Direct Observation by Carbon-13 Nuclear Magnetic Resonance of Membrane-Bound Human Myelin Basic Protein†

John G. Stollery, Joan M. Boggs, Mario A. Moscarello, and Charles M. Deber*

ABSTRACT: Carbon-13 enriched human myelin basic protein (MBP) was reconstituted in multilamellar liposomes. The protein contained ^{13}C-labeled methyl groups in the side chains of each of its two methionine residues. When the protein was bound to dipalmitoylphosphatidylglycerol (DPPG) or dimyristoylphosphatidic acid (DMPA) vesicles and the temperature was maintained below the lipid gel to liquid-crystalline phase transition (20–30 °C), ^{13}C NMR spectra were obtained which showed only a single peak, attributable to membrane-bound myelin basic protein. Values of (corrected) protein Met S–CH_3 line widths in association with DPPG were 21 Hz (20 °C, below phase transition) and 4 Hz (50 °C, above phase transition). By comparison, protein line widths in the presence of a (neutral) phospholipid, dipalmitoylphosphatidylcholine (DPPC), were 2 ± 0.5 Hz both below (30 °C) and above (55 °C) the DPPC phase transition. Loss of protein spectral intensity on passing from DPPG or DMPA liquid-crystalline to gel states suggested a distribution of environments for MBP molecules, with some near membrane surfaces (relatively mobile) and some incorporated into bilayer interiors (resonance broadened beyond detection). Qualitative analysis of the line widths of protein resonances yielded values of the overall correlation time, $\tau_c \simeq 1$ ns, for motion of membrane-bound protein chains in the vicinity of the ^{13}C-labeled probes. Protein line width data indicated that motion of the ^{13}C-labeled probe is sensitive to lipid phase transitions, thus establishing that motions in the bilayer interior can be "transmitted", via noncovalent bonds, to sites in membrane-bound MBP. These findings, coupled with the spectral behavior of DPPG glycerol head group carbons, support the notion that portions of the protein sequence reside at the membrane surface and/or in the adjoining regions of aqueous space between liposomes or between bilayers of their multilamellar structures. Myelin basic protein is thus well suited for positioning between folded myelin layers to facilitate their organization into a biologically functional structure.

To aid in elucidating the role of myelin basic protein (MBP)[1] in organizing the structure of native myelin, the membrane surrounding nerve axons in the central nervous system, it is essential to determine where the protein resides with respect to myelin membrane surfaces and bilayers. The hydrophilic sequence (Eylar et al., 1971) of this abundant myelin protein and its water solubility suggest the character of an extrinsic membrane protein, in which at least portions of its sequence would lie on or near myelin membrane surfaces or interfaces. Critical regions of MBP may be accessible to antibodies or sensitized cells and thus be implicated in triggering the "autoimmune destruction" of myelin. Such a process may be directly or indirectly involved in the onset of multiple sclerosis in humans and in induction of experimental allergic encephalomyelitis (EAE disease) in laboratory animals [for a review, see Maugh (1977)].

From a variety of studies on the conformation of MBP in aqueous solution [for a review, see Moscarello (1976)], a picture has emerged of an elongated molecule (Epand et al., 1974), perhaps with a hairpin bend near a proline-rich mid-region (Brostoff & Eylar, 1971), devoid of organized secondary structure (Chao & Einstein, 1970; Palmer & Dawson, 1969) but with the possibility of local pockets of tertiary structure (Chapman & Moore, 1976, 1978). Circular dichroism measurements have suggested the onset of additional ordered structure when the protein is in contact with a membrane surface (Keniry & Smith, 1979).

Carbon-13 nuclear magnetic resonance is a technique which can, in principle, provide dynamic information concerning interactions of membranes with macromolecules. It has afforded data concerning gradations of molecular motion as a function of carbon positions in phospholipid molecules [e.g., Levine et al. (1972) and Godici & Landsberger (1974)] and the effects on lipid spectra of added protein [e.g., Brown & Wuthrich (1977) and Fleischer et al. (1979)]. However, attempts to view membrane-bound proteins directly have been limited not only by their restricted mobility but also by the low effective concentration of natural abundance protein carbon resonances. The latter circumstance can be surmounted through specific ^{13}C enrichment of selected sites in the protein by using a method introduced by Jones et al. (1975, 1976) to produce ^{13}C-enriched samples of sperm whale myoglobin. Derivatization of the *S*-methyl side chain of protein methionyl (Met) residues by reaction with ^{13}C-enriched methyl iodide gives an "S-methylated protein" [side chain $-CH_2CH_2S^+(CH_3)_2$ with one $^{12}CH_3$ and one $^{13}CH_3$ substituent]; this protein derivative can be studied directly, or the Met residue can be reduced (using dithiothreitol) back to native methionine (with random loss of [^{12}C]- or [^{13}C]methyl groups). Selective ^{13}C enrichment by this procedure has been used in our laboratory to prepare bovine and human MBP S-methylated and "reduced" at its two Met residues (numbers 21 and 167) (Deber et al., 1978). Other proteins including horse cytochrome *c* (Schejter et al., 1978) and bovine pancreatic trypsin inhibitor (BPTI) (Harina et al., 1978) have been similarly studied.

