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Stoichiometry and Specificity of Lipid-Protein Interaction with Myelin Proteolipid Protein Studied by Spin-Label Electron Spin Resonance†

Peter J. Brophy,‡ László I. Horváth,§ and Derek Marsh*

ABSTRACT: The interaction of spin-labeled lipids with the myelin proteolipid apoprotein in complexes with dimyristoylphosphatidylcholine of varying lipid/protein ratios has been studied with electron spin resonance spectroscopy. A first shell of approximately 10 lipids per 25 000-dalton protein is found to be motionally restricted by the protein interface. This stoichiometry is consistent with a hexameric arrangement of the protein in the membrane. A selectivity of the various spin-labeled lipids for the motionally restricted component at the protein interface is found in the order stearic acid > phosphatidic acid > cardiolipin ≳ phosphatidylserine > phosphatidylglycerol ≈ phosphatidylcholine > phosphatidylethanolamine > androstanol ≳ cholestane.

Interactions between lipids and proteins in biological membranes can exert a major influence on membrane protein function (Sanderman, 1978). It is therefore of considerable interest to determine the stoichiometry of the lipid-protein interactions and whether particular membrane proteins display any specificity in their interactions with lipids. Jost et al. (1973) were the first to demonstrate the existence of motionally restricted lipid in the immediate environment of an integral membrane protein using electron spin resonance (ESR)[1] spectroscopy. From the stoichiometry of the interaction, the lipid that they observed to be motionally restricted on the conventional ESR time scale was described as boundary lipid. Such motionally restricted lipid has subsequently been demonstrated in other systems, including rod outer segment disk membranes (Watts et al., 1979, 1981), acetylcholine receptor membranes (March & Barrantes, 1978; Marsh et al., 1981), and Na,K-ATPase membranes (Brotherus et al., 1980; Marsh et al., 1982). Boundary lipid would be expected to be enriched in any lipids for which a membrane protein showed a preferential interaction, and this has been demonstrated in certain cases (Brotherus et al., 1980; Knowles et al., 1981; Marsh et al., 1982). Because of its characteristic time scale, ESR spectroscopy is therefore an attractive technique for identifying and quantifying the lipids that interact with membrane proteins [see Marsh & Watts (1982) for a review].

The proteolipid protein is the major integral membrane protein of central nervous system myelin. The same protein isolated from human myelin has been called lipophilin (Moscarello, 1976). The proteolipid protein can be readily purified and recombined with defined lipids, and this has made the protein a suitable subject for studying the interactions of lipids with an integral membrane protein [see Boggs et al. (1982) for a review]. The existence of motionally restricted lipid in lipophilin-egg phosphatidylcholine complexes has been demonstrated by the use of spin-labeled fatty acids (Boggs et al., 1976). Boggs and co-workers have investigated the nature of the lipid-lipophilin interactions further by calorimetric techniques. They have shown that the protein preferentially associates with negatively charged phospholipids (Boggs et al., 1977) and that these interactions are independent of fatty acid chain length between C-14 and C-18 (Boggs & Moscarello, 1978). In three different calorimetric studies the number of lipid molecules removed from the cooperative calorimetric transition has variously been estimated as 15, 21-25, and 20-35 per 25 000-dalton protein (Papahadjopoulos et al., 1975; Boggs & Moscarello, 1978; Boggs et al., 1980).

Since the proteolipid apoprotein is considerably more hydrophobic in character than the amphipathic integral membrane proteins that we have studied previously [see, e.g., Marsh et al. (1982) and Marsh & Watts (1982)], it is an attractive candidate for more detailed study. In this paper we have investigated phospholipid interactions with the proteolipid protein from bovine central nervous system myelin using ESR spectroscopy. The proteolipid protein has been reconstituted with dimyristoylphosphatidylcholine (DMPC) in the presence of a variety of spin-labeled phospholipids. In this way we have been able to investigate both the specificity and the stoichiometry of lipid interaction with the protein. An interesting pattern of lipid specificity emerges, and the relatively low lipid/protein stoichiometry suggests an oligomeric form for the protein in the membrane.

