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Lipid-Protein and Protein-Protein Interactions in Double Recombinants of Myelin Proteolipid Apoprotein and Myelin Basic Protein with Dimyristoylphosphatidylglycerol

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Received December 27, 1990; Revised Manuscript Received March 21, 1991

ABSTRACT: The integral proteolipid apoprotein (PLP) from bovine spinal cord has been reconstituted in dimyristoylphosphatidylglycerol (DMPG) bilayers, and the mutual interactions on binding the peripheral myelin basic protein (MBP) have been studied. Quantitation of protein and lipid contents in the MBP-PLP-DMPG double recombinants at different PLP:DMPG ratios led to the conclusion that MBP binds only to the DMPG lipid headgroups and is hindered from interaction with the first shell of lipids surrounding the PLP. No specific PLP-MBP association could be detected. Electron spin resonance (ESR) spectra of phosphatidylglycerol spin-labeled at position $n = 5$ in the *sn*-2 chain showed that complexation of MBP with the PLP-DMPG recombinants leads to a decrease in lipid chain mobility to an extent which correlates with the degree of MBP binding. At low DMPG:PLP ratios, the perturbations of lipid mobility by both proteins are mutually enhanced. In single recombinants of PLP with DMPG, the ESR spectra of phosphatidylglycerol spin-labeled at position $n = 14$ in the *sn*-2 chain indicated that approximately 10 lipids/protein are motionally restricted by direct contact with the intramembranous surface of the protein. This number is in agreement with earlier results for reconstitutions of PLP in dimyristoylphosphatidylcholine (DMPC) [Brophy, P. J., Horváth, L. I., & Marsh, D. (1984) *Biochemistry 23*, 860-865] and is consistent with a hexameric arrangement of the PLP molecules in DMPG bilayers. The selectivity of interaction of different spin-labeled lipids with PLP in single recombinants with DMPG is in the order cardiolipin ≈ stearic acid > phosphatidic acid > phosphatidylglycerol > phosphatidylethanolamine > phosphatidylserine > phosphatidylcholine, which differs from that found previously in recombinants with DMPC, due to differences in lipid-lipid interactions with the background lipid and shifts in the lipid pK_a due to the electrostatic surface charge. Binding of MBP to the PLP-DMPG recombinants decreased the specificity of interaction of the different lipids for the PLP, and modified the selectivity pattern which was found to be in the order cardiolipin > stearic acid > phosphatidylglycerol ≈ phosphatidic acid > phosphatidylethanolamine ≥ phosphatidylserine ≈ phosphatidylcholine. These changes in specificity can be attributed to the interaction of MBP with the bulk lipid regions of the double recombinants, and the extent of competition correlates with the previously measured selectivity of interaction of the different lipids with MBP bound to DMPG [Sankaram, M. B., Brophy, P. J., & Marsh, D. (1989) *Biochemistry 28*, 9699-9707].

Integral membrane proteins span the hydrophobic core of the lipid bilayer and present an apolar face to the surrounding acyl chain milieu. Peripheral proteins are associated with membrane surfaces through interactions with phospholipid headgroups or with other membrane proteins. These two different modes of membrane associations of proteins are manifest in their operational behavior. Relatively mild conditions, mostly high ionic strength buffers, release membrane-bound peripheral proteins whereas purification of in-

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0006-2960/91/0430-5866$02.50/0

tegral membrane proteins requires conditions under which the lipid bilayer structure is disrupted. The interactions between integral and peripheral proteins and their mutual influence on lipid–protein interactions are significant determinants of membrane structure and function. For instance, certain peripheral proteins, e.g., cytochrome *c*, are substrates of integral membrane enzymes or, e.g., the G-proteins, are involved with integral proteins in membrane signaling, whereas other peripheral proteins may have a purely structural association with integral protein membrane anchors, e.g., the spectrin cytoskeletal network of the erythrocyte.