Experiments involving MBP–phospholipid complexes have indicated not only that acidic phospholipids play an essential role in promoting "reconstitution" but also that MBP induces phase separation of lipids such as PS, PG, and PA in mixtures

† From The Research Institute, The Hospital for Sick Children, Toronto M5G 1X8, Ontario, Canada, and the Department of Biochemistry, University of Toronto, Toronto M5S 1A8, Ontario, Canada. *Received December 6, 1979.* Supported, in part, by grants (to C.M.D.) from the Multiple Sclerosis Society of Canada and (to M.A.M. and J.M.B.) from the Medical Research Council of Canada. J.G.S. is the recipient of an M.S. Society Studentship in 1978–1980.

[1] Abbreviations used: PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; MBP, myelin basic protein; NMR, nuclear magnetic resonance.

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with PC (Boggs et al., 1977). Native myelin consists of perhaps 10–20% of "acidic lipids" (e.g., sulfatides and PS) which could, by analogy, provide domains for the MBP–membrane electrostatic interactions anticipated for this Lys- and Arg-rich protein. NMR studies have already demonstrated the feasibility of observing ^{13}C-enriched resonances due to MBP resonances when the protein was reconstituted into multilamellar phosphatidic acid–phosphatidylcholine (PA/PC = 50:50) vesicles (Deber et al., 1978). This work also demonstrated (from line widths and spin–lattice relaxation data) that overall conformational properties of native ^{13}C-enriched MBP and its S-methylated derivative are essentially indistinguishable. Similar techniques have been applied to observe the binding of ^{13}C-enriched human encephalitogenic (EAE) nonapeptide (residues 114–122 of MBP) to phosphatidylglycerol vesicles (Deber & Young, 1979). Further, by using PA and PG derivatives with saturated fatty acid side chains (dimyristoylphosphatidic acid, DMPA; dipalmitoylphosphatidylglycerol, DPPG), it becomes possible to monitor ^{13}C NMR spectral effects of protein dynamics as the lipid passes through its gel to liquid-crystalline phase transition and thereby obtain direct information concerning the location of the protein vis-à-vis the lipid bilayer.

Materials and Methods

Materials. Human myelin basic protein was obtained from normal human white matter and purified by the method of Lowden et al. (1966). Dimyristoylphosphatidic acid (DMPA) was prepared by the procedure of Papahadjopoulos & Miller (1967), and dipalmitoylphosphatidylglycerol (DPPG) was prepared according to Papahadjopoulos et al. (1973). ^{13}C-Labeled methyl iodide (90% enriched) and deuterium oxide (99.8% D) were purchased from Merck Sharp & Dohme, Montreal. ^{14}C-Labeled methyl iodide was obtained from New England Nuclear, Cambridge, MA.

Carbon-13 Labeling of Myelin Basic Protein (MBP). Methylation of human MBP with [^{13}C]methyl iodide was carried out with minor modifications according to Jones et al. (1975, 1976); this procedure produces a sample which is completely and uniformly labeled at Met-21 and Met-167 *S*-methyl side-chain positions (Deber et al., 1978).

Preparation of NMR Samples. (a) Pure Lipid Samples. A chloroform solution of lipid was dried down in a (12-mm) NMR tube under nitrogen gas; final traces of solvent were removed by placing the tube under vacuum for 30 min. Large multilamellar vesicles were formed from lipid by vortexing the lipid in 1.2 mL of D_2O buffer (10 mM NaCl, 2 mM Hepes, and 0.1 mM EDTA, pD 6.8) for 5 min at 60 °C.