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[1] Abbreviations: ESR, electron spin resonance; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PLP, proteolipid apoprotein; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; 14-PCSL, -PESL, -PGSL, -PSSL, and -PASL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine, -phosphoethanolamine, -phosphoglycerol, -phosphoserine, and -phosphoric acid; 14-CLSL, 1-(3-*sn*-phosphatidyl)-3-[1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]glycero-3-phospho]-*sn*-glycerol; 14-SASL, 14-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid; ASL, 17β-hydroxy-4′,4′-dimethylspiro[15α-androstane-3,2′-oxazolidin]-3′-yloxy; CSL, 4′,4′-dimethylspiro[5α-cholestane-3,2′-oxazolidin]-3′-yloxy. The stearoyl component of 14-PCSL etc. should more systematically be named 13-(2-butyl-4,4-dimethyl-3-oxyoxazolidin-2-yl)tridecanoyl.

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Materials and Methods

Dimyristoylphosphatidylcholine (DMPC) was from Fluka (Buchs, Switzerland). Hepes was from Sigma (St. Louis, MO). The 14-SASL spin-labeled on the C-14 atom position was prepared essentially following the methods of Hubbell & McConnell (1971). The corresponding phosphatidylcholine spin-label, 14-PCSL, acylated on the *sn*-2 chain, was prepared according to Boss et al. (1975). The 14-PESL, 14-PGSL, 14-PSSL, and 14-PASL spin-labels were prepared from the 14-PCSL label by transphosphatidylation catalyzed by phospholipase D (Comfurius & Zwaal, 1977). The 14-CLSL cardiolipin spin-label was prepared according to Cable et al. (1978). A more detailed description of the spin-label synthesis can be found in Marsh & Watts (1982).

Myelin was isolated from bovine spinal cord by the procedure of Benjamins et al. (1976). The proteolipid protein was extracted and delipidated by chromatography on Sephadex LH-20 in chloroform–methanol–0.01 M HCl (50:50:1 by volume) as described by Brophy (1977). Chromatography was repeated in order to ensure complete delipidation as judged by thin-layer chromatography and phosphorus analysis. The purified protein contained less than 1 mol of lipid phosphorus/mol of protein.

The proteolipid protein was reconstituted with DMPC by dialysis from 2-chloroethanol as described previously (Brophy, 1977). The buffer used throughout was 100 mM NaCl, 1 mM EDTA, and 2 mM Hepes adjusted to pH 7.4 at 20 °C (reconstitution buffer). Samples in 2-chloroethanol (1–5-mL volume) were dialyzed exhaustively against three 5-L changes of reconstitution buffer. Dialyzed complexes were analyzed by sucrose density gradient centrifugation (10–55%, sucrose in reconstitution buffer) for 3 h at 40000 rpm (Beckman 3W40 rotor). Each sample was recovered as a single band whose position in the gradient was determined by the lipid/protein ratio. Lipid phosphorus was measured by the method of Eibl & Lands (1969), and protein was measured by the procedure of Lowry (1951). The monomer molecular weight of the protein was assumed to be 25000.

Samples were spin-labeled either before or after dialysis, and the spin-label was present as 1.5 mol % of DMPC. For comparison of the interaction of various phospholipid labels with the proteolipid protein, a large sample was prepared and divided into several aliquots in order to ensure identical lipid/protein ratios. Each complex was then labeled by the addition of 10 μL of an ethanolic solution of spin-label to 1 mL of sample in reconstitution buffer. After 10 min of incubation at room temperature the complex was centrifuged (45 min, 90000*g*) to remove unincorporated label, transferred to a 100-μL capillary tube, and sealed. The sample was then concentrated by centrifugation (30 min, 3000*g*) before ESR measurement. Spectra obtained from complexes labeled by this method were identical with those obtained from samples labeled before dialysis, indicating that the incorporated label was uniformly distributed throughout the lipid (by lateral diffusion).

ESR spectra were recorded on a Varian E-12 Century Line spectrometer with nitrogen gas flow temperature regulation. Spectra were digitized by using a Digital Equipment Corp. LPS system and dedicated PDP 11/10 computer with VT-11 display. Spectra were analyzed with interactive graphics using software written by Dr. W. Möller of this Institute. For further details of the ESR spectroscopic methods, see Marsh (1982).

