexylated peptide in other solvents, with a θ_{222} value of about −7500 deg cm^2 dmol^{-1}. However, when the carbon chain length reached 8 and 10 carbons, the θ_{222} values were similar to those in 90% 2-propanol. Although we cannot assign any particular ordered structure to the changes in θ_{198}, the large negative θ_{222} ellipticies suggest α-helical structure. The general shapes of the spectra in Figure 3 are in agreement with this conclusion, especially the spectrum of the butylated peptide, which has minima at 208 and 222 nm.

The N-terminus of MBP (*i.e.*, the first 21 residues) contains a number of sites which are posttranslationally modified, including N-terminal acylation, deimination of arginyl residues, ADP-ribosylation, and phosphorylation. To explore the role of local or general conformational changes in peptide 1–21, ADP-ribosylation with cholera toxin and phosphorylation with a Ca^{2+}-, phospholipid-dependent protein kinase C were selected because several of the sites modified in the intact MBP are found in the first 21 amino acids. Since CD is an averaging method, correlations between structure and enzymic reactions at specific sites are difficult to substantiate. Phosphorylation at Ser^{12}, which increased from zero for the unblocked peptide to 0.95 mol of PO_4/mol of peptide for the C_{10}-blocked N-terminus, correlated with θ_{198} (Figure 8) in 1000 μM LPG and 90% and 95% 2-propanol. Although the correlations of ADP-ribosylation at Arg^9 and phosphorylation at Ser^7 with θ_{222} are less defined, maximal ADP-ribosylation and phosphorylation occurred when peptide 1–21 was blocked with C_4 or C_6 alkyl carboxylates. These peptides showed the largest increase in helical structure in 95% 2-propanol. Other methodologies such as NMR are being pursued to define more accurately local structural changes under different experimental conditions.

In another study, the immunological reactivity of peptide 1–21 used in all the studies in this paper was influenced by the number of carbon atoms at the N-terminus. Three of six monoclonal antibodies reacted only with acetyl 1–21 and not with any of the other peptides. One Mab reacted with all peptides. Mab raised with the citrullinated component of MBP reacted better with peptide 1–21 containing longer alkyl chains. The T-cell line PL11 reacted with acetyl 1–9 and failed to respond to the unblocked peptide but responded to the peptide blocked with longer alkyl carboxylates (Zhou et al., 1993). These studies extend the earlier studies of Zamvil et al. (1986), who showed that the induction of EAE in the mouse required an acetylated N-terminus, since the unblocked peptide was inactive. Our present results suggest that a partial explanation for this observation may involve the secondary structure of the peptide, through which the immune response may be modulated.

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REFERENCES

Aitken, A., Cohen, P., Santkarn, S., Williams, D. H., Calder, A. G., Smith, A., & Klee, C. B. (1982) *FEBS Lett. 150*, 314–318.
Boulias, C., & Moscarello, M. A. (1993) *J. Neurochem.* (in press).
Bryant, M. L., & Ratner, L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 523–527.

Carnegie, P. R. (1969) *Biochem. J. 111*, 240–242.
Carr, S. A., Bieman, K., Shoji, S., Parmelee, D. C., & Titani, K. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6128–6131.
Dizhoor, A. M., Ericsson, L. H., Johnson, R. S., Kumar, S., Olshevskaya, E., Zozulya, S., Neubert, T. A., Stryer, L., Hurley, J. B., & Walsh, K. A. (1992) *J. Biol. Chem. 267*, 16033–16036.
Dizhoor, A. M., Che, C. K., Olshevskaya, E., Sineinikova, V. V., Phillipov, P., & Hurley, J. B. (1993) *Science 259*, 829–832.
Edashige, K., Utsumi, T., Sato, E. F., Ide, A., Kasai, M., & Utsumi, K. (1992) *Arch. Biochem. Biophys. 296*, 296–301.
Gottlinger, H. G., Sodroski, J. G., & Haseltine, W. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5781–5785.
Hashim, G., & Eylar, E. H. (1969) *Arch. Biochem. Biophys. 135*, 324–333.
Henderson, L. E., Krutzch, H. C., & Oroszlan, S. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 339–343.
Kokame, K., Fukada, Y., Yoshizawa, T., Takao, T., & Shimonishi, Y. (1992) *Nature 359*, 749–752.
Moscarello, M. A. (1990) in *Dynamic Interactions of Myelin Proteins* (Hashim, G. A., & Moscarello, M. A., Eds.) pp 25–48, Alan R. Liss Inc., New York.
Moscarello, M. A., Pang, H., Pace-Asciak, C. R., & Wood, D. D. (1992) *J. Biol. Chem. 267*, 9779–9782.
Neubert, T. A., Johnson, R. S., Hurley, J. B., & Walsh, K. A. (1992) *J. Biol. Chem. 267*, 18274–18277.
Ozols, J., Carr, S. A., & Stritmatter, P. (1984) *J. Biol. Chem. 259*, 13349–13354.
Ramwani, J., & Moscarello, M. A. (1990) *J. Neurochem. 55*, 1703–1710.
Ray, S., Zozulya, S., Niemi, G. A., Flaherty, K. M., Brolley, D., Dizhoor, A. M., McKay, D. B., Hurley, J., & Stryer, L. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 5705–5709.
Salisbury, S. A., Tremeer, E. J., Davies, J. W., & Owen, D. E. I. A. (1990) *J. Chem. Soc. Commun.* (London) 538–540.
Schultz, A. M., & Oroszlan, S. (1983) *J. Virol. 46*, 355–361.
Wood, D. D., & Moscarello, M. A. (1989) *J. Biol. Chem. 264*, 5121–5127.
Zamvil, S. S., Mitchell, D. J., Moore, A. C., Kitamura, K., Steinman, L., & Rothbard, J. (1986) *Nature 324*, 258–260.
Zhou, S. R., Whitaker, J. N., Wood, D. D., & Moscarello, M. A. (1993) *J. Neuroimmunol. 46*, 91–96.