(b) Incorporation of Myelin Basic Protein into Vesicles. The lipid was dried in an NMR tube under vacuum, as described in (a) above, and multilamellar vesicles were formed by vortexing the lipid in 0.6 mL of D_2O buffer for 5 min at 60 °C. ^{13}C-Enriched $Met_{21,167}$-S-methylated human myelin basic protein was dissolved in D_2O buffer (0.6 mL) and added to the lipid suspension at 4 °C. Samples were kept in an ice bath until being placed in the NMR spectrometer. $^{13}CH_3CN$ (2 μL) was usually added as an internal standard. Protein and lipid concentrations for individual samples are given in the figure legends. After each experiment, protein concentrations were determined by amino acid analysis on a Durrum D-500 analyzer after hydrolysis with 5.7 N HCl for 24 h at 110 °C, and lipid phosphorus was assayed by the method of Bartlett (1959).

NMR Methods. Carbon-13 nuclear magnetic resonance spectra were recorded on a Varian XL-100 spectrometer operating at 25.16 MHz equipped with a variable temperature accessory. Probe temperature was 30 ± 1 °C. Spectral parameters are given in the figure legends for individual lipid and protein spectra. Line width (LW) values reported are the results of measurements of the widths at half-height of indicated resonances, taken directly from expanded plots of experimental spectra. Values given are already adjusted for (a) broadening due to signal enhancement during data processing [typically, a sensitivity enhancement (SE) of 0.1 s, giving $1/(\pi \times SE) = 3.2$ Hz broadening] and (b) broadening due to instrumental inhomogeneities as monitored by the observed line width of the internal standard $^{13}CH_3CN$ (typically, the line width of this resonance was 4 to 5 Hz in the presence of either protein or lipid alone and 5 to 6 Hz in protein–lipid samples). Relative areas of protein resonances were determined by cutting out and weighing peaks from expanded versions of spectra.

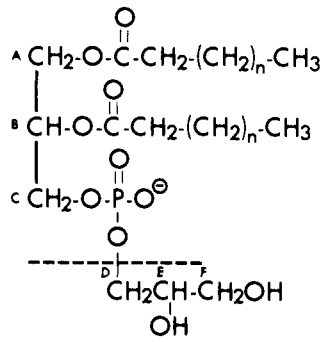

FIGURE 1: Structure of dipalmitoylphosphatidylglycerol (DPPG) (n = 13). Dimyristoylphosphatidic acid (DMPA) is obtained from this structure when n = 11 and the glycerol head group (below the dashed line) is replaced by H. Note the distinction between *glycerol backbone carbons* (A, B, and C) (present in both DPPG and DMPA) and *glycerol head group carbons* (D, E, and F) in DPPG.

Results

Phase Transition of Dipalmitoylphosphatidylglycerol (DPPG). Carbon-13 resonance line widths of phospholipids in vesicles depend upon a combination of the overall motional characteristics of the vesicles and of segmental motion of individual carbon atoms, the latter being governed largely by their location in the phospholipid molecule (Figure 1). These phenomena are illustrated in Figure 2, which presents the natural abundance ^{13}C NMR spectra of DPPG at 20–50 °C. Over this temperature range, DPPG undergoes a phase transition (at ~42 °C) from the gel to liquid-crystalline state. ^{13}C resonances at 50 °C display relatively narrow line widths (4–8 Hz), but where carbons of the DPPG lipid bilayer have become relatively immobilized (in the gel state at 20 °C), only a residual lipid resonance envelope remains near 160 ppm (Figure 2a). Most carbon atoms in the bilayer now have resonances which have been "broadened beyond detection"; in the present manuscript, experimental spectra suggest that a resonance of line width >75–100 Hz would become undetectable by conventional methods. While DPPG bilayer carbons have lost most of their resonance intensity at 20 °C, the resonances corresponding to the three glycerol head group carbons (D, E, and F, Figure 1) are still observed (LW = 8–10 Hz). Note the distinction between glycerol head group carbons and glycerol backbone carbons A, B, and C; these latter atoms, located in the surface of the vesicle, are known to display the shortest spin–lattice relaxation times (T_1 values) ("slowest motion") of all carbons in phospholipid molecules (Levine et al., 1972; Godici & Landsberger, 1974) and often give rise to broadened resonances which would not be detected readily (particularly in spectra of DPPG where resonances of carbons