Results

The ESR spectra of the 14-PASL spin probe in myelin proteolipid apoprotein–dimyristoylphosphatidylcholine recombinants are shown in Figure 1. The spectra were recorded at 30 °C, which is above the gel-to-fluid phase transition of all the recombinants, as illustrated for the lipid alone by the extensively motionally averaged, sharp, three-line spectrum. The spectra of the recombinants all contain a second, broader component, in addition to the motionally averaged component characteristic of the fluid lipid. The broader component very strongly resembles the spectrum of the 14-PASL probe associated with apoprotein alone and corresponds to spin probes whose motion is strongly restricted on the ESR time scale [see, e.g., Marsh (1982)]. The relative proportion of the broader component increases systematically with increasing protein content of the recombinants, and by analogy with the interpretation of similar two-component spectra in other lipid–protein systems [see, e.g., Marsh & Watts (1982)], it is attributed to spin-labeled lipids interacting directly with the hydrophobic surface of the membrane protein. Qualitatively similar, although quantitatively different, results have been obtained with recombinants of different lipid/protein ratios labeled with the 14-PCSL and the 14-SASL spin probes. It should be noted here that the two-component nature of the ESR spectra of the recombinants does not correspond to the presence of unincorporated protein, since continuous sucrose density gradient centrifugation showed the recombinants to be of uniform composition and free of contamination by protein aggregates or pure lipid liposomes. Previous experiments employing the same reconstitution procedure (Brophy, 1977) have revealed that the recombinants are also reasonably uniform morphologically.

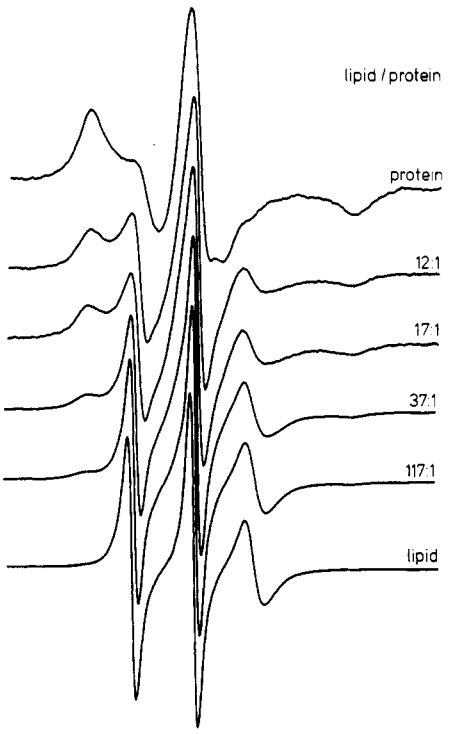

FIGURE 1: ESR spectra of the 14-PASL spin-label in myelin proteolipid apoprotein–dimyristoylphosphatidylcholine recombinants of various lipid/protein mole ratios. T = 30 °C. The upper spectrum corresponds to the protein alone and the lower spectrum to the lipid alone. Total scan width = 100 G.

The quantitative evaluation of the two-component spectra can be performed by spectral subtraction or, alternatively, by spectral addition, as illustrated in Figure 2 [see, e.g., Jost & Griffith (1978) and Marsh (1982)]. For subtraction of the motionally restricted component from the 30 °C spectrum to yield a mobile end point, the "protein-alone" spectrum taken at 30–32 °C was used as the reference spectrum. For the

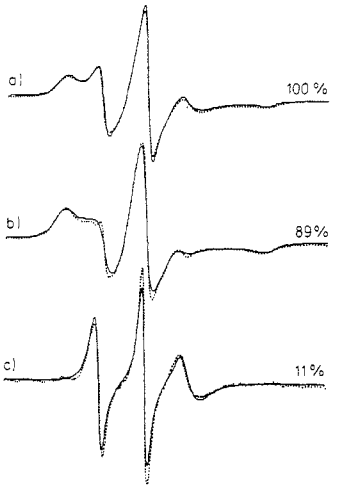

FIGURE 2: Spectral subtraction and addition with 14-PASL spin-label. Full lines are original spectra: (a) myelin proteolipid apoprotein–dimyristoylphosphatidylcholine recombinant of lipid/protein ratio 12/1 at 30 °C; (b) proteolipid apoprotein alone at 32 °C; (c) dimyristoylphosphatidylcholine alone at 25 °C. Dotted lines are summed spectra and difference spectra: (a) 11% lipid-alone spectrum plus 89% protein-alone spectrum; (b) recombinant spectrum minus 11% lipid-alone spectrum; (c) recombinant spectrum minus 89% protein-alone spectrum.