A wide range of biophysical measurements have been undertaken on lipid–protein interactions in reconstituted membranes containing single proteins [see, e.g., Watts and De Pont (1985, 1986)]. Among these, spin-label electron spin resonance (ESR)[1] studies have proved to be especially fruitful [see, e.g., Marsh (1985, 1990)]. By contrast, relatively few investigations have been conducted on double reconstitutions involving both integral and peripheral proteins. Recombinant systems of this type are necessary for a detailed characterization of peripheral protein–integral protein interactions and of higher order lipid–protein interactions in membranes. As such, they represent an important further step in approaching the molecular complexity of intact biological membranes.

The myelin sheath is one system suitable for investigation of the mutual influence of peripheral and integral membrane proteins on one another [see Boggs et al. (1982) for a review]. This membrane contains approximately 24 wt % protein. Most of the protein component (≈85 wt %) comprises two proteins: the integral proteolipid apoprotein (PLP) and the peripheral myelin basic protein (MBP). Both these proteins have been isolated, purified, and reconstituted into bilayers formed from chemically well-defined phospholipids (Boggs & Moscarello, 1978; Boggs et al., 1976; Brophy, 1977). Spin-label ESR (Brophy et al., 1984) and ^2H nuclear magnetic resonance (Meier et al., 1987) studies on PLP–dimyristoylphosphatidylcholine (DMPC) recombinants are consistent with a hexameric arrangement of the protein in the membrane. The monomeric protein probably assumes a conformation in which three hydrophobic α-helical segments traverse the membrane (Stoffel et al., 1984; Laursen et al., 1984). The MBP, on the other hand, interacts strongly with acidic lipids (Palmer & Dawson, 1969; Sankaram et al., 1989a). The interaction is predominantly electrostatic between the lipid headgroups and the basic amino acid side chains (Sixl et al., 1984), although some hydrophobic interaction with the lipid chains has also been found (Sankaram et al., 1989a). Models have been proposed in which the MBP may interact directly both with the PLP and with the membrane lipid in the structure of compact myelin (Laursen et al., 1984; Rumsby, 1978).

In the present work, we have undertaken double reconstitutions of PLP and MBP in DMPG bilayers to characterize the protein-induced acyl chain motional restriction using spin-label ESR spectroscopy. This approach is particularly appropriate since the ESR technique is capable of resolving the spectral components of fluid lipid populations from those of any motionally restricted lipid populations that are induced by direct hydrophobic lipid acyl chain–protein associations. In addition, chemical binding assays have been found to yield direct insight into the interactions between the different molecular components of the protein–lipid double recombinants. These studies provide a detailed description of the interactions of the two proteins and the membrane lipids in the reconstituted system and have particular relevance to molecular models of the myelin structure.

Materials and Methods

Materials. DMPG was synthesized by transphosphatidylation of DMPC (Fluka, Buchs, Switzerland) catalyzed by phospholipase D (Comfurius & Zwaal, 1977). Spin-labeled positional isomers of stearic acid, *n*-SASL, were synthesized and used to acylate lysophosphatidylcholine to yield the corresponding spin-labeled phosphatidylcholines, *n*-PCSL, following the methods given in Marsh and Watts (1982). Other spin-labeled phospholipid species, *n*-PGSL, 14-PASL, 14-PSSL, and 14-PESL, were synthesized from spin-labeled phosphatidylcholine, using headgroup exchange, catalyzed by phospholipase D (Comfurius & Zwaal, 1977; Marsh & Watts, 1982). Spin-labeled cardiolipin, 14-CLSL, was synthesized according to Cable and Powell (1980). Hepes, 2-chloroethanol, and EDTA were from Sigma (St. Louis, MO).