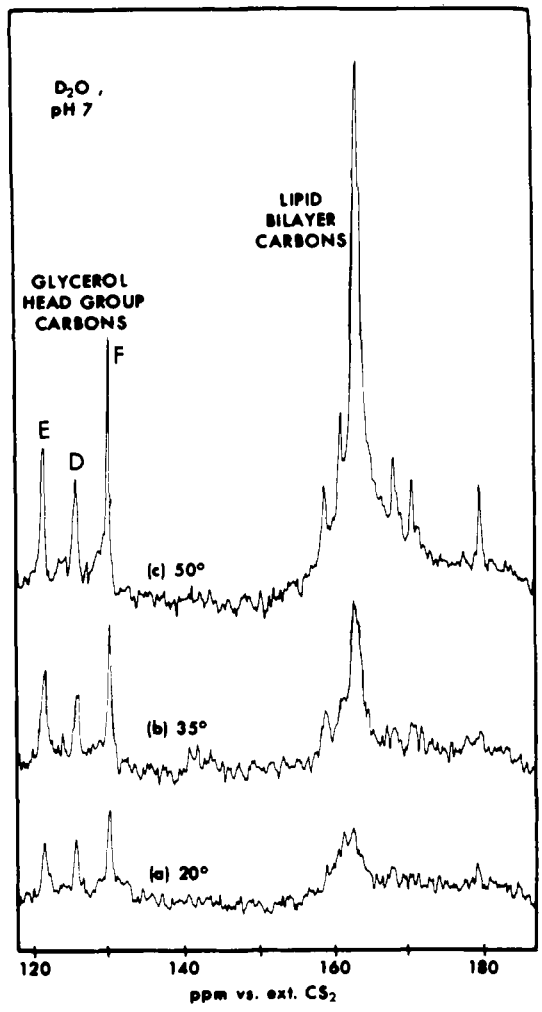

FIGURE 2: Natural abundance Fourier transform ^{13}C NMR spectra (25.16 MHz) of unsonicated dipalmitoylphosphatidylglycerol (DPPG) (50 mg/mL) in D_2O buffer (see Materials and Methods). In spectrum a, at 20 °C, the lipid is in the gel state; in spectrum b, at 35 °C, the lipid is just below its phase transition; in spectrum c, at 50 °C, the lipid is in the liquid-crystalline state. Spectra are the result of ~100 000 scans, recycle time 0.6 s. A sensitivity enhancement of 0.1 s was applied during data processing. $^{13}CH_3CN$ was added as an internal standard (2 μL). Chemical shifts can be converted to values relative to external tetramethylsilane by subtracting the values given from 193.8 ppm. Assignments of resonances to carbons D, E, and F were made on the basis of empirical correlations with glycerol backbone carbon chemical shifts (Godici & Landsberger, 1974) and from relative line widths observed for carbons D, E, and F in the present work.

D, E, and F may be superimposed over them). Through the phase transition, carbon line widths of carbons D, E, and F narrow to 4–6 Hz (Figure 2c).

Interaction of ^{13}C-Enriched Myelin Basic Protein with Dipalmitoylphosphatidylglycerol (DPPG) Liposomes. Previous investigations on MBP–lipid samples similar to those employed here have shown, based on assays of centrifuged pellets, that essentially all protein present is bound to lipid under these experimental conditions (Boggs & Moscarello, 1978). When myelin basic protein is reconstituted into multilamellar DPPG liposomes (43% w/w), the spectra shown in Figure 3 result. *The only spectrum observable below the lipid phase transition (Figure 3a) is a single resonance near 168 ppm, attributable to protein.* All resonances due to phospholipid carbons have been broadened beyond detection at 20 °C (>75 Hz). As the sample is warmed through its phase transition, the resonance attributable to myelin basic protein narrows in line width from ~21 Hz at 20 °C to ~4 Hz at 50 °C (see Table I). Concomitantly, the lipid spectrum recovers intensity through its phase transition, but it is obvious