complementary subtractions, a pure lipid spectrum taken at 25–27 °C was used for the mobile component reference spectrum. In spectral additions the optimal fit of the composite line shape from the two added reference spectra was found by minimizing the mean square deviation between this and the experimental spectrum. To within experimental error, all three methods of analysis gave identical results (cf. Figure 2).

The results of the lipid–protein titration experiments with the 14-PASL, 14-PCSL, and 14-SASL spin probes were analyzed by spectral subtraction/addition with double integration of the fluid and motionally restricted components. The titrations were fitted to the approximate equation for lipid–protein association introduced previously (Brotherus et al., 1981; Knowles et al., 1981):

$$n_f^*/n_b^* = n_t/(n_1K_r) - 1/K_r \qquad (1)$$

where n_f^*/n_b^* is the ratio of the double-integrated intensities of the fluid and motionally restricted components in the ESR spectrum, n_t is the total lipid/protein ratio of the recombinant, n_1 is the number of lipid association sites on the protein, and K_r is the average association constant of the lipid probe with the protein *relative* to that of the background host lipid (DMPC). The data for 14-PASL, 14-PCSL, and 14-SASL are given in Figure 3, and all conform well to the lipid/protein ratio dependence of eq 1. For the 14-PCSL probe the intercept on the ordinate yields $K_r^{PC} = 1.0$, indicating no selectivity relative to the parent, unlabeled DMPC, as found previously in other systems (Knowles et al., 1979; Brotherus et al., 1981). The intercept on the abscissa yields an effective number of sites $n_1 = 9$ on the protein. The 14-PASL and 14-SASL probes display a definite selectivity over phosphatidylcholine with average relative association constants $K_r^{PA} = 3.2$ and $K_r^{SA} = 5.2$. The corresponding numbers of sites are $n_1 = 10$ and 9 per monomer protein for 14-PASL and 14-SASL, respectively, and are not significantly different from that of phosphatidylcholine.

Since the number of sites, n_1, remains approximately the same for the three spin-labeled lipids with different specificities for the protein, the selectivities for other lipids may be determined simply by comparing the spectra of the different spin-labels in a complex of fixed lipid/protein ratio. The ESR

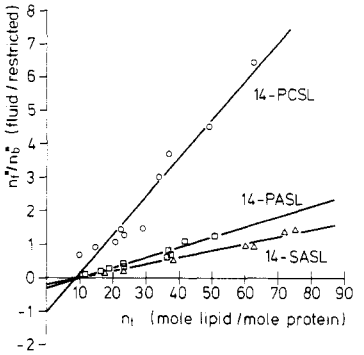

FIGURE 3: Lipid/protein titration of myelin proteolipid protein–dimyristoylphosphatidylcholine recombinants from the ESR difference spectra of the 14-PCSL phosphatidylcholine (○), 14-PASL (□), and 14-SASL (△) spin-labels at 30 °C. n_f^*/n_b^* is the ratio of the double-integrated intensity of the fluid and motionally restricted components in the ESR spectra of the recombinants and n_t is the total lipid/protein ratio.