Protein Isolation. Myelin was isolated from bovine spinal cord by the procedure of Benjamins et al. (1976). The PLP was extracted into chloroform–methanol (2:1 v/v) and delipidated by chromatography on Sephadex LH-20 in chloroform–methanol–0.01 M HCl (50:50:1 v/v) as described in Brophy (1977). Column chromatography was repeated until thin-layer chromatography and phosphate analyses revealed complete delipidation. The purified protein (M_r 25 000) contained less than 1 mol of lipid phosphorus/mol of protein. The protein was stored refrigerated in chloroform–methanol (1:1 v/v). Myelin basic protein was extracted and purified as described by Deibler et al. (1972). The protein was further purified by chromatography on a Sephadex G-75 superfine column (3 × 90 cm) equilibrated with 10 mM HCl. The protein (M_r 18 300) was pure as judged by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Laemmli, 1970).

PLP Reconstitution. Lipid–protein recombinants of PLP with DMPG were prepared by dialysis from 2-chloroethanol (Boggs et al., 1976; Brophy, 1977), essentially as described previously for recombinants with DMPC (Brophy et al., 1984). The required amount of PLP was precipitated by adding diethyl ether at −20 °C to the PLP solution in chloroform–methanol (1:1 v/v). After centrifugation and discarding the supernatant, the protein was dissolved in 2-chloroethanol to a concentration of 1 mg/mL, together with the required amount of DMPG. Samples in 2-chloroethanol were dialyzed exhaustively against three 5-L changes of reconstitution buffer (2 mM Hepes, 1 mM EDTA, and 10 or 100 mM NaCl at pH 7.4). Dialyzed complexes were loaded onto a sucrose density gradient (10–55%) and centrifuged for 3 h at 40 000 rpm in a Beckman Ti50 rotor. Each sample was recovered as a single band whose position on the gradient was determined by the lipid:protein ratio. In all cases, some free lipid fraction was obtained at the top of the gradient and was discarded. Protein and phosphate contents of the recombinants were assayed as described by Lowry et al. (1951) and Eibl and Lands (1969), respectively.

[1] Abbreviations: ESR, electron spin resonance; MBP, bovine spinal cord myelin basic protein; PLP, bovine spinal cord myelin proteolipid apoprotein; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; *n*-PGSL, 1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphoglycerol; 14-PASL, -PSSL, -PESL, and -PCSL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphoric acid, -phosphoserine, -phosphoethanolamine, and -phosphocholine, respectively; 14-CLSL, 1-(3-*sn*-phosphatidyl)-3-[1-acyl-2-*O*-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phospho]-*sn*-glycerol; 14-SASL, 14-(4,4-dimethyloxazolidine-*N*-oxyl)-stearic acid.

Double Reconstitution. The single PLP–DMPG recombinants of known lipid:protein ratio were dispersed in the appropriate buffer to which a solution of MBP in the same buffer (2.5 mg MBP/mg of DMPG) was added at 30 °C. Upon vortexing and incubation at 35 °C for 30 min, the complexes were pelleted by ultracentrifugation at 40000 rpm (Beckman Ti50 rotor), recovered, and used for ESR experiments. Both the supernatants and the pellets were analyzed for their lipid and protein contents as described above. No lipid was found in the supernatants.

ESR Spectroscopy. The single PLP–DMPG and the double MBP–PLP–DMPG recombinants were both spin-labeled by adding 10 μL of an ethanolic solution of the spin-label to 1 mL of the single-recombinant sample in the reconstitution buffer. After incubation for 10 min at room temperature, the recombinants were centrifuged (45 min, 90000*g*) to remove any unincorporated label, transferred to a 100-μL capillary tube, and sealed (after complexation with MBP in the case of the double recombinants). The samples were then concentrated by centrifugation before ESR measurements. ESR spectra were recorded on a Varian E-12 Century Line spectrometer equipped with a nitrogen gas flow temperature regulation system. Spectra were digitized by using a DEC-LPS system and a dedicated PDP 11/10 computer with VT-11 display. Digital spectral subtractions were performed as described earlier (Marsh, 1982). Apparent order parameters were calculated from the expression:

$$S^{\text{eff}} = (A_{\parallel} - A_{\perp})/[A_{zz} - \tfrac{1}{2}(A_{xx} + A_{yy})](a'_0/a_0) \quad (1)$$