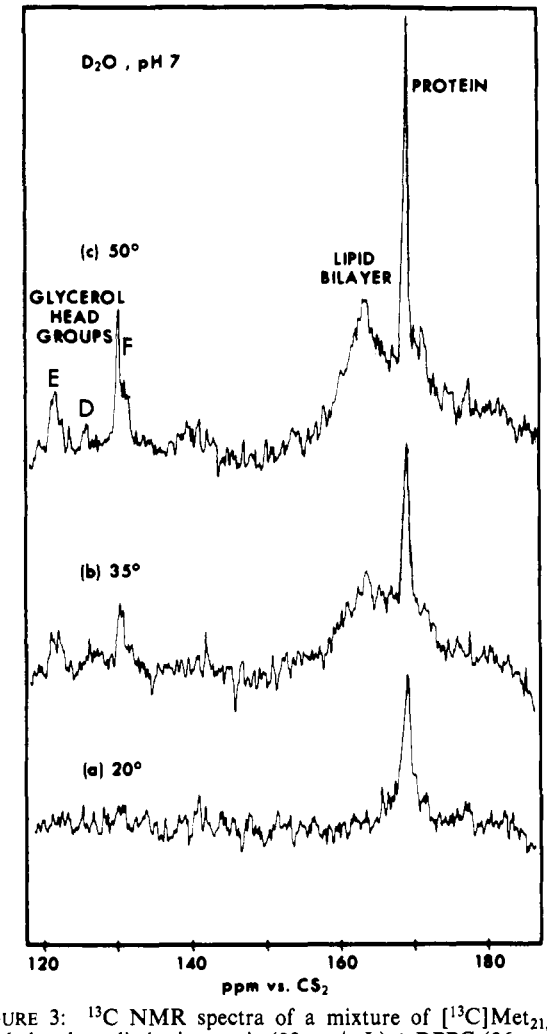

FIGURE 3: ^{13}C NMR spectra of a mixture of [^{13}C]$Met_{21,167}$-S-methylated myelin basic protein (20 mg/mL) + DPPG (36 mg/mL) (protein/lipid 43% w/w) in D_2O buffer, recorded at (a) 20, (b) 35, and (c) 50 °C. Other spectral parameters were as given in the caption to Figure 2.

Table I: Experimentally Measured Line Widths ($\Delta\nu_{1/2}$) of [^{13}C]$Met_{21,167}$-S-Methylated Human Myelin Basic Protein Reconstituted into Liposomes[a]

lipid	temp (°C)	protein $\Delta\nu_{1/2}$ (Hz)[b]
–	30	2 ± 0.5
DPPC[c]	30	2 ± 0.5
DPPC	55	2 ± 0.5
DMPA[c]	30	22 ± 2
DMPA	55	7 ± 1
DPPG[c]	20	21 ± 2
DPPG	35	15 ± 1
DPPG	50	4 ± 1
egg PA-PC (50:50)[d]	30	15 ± 1

[a] Samples (prepared as described under Materials and Methods) consisted of ~40% protein (w/w) associated with unsonicated multilamellar vesicles. [b] Line widths ($\Delta\nu_{1/2}$) have been corrected for broadening during data processing and for instrumental inhomogeneity as described under Materials and Methods. [c] Gel-liquid-crystalline transition temperatures are the following: 41 °C (pure DPPC); 52.6 °C (pure DMPA); 48 °C (DMPA + 20% MBP w/w); 42.4 °C (pure DPPG); 37.7 or 41.3 °C (DPPG + 50 or 28% MBP w/w) (Boggs & Moscarello, 1978). [d] Data from Deber et al. (1978). This lipid system is in the liquid-crystalline state at 30 °C.

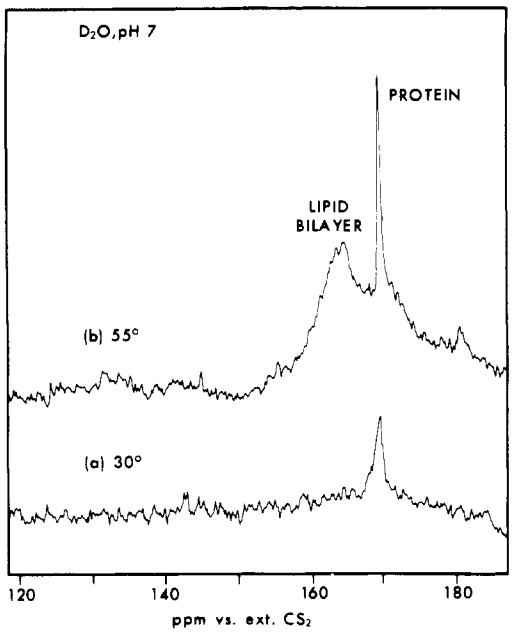

FIGURE 4: ^{13}Carbon NMR spectra of a mixture of [^{13}C]Met$_{21,167}$-S-methylated myelin basic protein (21 mg/mL) + dimyristoylphosphatidic acid (DMPA) (50 mg/mL) (protein/lipid 42% w/w) in D_2O buffer, recorded at (a) 30 and (b) 55 °C. Other spectral parameters were as given in the caption to Figure 2.

that resonances due both to bilayer carbons and glycerol head group carbons are broadened significantly vs. the spectrum of pure DPPG at 50 °C (compare Figure 2c vs. Figure 3c). Resonances of DPPG head group carbons D, E, and F appear to be recovering intensity in stepwise fashion; carbon D, adjacent to the phosphate group, is the least recovered, while carbon F, the terminal glycerol carbon, is the most recovered. In spectrum of Figure 3c, this latter resonance (near 130 ppm) appears as a sharper spike over a broadened background; the spike has the same chemical shift as carbon F in Figure 2c. This observation may suggest that "two types" of lipid, in slow exchange on the NMR time scale, are produced upon interaction with protein.