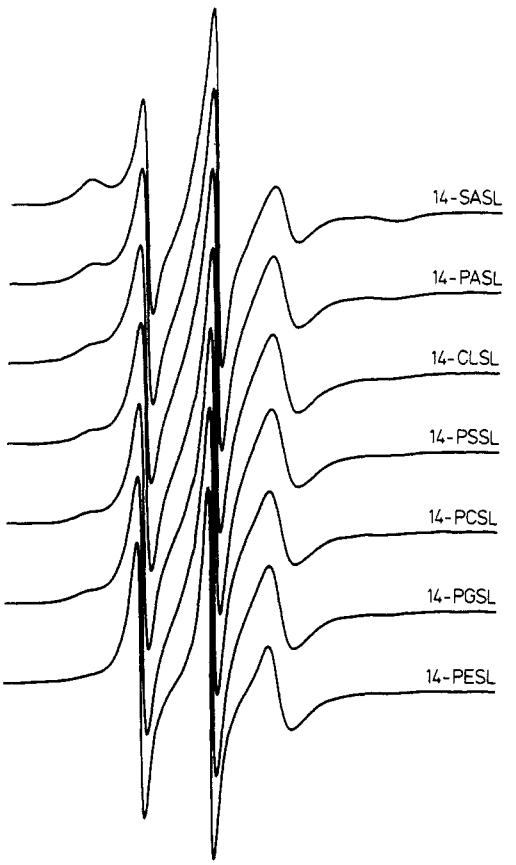

FIGURE 4: ESR spectra of the different head-group spin-labels in myelin proteolipid apoprotein–dimyristoylphosphatidylcholine recombinants of lipid/protein ratio 23/1 mol/mol, at $T = 30$ °C. Total scan width = 100 G.

spectra of various C-14 spin-labeled phospholipids in aliquots of a proteolipid apoprotein–DMPC sample of lipid/protein ratio 23/1 are given in Figure 4. All of the spectra consist of two components, with the different proportions of the fluid and motionally restricted components indicating the relative selectivities of the different phospholipids. The different fractions, f, of the motionally restricted component obtained by spectral subtraction/addition are listed in Table I. Assuming that n_1 is constant, for recombinants of a given lipid/protein ratio, one obtains

$$\frac{(n_f^*/n_b^*)^{PC}}{(n_f^*/n_b^*)^{L}} = \frac{K_r^{L}}{K_r^{PC}} \qquad (2)$$

Table I: Selectivity for the Motionally Restricted Spin-Labeled Lipids in Myelin Proteolipid Apoprotein-Dimyristoylphosphatidylcholine Recombinants of Lipid/Protein Ratio 23/1, $T = 30\ ^{\circ}C$[a]

L	f	K_r^L/K_r^{PC}	$\Delta G^{\circ}_L - \Delta G^{\circ}_{PC}$ (cal/mol)
SA*	0.83	7.0	−1170
PA*	0.67	2.9	−650
CL*	0.51	1.5	−240
PS*	0.49	1.4	−200
PG*	0.44	1.1	−70
PC*	0.41	1.0	0
PE*	0.25	0.48	440
ASL	0.19	0.34	650
CSL	0.13	0.22	930

[a] f, fraction of motionally restricted spin-label; K_r, relative average association constant, assuming a fixed number of sites.

Thus, the association constant, K_r^L, for a particular spin-labeled lipid, L, can be obtained relative to that for spin-labeled PC simply from the ratio of the values of $n_f^*/n_b^* = (1 - f)/f$ deduced from the spectral subtractions/additions. These relative association constants, and the differential free energies of association $\Delta G^{\circ}_L - \Delta G^{\circ}_{PC} = -RT \ln (K_r^L/K_r^{PC})$ deduced from them are given in Table I. (Since it was found from the titration in Figure 3 that there was no selectivity of the spin-labeled PC relative to the unlabeled DMPC, $K_r^{PC} = 1.0$, the association constants in Table I are referred not merely relative to the spin-labeled PC but directly to the host DMPC.) For phosphatidic acid, the value obtained for the relative association constant, $K_r^{PA}/K_r^{PC} = 2.9$ from Table I, agrees well with the values obtained from the titrations in Figure 3: $K_r^{PA} = 3.2$; $K_r^{PC} = 1.0$. For stearic acid the agreement is not quite so good: $K_r^{SA}/K_r^{PC} = 7.0$ from Table I, as compared with $K_r^{SA} = 5.2$ deduced from the titration. This discrepancy most probably reflects the inaccuracy of the single-point method for samples with intrinsically high selectivity.