where $2A_{\parallel}$ is given by the maximum outer hyperfine splitting, $2A_{\text{max}}$, and $A_{\perp}$ is given by

$$A_{\perp}(G) = A_{\text{min}}(G) + 1.4\{1 - (A_{\parallel} - A_{\text{min}})/[A_{zz} - \tfrac{1}{2}(A_{xx} + A_{yy})]\} \quad (2)$$

where $2A_{\text{min}}$ is the inner hyperfine splitting. a_0 is the effective isotropic hyperfine splitting constant and is given by

$$a_0 = (1/3)(A_{\parallel} + 2A_{\perp}) \quad (3)$$

a'_0 is the isotropic hyperfine splitting constant in the single-crystal environment in which the hyperfine tensor was determined and is given by

$$a'_0 = (1/3)(A_{xx} + A_{yy} + A_{zz}) \quad (4)$$

A_{xx}, A_{yy}, and A_{zz} being the principal values of the hyperfine tensor for doxylpropane (Jost et al., 1971). Detailed ESR lineshape simulations have shown that the spectra of lipid spin-labels contain important contributions from slow molecular motions (Lange et al., 1985; Moser et al., 1989). Therefore, the effective order parameters calculated by using eq 1, which assumes the fast motional limit, can only be considered as apparent values but are nonetheless useful for investigating the effects of binding the different proteins.

Results

MBP Binding in Double Recombinants. The saturation binding of MBP to DMPG–PLP single recombinants of different lipid:protein ratios was determined from assays of the total protein content of the resulting double recombinants. The PLP:DMPG ratio of the double recombinants was determined by lipid and protein assays of the single recombinants prior to binding MBP. The results are shown in Figure 1. At low PLP contents (PLP/DMPG ≤ 1.0 w/w), the binding stoichiometry for the MPB remains approximately constant. At higher PLP contents (PLP/DMPG > 1.0 w/w), the MBP binding decreases approximately linearly with increasing PLP:DMPG ratio, extrapolating to zero at a value of ca. 3.4 w/w.

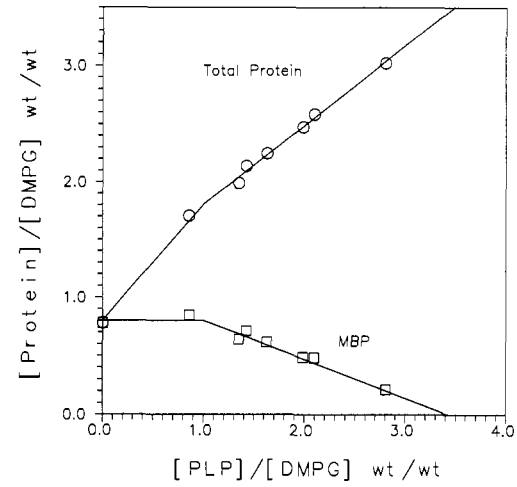

FIGURE 1: Total protein content (○) of PLP–DMPG recombinants, to which a saturating quantity of MBP is bound, as a function of the PLP:DMPG ratio of the original recombinant. Double recombinants were prepared as described under Materials and Methods. The MBP:DMPG ratio (□) is obtained from the difference between the total lipid:protein ratio and the PLP:DMPG ratio. The solid line given for the MBP/DMPG binding stoichiometry in the lower curve is calculated from eq 6, using values of N_1 = 10.7 mol/mol, N_c = 37.4 mol/mol, and $(\text{MBP/L})_0$ = 0.82 w/w. The solid line for the upper curve is derived from that for the lower curve plus the measured PLP/DMPG values given by the abscissa.