Throughout these experiments, chemical shifts of MBP *S*-methylmethionyl resonances were essentially invariant (<0.1 ppm) from their position at 167.8 ppm. Further, a single protein resonance was consistently observed for the two (Met-21 and Met-167) ^{13}C-enriched sites per molecule.

Interaction of Myelin Basic Protein with Dimyristoylphosphatidic Acid (DMPA). DMPA is also a saturated, acidic phospholipid but lacks a head group other than the negatively charged phosphate moiety. Only a hint of resonance intensity is observed for glycerol backbone carbons A, B, and C (Figure 1) in the region near 130 ppm in Figure 4b. As seen in parts a and b of Figure 4, spectral effects of myelin basic protein bound to DMPA are similar to those for DPPG; below the DMPA phase transition, the only discernible resonance in the spectrum is that attributable to the ^{13}C-enriched protein. Line widths of MBP resonances as a function of temperature in the presence of DMPA are comparable to those of the DPPG system (Table I).

Integrated Intensities of Protein Resonances. In NMR spectra of phospholipids, particularly upon addition of protein [see, for example, Brown & Wuthrich (1977)], the observed spectrum of the lipid carbons is diminished in integrated intensity. In the present study, we noted similar phenomena with respect to protein spectral intensity. By obtaining the integrated intensity of a known MBP sample (14.3 mg in 1.2 mL of D_2O at pH 7, 30 °C; assayed for protein on the amino acid analyzer) and comparing this value to those obtained from Figures 3 and 4, it was estimated that (allowing for the exponential dependence of resonance intensity on absolute protein concentration) the observed protein resonance intensities for DPPG (50 °C, Figure 3c) and DMPA (55 °C, Figure 4b) correspond to 80 and 73%, respectively, of the calculated full intensity. Although a significant percentage of protein is not observed spectrally, the overall observations thus apply to the bulk of MBP present.

In DPPG over the temperature range 20–50 °C, the integrated intensities of protein resonances, determined by setting the resonance area at 50 °C in Figure 3 to a relative intensity of 1.0, displayed values of 0.84 at 20 °C and 0.80 at 30 °C. In the DMPA system, similar procedures with the integrated intensity of the MBP resonance in Figure 4b (55 °C) set at 1.0 yielded an intensity for the corresponding resonance at 30 °C (Figure 4a) which was estimated (from three separate measurements) to be 0.58 ± 0.05.

Myelin Basic Protein in the Presence of Dipalmitoylphosphatidylcholine (DPPC). ^{13}C spectra (not shown) were recorded under conditions similar to those described above for DPPG and DMPA to determine spectral effects, if any, on MBP resonances upon addition of DPPC, a neutral phospholipid which is essentially noninteractive with this highly charged protein. Spectra of pure unsonicated DPPC (50 mg/mL) at 30 °C displayed only a broadened $^{+}N(CH_3)_3$ head group methyl resonance (~50-Hz line width) and little or no bilayer spectral intensity. Addition of [^{13}C]MBP to this lipid, either below (30 °C) or above (55 °C) its gel to liquid-crystalline phase transition, resulted in clear spectra of the protein, including most of its α-carbon and aromatic side-chain resonances as well as the expected [^{13}C]Met S–CH_3 resonance. Protein line widths were not affected by DPPC and remained the same as those observed for MBP in free solution (see Table I).

Discussion

Broadening of line widths of ^{13}C NMR spectra generally arises from chemical shift nonequivalence, incomplete C–H decoupling, and inhomogeneities due to (nonoptimal) instrument conditions and in the present system additionally from dipolar broadening due to anisotropic motions of the macromolecular protein–lipid system (Lee et al., 1974). These motions, which include tumbling of the whole vesicles, lateral diffusion of phospholipid molecules within the bilayer, rotation of the lipid molecules around their long axis, and motion about individual single (C–C and O–P) bonds, hinder interpretation of experimentally determined *absolute* line widths. Nevertheless, in the present investigation, *relative* line widths within a series of related lipid and protein spectra may be used to obtain a qualitative description of some details of myelin basic protein binding to phosphatidylglycerol and phosphatidic acid multilamellar liposomes.