Measurements have also been made with two spin-labeled steroid analogues, the cholestane spin-label, CSL, and the androstanol spin-label, ASL. The spectra are given in Figure 5. Although the line shapes are considerably different from those of the phospholipid labels, a second more motionally restricted component (indicated by the arrows) is clearly distinguishable in the outer wings of the narrower, fluid component characteristic of the DMPC lipids alone. Quantitation of the relative amounts of the fluid and motionally restricted components by spectral subtraction/addition indicates that the steroids are to a certain extent excluded from the lipid population interacting with the protein, i.e., $K_r^{ASL}/K_r^{PC} < 1$ (see Table I).

Discussion

The results of Figures 1, 2, 4, and 5 convincingly demonstrate the presence of a motionally restricted lipid component, in addition to the fluid lipid component, in the ESR spectra from the spin-labeled lipids in the lipid–protein complexes. The combination of spectral addition plus spectral subtraction illustrated in Figure 2 provides one of the most exacting tests to date of the two-component nature of this type of spectra. Essentially all of the spectral intensity is accounted for by the two components. Similar two-component spectra have previously been observed from fatty acid and steroid spin-labels in complexes of human myelin proteolipid protein with egg phosphatidylcholine (Boggs et al., 1976). The lipid–protein titrations of Figure 2 indicate that the motionally restricted spin-label component corresponds to lipids interacting directly with the intramembranous surface of the protein, as concluded previously for other systems (Marsh & Watts, 1982).

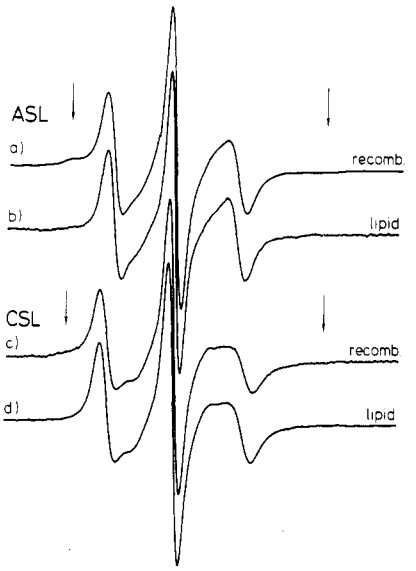

FIGURE 5: ESR spectra of the androstanol, ASL, and the cholestane, CSL, spin probes in myelin proteolipid apoprotein–dimyristoylphosphatidylcholine recombinants of lipid/protein ratio 23/1 mol/mol at $T = 30\ ^{\circ}C$. (a) ASL in 23/1 recombinant; (b) ASL in DMPC alone; (c) CSL in 23/1 recombinant; (d) CSL in DMPC alone. $T = 30\ ^{\circ}C$; total scan range = 100 G.

The stoichiometry of the interaction suggests that approximately 10 lipids per 25 000-dalton protein are associated with the first shell of motionally restricted lipids. Previous calorimetric and dilatometric measurements (Papahadjopoulos et al., 1975; Boggs & Moscarello, 1978; Boggs et al., 1980; Fodor et al., 1982) have indicated that a rather larger number of lipids are removed from the chain melting-phase transition by the protein. It is not surprising that the protein has a longer range effect on the highly cooperative lipid phase transition, presumably extending beyond the first shell. Curatolo et al. (1977) have estimated the number of DMPC molecules that could be accommodated around the PLP molecule. For a right circular cylinder the estimated values of n_1 range from 23 to 30 lipids per 25 000-dalton protein, depending on whether an appreciable portion of the protein protrudes into the aqueous phase. These higher values can be reconciled with our measured number of first shell lipids if it is assumed that the protein is oligomeric in the membrane, hence reducing the number of available surface sites per monomer. Light-scattering experiments have indicated that the protein is not monomeric in 2-chloroethanol, from which the lipid–protein complexes were formed (Lavialle et al., 1979). Sedimentation–equilibrium experiments have also indicated that the protein is a hexamer in nondenaturing detergents (Smith et al., 1982). For a hexamer, the above estimates for the number of lipids accommodated around the protein perimeter would be reduced to $n_1 \sim 10$–12 lipids per 25 000-dalton protein, in good accord with the values obtained from spin-label spectroscopy.