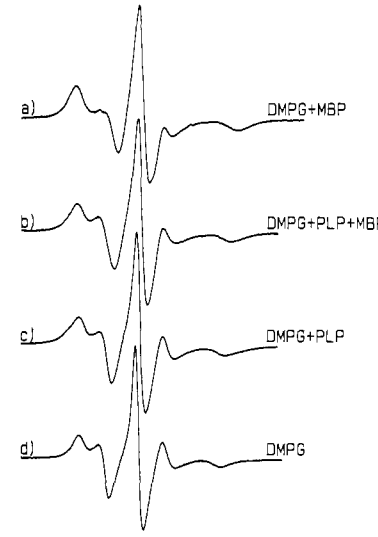

FIGURE 2: ESR spectra recorded at 30 °C of the 5-PGSL phosphatidylglycerol spin-label in (a) DMPG–MBP (36:1 mol/mol) complex, (b) DMPG–PLP–MBP double recombinant with a 25:1 DMPG:PLP mole ratio, (c) DMPG–PLP (25:1 mol/mol) single recombinant, and (d) DMPG alone. Buffer: 2 mM Hepes, 10 mM NaCl, and 1 mM EDTA, pH 7.4. Total spectral width = 100 G.

5-PGSL ESR Spectra. The ESR spectra of the C-5 atom position phosphatidylglycerol spin-label in complexes of MBP with DMPG and with PLP–DMPG recombinants, as well as in single PLP–DMPG recombinants and DMPG bilayers alone, are shown in Figure 2. The spectra were recorded at 30 °C, which is above the gel to liquid-crystalline phase transition temperature for all the systems. The fifth position in the acyl chain, being close to the phospholipid headgroup region, does not lead to spectra displaying extensive motional averaging of the hyperfine and **g** tensors. The spectral anisotropy for this position is a good indicator of the electrostatic headgroup–MBP interactions [cf. Sankaram et al. (1989a)]. The spectral anisotropy of the 5-PGSL label is seen to increase

Table I: Outer Hyperfine Splitting Constants (A_{max}) and Apparent Order Parameters (S^{eff}) of 5-PGSL in DMPG Bilayers, DMPG–PLP Single Recombinants, DMPG–MBP Complexes, and DMPG–PLP–MBP Double Recombinants[a]

lipid/protein (ratio)	10 mM NaCl		100 mM NaCl	
	A_{max} (G)	S^{eff}	A_{max} (G)	S^{eff}
DMPG	25.0	0.54	24.7	0.52
DMPG + MBP	28.6	0.71	28.0	0.69
DMPG + PLP (25:1)	26.4	0.60	26.2	0.61
DMPG + PLP (17:1)	26.7	0.63	26.75	0.62
DMPG + PLP (25:1) + MBP	27.4	0.65	26.9	0.64
DMPG + PLP (17:1) + MBP	29.0	0.68	28.0	0.67

[a] In 10 and 100 mM NaCl for DMPG:PLP ratios of 25:1 and 17:1 mol/mol. T = 30 °C.

on going from the pure DMPG bilayers to the various lipid–protein recombinants. The outer hyperfine splitting constant and effective order parameter, A_{max} and S^{eff}, respectively, are given in Table I at two different DMPG:PLP ratios and salt concentrations. Similar systematic changes to those given in Table I are also seen in the spectra recorded at 40 °C (data not shown). Both A_{max} and S^{eff} increase with decreasing lipid:protein ratio in the single (DMPG–PLP) recombinants, presumably due to interaction with the hydrophobic surface of the PLP. These increases are not as great as those arising from electrostatic interaction on complexing MBP with DMPG alone. A further increase in these parameters for the DMPG–PLP single recombinants is observed on subsequent complexation with MBP. At a DMPG:PLP ratio of 25:1, the increase in spectral anisotropy is still not as great, however, as that observed for DMPG–MBP complexes alone. This attenuation presumably results from the reduced binding of MBP in the presence of PLP, as is indicated in Figure 1.