The spectral line broadening observed upon addition of protein is partially due to protein-dependent aggregation of lipid vesicles. Such aggregation of liposomes by MBP can also be noted visually (Smith, 1977), as the vesicle suspension becomes milky and particulate immediately upon addition of protein. [Calcium also aggregates DPPG, an effect which similarly gives rise to extreme broadening of ^{31}P NMR resonances (Cullis & DeKruyff, 1976)]. Since each protein molecule acts as a macromolecular cation, the protein can bridge vesicles via negative phosphate groups, utilizing its ~30 positively charged side chains (Boggs et al., 1977). Protein molecules situated in the aqueous space *between* bilayers of a multilamellar vesicle could similarly bridge these inner bi-

layers. Integrated intensities of protein ^{13}C spectra suggest that some (very high molecular weight) protein–lipid aggregates may have "precipitated" to such a degree that they no longer contribute to the observed spectra. The additional loss of protein resonance intensity observed upon transition from the liquid-crystalline to the lipid gel state (Figures 3a and 4a) (which does *not* involve a change in the degree of aggregation) may indicate a distribution of environments for MBP reconstituted into DPPG and particularly DMPA vesicles. Thus, some protein molecules would be situated relatively near membrane surfaces (resonance observed) while others may be substantially "buried" in the bilayer (resonance broadened beyond detection). It is interesting to speculate how closely this would resemble MBP in native myelin, where one might initially suppose a common environment for each MBP molecule.[2]

Once bound to liposomes, the overall motion of the protein contains components attributable to the tumbling of the protein in unison with these (much larger) multilamellar vesicular particles. Thus, a key result is that the *resonance attributable to protein is the only resonance not broadened beyond detection when the protein is bound to DPPG or DMPA in the lipid gel states.* By use of the relationship between line width and the spin–spin relaxation time T_2 that LW = $(\pi T_2)^{-1}$, the protein line width of 21 to 22 Hz while bound to DPPG or DMPA in the lipid gel state leads to an estimate (Allerhand & Oldfield, 1973) of the effective correlation time, τ_c, of the Met S–CH_3 group of τ_c = 1 ns. (It should be noted that this group is an ϵ substituent on the protein side chain and will experience more segmental motion than a probe located in the protein peptide backbone. Thus, its τ_c is likely to be among the shortest in the range of correlation times which describe protein motions.) The corresponding calculation, assuming a "minimum" line width of a lipid resonance of $\simeq$75 Hz, gives a *lower limit* for a vesicle midbilayer carbon of $\tau_c = \sim 30$ ns. These results suggest that the protein (at least insofar as the protein environment can be sensed by the ^{13}C-enriched Met sites) cannot be completely entrapped in the interior of the phospholipid bilayer but rather is likely to reside at or in the vicinity of membrane surfaces.

Previous investigations, employing differential scanning calorimetry and electron spin resonance (Papahadjopoulos et al., 1975; Boggs et al., 1977; Boggs & Moscarello, 1978), have shown that the presence of MBP lowers phospholipid phase transitions, induces phase separation of acidic phospholipids, and tends to distort the packing of lipid chains near the head group region. These observations suggest the presence of other types of interactions in addition to protein binding via electrostatic attraction to lipid phosphate groups. Preliminary evidence for such further interaction is obtained from aspects of the present ^{13}C NMR data. First, protein-bound DPPG molecules apparently display a chemical shift change and differential line broadening of the resonance arising from the head group moiety –CH_2OH (carbon F) (see Figure 3c). Also, in the presence of protein, DPPG glycerol head group carbons recover intensity as a function of their distance from phosphate groups (Figure 3c), with the carbon nearest the phosphate still "immobilized" at 50 °C. Since molecular motion in phospholipid molecules generally increases with distance from the glycerol backbone, stepwise sharpening of resonances out along head group carbons D, E, and F would be consistent with known motional characteristics of liposomes and may not be protein dependent. Nevertheless, since carbons D, E, and F all appear relatively sharp (LW < 10 Hz) even for DPPG in the gel state (Figure 2a), the present data may also indicate involvement of the protein directly in the DPPG glycerol head group region.

Spectral line widths normally narrow on heating, due to thermally induced increases in molecular motion (see, for example, spectra of [^{13}C]Met-myoglobin; Jones et al., 1976) and possibly to increased "on–off" protein exchange rates at higher temperatures. However, data in Table I indicate that protein line widths are also a function of the physical state of the bilayer as well as of the absolute temperature of the sample, since approximately 5- to 6-Hz line broadening is clearly attributable to protein binding to the lipid in its gel state. This finding, that motions in the bilayer interior can be "transmitted" via *noncovalent bonds* to sites in membrane-bound MBP, extends observations from ^{31}P NMR (Cullis & DeKruyff, 1976) in which the motion of DPPG phosphate groups was found to be sensitive to motion within the bilayer. It is also consistent with electron spin resonance studies using MBP samples spin-labeled at Met residues (Boggs et al., 1980), wherein the spectral probe was similarly responsive to bilayer phase transitions.