From the quantitative spectral subtractions an interesting picture also emerges as to the head-group selectivity of the phospholipid–proteolipid protein interaction (Figures 3–5). There is a clear preference for acidic phospholipids, in line with previous, more restricted, calorimetric data (Boggs et al., 1982). Of particular importance is the preference for phosphatidylserine, one of the quantitatively significant lipids in myelin. It can be conjectured that in myelin there might also be some selective interaction with the negatively charged cerebroside sulfate. Significant differences are, however, observed between the different negatively charged lipids, in-

dicating that the selectivity is not totally electrostatic in origin. For instance, phosphatidylserine, phosphatidylglycerol, and cardiolipin all bear the same formal charge (per two chains) at neutral pH but display different selectivities. The order of lipid selectivity found here for the proteolipid protein is also different from that found for other integral membrane proteins: cytochrome oxidase (Knowles et al., 1981), rhodopsin (Watts et al., 1979), and Na^+,K^+-ATPase (Marsh et al., 1982; M. Esmann, A. Watts, and D. Marsh, unpublished experiments). As might be expected, the specificity depends on the detailed conformation and structure of the protein polar groups as well as that of the phospholipids. Notable is the apparent discrimination against the zwitterionic phospholipid, phosphatidylethanolamine, which is also one of the major phospholipids of myelin. This presumably is a result of the different hydration and hydrogen-bonding potentialities of this phospholipid relative to those of phosphatidylcholine. A strong discrimination against the steroid probes, ASL and CSL, is also observed, which will have important implications for the structure and dynamics in the myelin membrane, if extrapolation can be made to the native sterol, cholesterol. A negative selectivity of cholesterol has previously been suggested for the Ca^{2+}-ATPase, from both activity measurements (Warren et al., 1975) and structural studies (Griffith et al., 1982). A possible explanation for this partial exclusion is that the rigid steroid nucleus may not be so easily accommodated at the protein surface and also, in view of the well-known condensing effect of cholesterol, may be better solubilized in the fluid lipid environment.

As previously stated, the results of the titrations in Figure 3 suggest that the selectivities for the different phospholipids arise from changes in relative association constant rather than changes in the total number of available interfacial sites. It should be noted, however, that the values for relative association constants and free energies of association given in Table I represent averages over the total number of available sites (Brotherus et al., 1981). It is not yet known whether the values truly represent a uniform increase in affinity for all sites or a larger increase in affinity for fewer sites or even for a single site. In the latter case the relative association constant for a single site could be up to 10 times larger, with a corresponding −1400 cal/mol increase in free energy of association. This would imply that the site would be mostly occupied by the specific lipid, even if this were present in relatively low abundance.

The line shapes of the difference spectra (cf. Figure 2) yield information about the dynamics of the lipid–protein interactions, in contrast to the thermodynamic features discussed above. Good agreement is obtained between the line shapes of the delipidated sample and the motionally restricted subtraction end point, apart from a small mismatch in the mobile triplet region to be discussed below. The outer hyperfine splitting is 62.8 ± 0.8 G, and the half-widths of the low-field and high-field extrema are 3.9 ± 0.1 and 4.9 ± 0.6 G, respectively, at 30 °C. The above parameters showed relatively little temperature dependence over the range 30–40 °C (data not shown). There was no significant difference between the spectral parameters of the delipidated samples (four determinations) and the motionally restricted subtraction end points (28 determinations). The lack of an appreciable temperature dependence (for the delipidated samples over a considerably greater range) suggests a relatively high degree of motional restriction for the protein-interacting lipids. By use of the empirical calibrations for slow isotropic spin-label motion (Freed, 1976), an estimate can be given for the rotational correlation time of the motionally restricted lipids of ~60–70 ns from the outer splittings and 20–30 ns from the line widths.[2] Since the spectra are close to the limits of motional sensitivity, and in view of the assumptions required, it is possible that these estimates may provide only a lower limit.