PLP Lipid Stoichiometry and Selectivity. The ESR spectra of different phospholipids, each spin-labeled at the C-14 atom of the *sn*-2 chain, in PLP–DMPG single recombinants are shown in Figure 3. Cardiolipin, stearic acid, phosphatidylserine, phosphatidic acid, phosphatidylglycerol, phosphatidylethanolamine, and phosphatidylcholine spin-labels were used to determine the selectivity of interaction of these lipids with PLP in the DMPG host. All the spin-labels yield two-component ESR spectra, but the relative amounts of the two components vary with the particular spin-labeled lipid. One of the spectral components has a narrow hyperfine splitting and represents the fluid bilayer lipids. The other, broader component, which is visible in the wings of the spectrum, corresponds to lipids whose mobility is considerably restricted by direct interaction with the intramembranous section of the protein [see, e.g., Marsh (1985)]. These two components could be resolved and quantitated by digital spectral subtractions where an appropriate fluid component spectrum was chosen from a library of 14-PGSL spectra in pure DMPG bilayers at different temperatures in the fluid phase. The spectrum

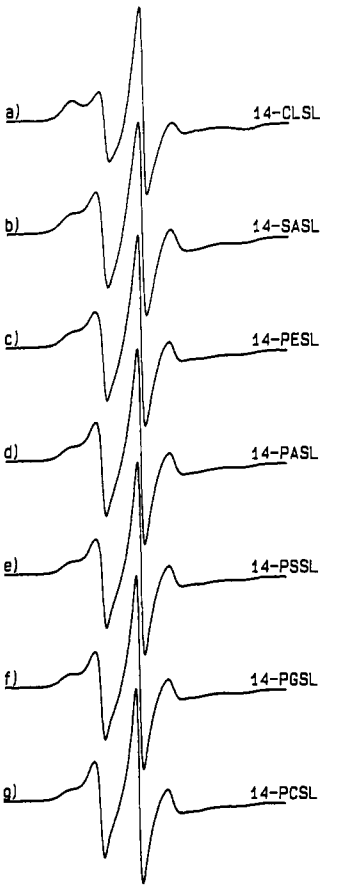

FIGURE 3: ESR spectra of phospholipid spin-labels labeled at the C-14 atom of the *sn*-2 chain in DMPG–PLP single recombinants (DMPG:PLP mole ratio = 13:1) at 30 °C. (a) Cardiolipin spin-label, 14-CLSL; (b) stearic acid spin-label, 14-SASL; (c) phosphatidylethanolamine spin-label, 14-PESL; (d) phosphatidic acid spin-label, 14-PASL; (e) phosphatidylserine spin-label, 14-PSSL; (f) phosphatidylglycerol spin-label, 14-PGSL; (g) phosphatidylcholine spin-label, 14-PCSL. Spectral width = 100 G.

at 26 °C was found to correspond best with the fluid component in the two-component PLP–DMPG spectrum at 30 °C. The motionally restricted component reference spectrum for subtractions was chosen from a set of 14-label spectra from an extensively delipidated protein sample recorded at several temperatures [cf. Brophy et al. (1984)]. The values for the fraction of motionally restricted lipid, f, derived from the spectral subtractions for the different spin-labeled lipids in PLP–DMPG single recombinants are given in Table II. The values of f display a well-defined pattern of specificity for the interaction of the different lipids with PLP in the DMPG single recombinants.

Spectral subtractions were also performed for PLP–DMPG recombinants of different lipid:protein ratios with the 14-PGSL label incorporated. The results were analyzed according to

Table II: Selectivity for the Motionally Restricted Spin-Labeled Lipids in Myelin Proteolipid Apoprotein (PLP)–Dimyristoylphosphatidylglycerol (DMPG) and in Myelin Basic Protein (MBP)–PLP–DMPG Recombinants of Lipid:PLP Ratio 13:1 mol/mol (T = 30 °C)[a]

lipid	f		K_r^L/K_r^{PC}		$\Delta G°_L - \Delta G°_{PC}$ (kJ/mol)	
	PLP	PLP + MBP	PLP	PLP + MBP	PLP	PLP + MBP
14-SASL	0.84	0.80	2.9	2.3	−2.7	−2.1
14-CLSL	0.84	0.82	3.0	2.7	−2.8	−2.5
14-PSSL	0.71	0.65	1.4	1.1	−0.8	−0.2
14-PGSL	0.78	0.76	2.0	1.8	−1.8	−1.5
14-PASL	0.81	0.75	2.4	1.7	−2.2	−1.4
14-PESL	0.74	0.67	1.7	1.2	−1.3	−0.4
14-PCSL	0.64	0.64	1.0	1.0	0	0