Interpreted ^{13}C NMR data from the present study embellish the prevailing view of myelin basic protein as an extrinsic membrane protein bound principally via electrostatic attractions into the head group regions of phospholipid surfaces. These results tend to confirm the role of MBP in stabilizing whole myelin by its positioning in regions between folded myelin layers, where it can anchor these layers together, and thereby aid in organizing the myelin sheath into its biologically functional structure.

[2] Since the ^{13}C-enriched protein contains two enriched Met sites at opposite ends of the molecule and the ratio of integrated intensities for MBP resonances is 0.58 for the DMPA system below/above the lipid phase transition, it is tempting, especially in the DMPA case, to attribute the intensity loss to some preferential immobilization of either the N- or C-terminal region of membrane-bound protein.

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New Calcium Indicators and Buffers with High Selectivity against Magnesium and Protons: Design, Synthesis, and Properties of Prototype Structures†

Roger Y. Tsien

ABSTRACT: A new family of high-affinity buffers and optical indicators for Ca^{2+} is rationally designed and synthesized. The parent compound is 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), a relative of the well-known chelator EGTA [ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] in which methylene links between oxygen and nitrogen are replaced by benzene rings. BAPTA and its derivatives share the high (>10^5) selectivity for Ca^{2+} over Mg^{2+} of EGTA but are very much less affected by pH changes and are faster at taking up and releasing Ca^{2+}. The affinity of the parent compound for Ca^{2+} (dissociation constant 1.1×10^{-7} M in 0.1 M KCl) may be strengthened or weakened by electron-releasing or -withdrawing substituents on the aromatic rings. The Ca^{2+} and Mg^{2+} affinities may further be altered by replacing the ether oxygens by heterocyclic nitrogen atoms. The compounds described are fluorescent Ca^{2+} indicators absorbing in the ultraviolet region; the very large spectral shifts observed on binding Ca^{2+} fit the prediction that complexation should hinder the conjugation of the nitrogen lone-pair electrons with the aromatic rings. Derivatives with quinoline nuclei are notable for their high sensitivity of fluorescent quantum yield to the binding of Ca^{2+} but not of Mg^{2+}. Preliminary biological tests have so far revealed little or no binding to membranes or toxic effects following intracellular microinjection.

It is scarcely necessary any longer to stress the importance of intracellular free Ca^{2+} as a second messenger for external stimuli and as a regulatory ion. There is vast literature on this topic, and several recent symposia have been devoted to the cellular functions of Ca^{2+} (Duncan, 1976; Scarpa & Carafoli, 1978). A major technical challenge has been to devise satisfactory means for nondestructively measuring intracellular free Ca^{2+} with good time resolution [for reviews see Kretsinger & Nelson (1976) and Ashley & Campbell (1979)]. The most popular technique has been to use dyes or proteins which change their absorption or luminescence upon binding Ca^{2+} ions. In the past, these indicators have suffered from several problems (Brown et al., 1977; Thomas, 1979; Moisescu et al., 1975; Blinks et al., 1976, 1978): (1) insufficient selectivity against competing cations, particularly H^+ and Mg^{2+}; (2) complex stoichiometries of interaction with Ca^{2+}, for example, 1 Ca^{2+}/2 dyes or several Ca^{2+}/1 protein; (3) inflexibility of molecular design, or the difficulty of rationally adjusting indicator properties by tinkering with the molecular structure. This paper demonstrates that these problems can be largely overcome by new rational designs and chemical syntheses. The resulting simple compounds are both fluorescent indicators, absorbing in the ultraviolet region, and buffers with distinct advantages over previous buffers. Later papers will describe substantial progress toward the goal of superior visible Ca^{2+} indicators which can give fast quantitative readout of intracellular free Ca^{2+} concentrations in a wide variety of interesting physiological systems.

Consider EGTA[1] (ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) (**1**). This compound is the only buffer with high selectivity for Ca^{2+} over Mg^{2+} to have found significant use in biological research. The Ca^{2+}/Mg^{2+}

† From the Physiological Laboratory, University of Cambridge, Cambridge CB2 3EG, England. *Received October 26, 1979.* This work was supported by grants from the Science Research Council.

[1] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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