Although the agreement between the subtracted end points and the corresponding single-component spectra is very good, there are slight mismatches (cf. Figure 2). In addition, the best fits are obtained with a delipidated sample recorded at a few degrees higher, and a pure lipid sample recorded at a few degrees lower, than the temperature at which the spectrum of the recombinant is taken. This adjustment is necessary to allow for any perturbation of the lipids beyond the first boundary layer shell and also to take into account the exchange of lipid molecules between the two components (Knowles et al., 1979; Marsh et al., 1982). The effect of slow exchange will be a decreasing spectral anisotropy in the motionally restricted component, accompanied by a gradual broadening of the mobile component. This is qualitatively in accord with the above observations, suggesting the possibility of exchange in the microsecond time range. Exchange at such a rate would also be consistent with the observation of a single spectral component in the NMR spectra of deuterated phospholipids combined with the myelin proteolipid protein (Rice et al., 1979). A more detailed analysis of the dynamic properties of the protein-interacting lipids will be the subject of a future publication.

Registry No. DMPC, 18194-24-6; stearic acid, 57-11-4; androstanol, 58855-92-8; cholestane, 14982-53-7.

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Diamagnetism of Human Apo-, Oxy-, and (Carbonmonoxy)hemoglobin†

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ABSTRACT: In recent years, a controversy has arisen over the magnetic properties of oxyhemoglobin (HbO_2) and (carbonmonoxy)hemoglobin (HbCO). At present, it is unclear which, if any, conditions give a completely diamagnetic state for the heme–ligand complex which can be used as a diamagnetic reference state. In order to establish a diamagnetic reference independent of assignments of electronic configurations, we have measured the magnetic susceptibilities of apohemoglobin solutions and powdered iron-free protoporphyrin IX. We have also reexamined the magnetism of HbO_2 and HbCO solutions at 20 °C and at several ionic strengths. We find no difference in magnetism between HbO_2 and HbCO and no changes in their magnetism with solution conditions. Furthermore, relative to the new (apohemoglobin + porphyrin) diamagnetic reference, our data are consistent with complete diamagnetism for both HbO_2 and HbCO under all conditions we have studied. Our data imply that any low-lying triplet state must lie at least 900 cm^{-1} above the diamagnetic ground states. These results disagree strongly with reports of substantial room temperature paramagnetism for HbO_2 and a smaller paramagnetism for HbCO which disappears at high ionic strength [see Cerdonio, M., Morante, S., Vitale, S., Giacometti, G., & Brunori, M. (1982) in *Hemoglobin and Oxygen Binding* (Ho, C., Ed.) pp 63-68, Elsevier/North-Holland, Amsterdam, and references cited therein].

In recent years, there has been considerable interest in the magnetic properties of oxyhemoglobin (HbO_2) and (carbonmonoxy)hemoglobin (HbCO). For many years, both had been regarded as "diamagnetic", i.e., as having no unpaired electron spins either on the heme iron or on its ligand. However, in 1977, Cerdonio et al. reported evidence for unpaired spins (paramagnetism) in frozen human HbO_2 solutions at temperatures above 50 K. Since then, they have also reported paramagnetism at room temperature for human HbO_2 in solution, and for human and carp HbCO under certain solution conditions (Cerdonio et al., 1978, 1980, 1982, 1983). These results conflict with the early assignments of HbO_2 and HbCO as completely diamagnetic, spin = 0, compounds by Linus Pauling and co-workers (Pauling & Coryell, 1936) as well with later studies by Havemann et al. (1961).

The recent reports of paramagnetism in HbO_2 and HbCO raise an important question for both past and future studies of the magnetism of heme proteins in solution: What can be used as a "diamagnetic reference" state? In studies of the magnetism of metalloprotein solutions near room temperature, the diamagnetism of the solvent and polypeptide is generally much larger than any paramagnetism of the metallic ions. Thus, when we speak of a metalloprotein being "paramagnetic" what we mean is that the protein solution is found to be less diamagnetic than some diamagnetic reference state. This diamagnetic reference is usually a solution of the protein in a form known to have no unpaired spins. For hemoglobin, this diamagnetic reference has traditionally been a HbO_2 or HbCO solution. Clearly, if either HbO_2 or HbCO has nonzero paramagnetism (or magnetism which varies with solution conditions), then earlier studies which used them as a diamagnetic reference may be in error, and future studies require

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