[a] The ratio of relative association constants is given by $K_r^L/K_r^{PC} = [(1-f)/f]^{PC}/[(1-f)/f]^L$, and the relative free energies of association are given by $\Delta G°_L - \Delta G°_{PC} = -RT \ln (K_r^L/K_r^{PC})$; see, e.g., Marsh (1985).

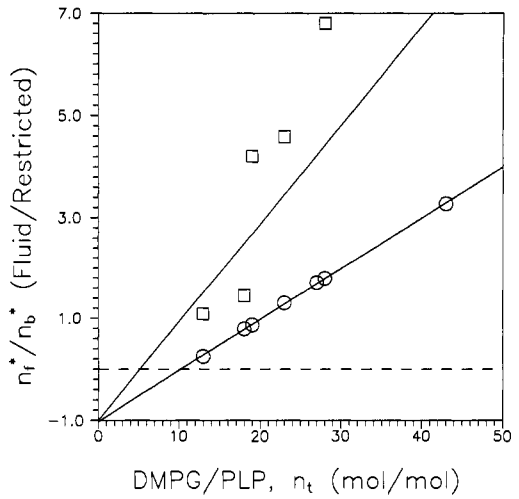

FIGURE 4: Lipid–protein titration of DMPG–PLP (○) and DMPG–PLP–MBP (□) recombinants obtained from the ESR difference spectra of the 14-PGSL spin-label. n_f^*/n_b^* is the ratio of the double-integrated intensity of the fluid and motionally restricted components in the ESR spectra of the type shown in Figure 3. n_t is the total lipid:PLP ratio in the complexes. The linear regression for DMPG–PLP (○) according to eq 5 yields values of $N_1 = 10$ sites and a relative association constant of $K_r = 1$. The line for DMPG–PLP–MBP is the predicted dependence with $N_1 = 5$ and $K_r = 1$ but with a fluid lipid reference spectrum corresponding to the MBP–DMPG complexes (see text).

the equation for lipid–protein association [see, e.g., Marsh (1985)]:

$$n_f^*/n_b^* = n_t/N_1K_r - 1/K_r \quad (5)$$

where n_t is the total lipid:protein ratio in the PLP–DMPG recombinant, n_f^* is the fraction of the fluid spin-label component obtained by subtraction, n_b^* is the fraction of the motionally restricted spin-label component obtained by subtraction, N_1 is the number of lipid association sites on the protein, and K_r is the relative association constant for the spin-label with respect to the background host lipid (DMPG). The resulting plot of n_f^*/n_b^* [$=(1-f)/f$] vs n_t is shown in Figure 4, the slope and intercept of which yield values for N_1 and K_r. The relative association constant K_r is unity for 14-PGSL, indicating no selectivity in interaction of the spin-label with the protein compared with the parent DMPG. The number of motionally restricted lipids corresponds to 10 per PLP monomer.

Double Recombinants. Similar two-component spectra were observed for the $n = 14$ position spin-labels in MBP–PLP–DMPG double recombinants. For a given PLP:DMPG ratio, the fraction of motionally restricted lipid component was found to be less in the presence of MPB at saturation binding than in the absence of MBP. This can be seen in Figure 5 from intersubtractions [see, e.g., Knowles et al. (1981)] between the spectra of the 14-PGSL label in single and double recombinants at a DMPG:PLP ratio of 13:1 mol/mol. Subtraction of the double-recombinant spectrum from that of the single recombinant yields a motionally restricted difference spectrum as end point, and the reverse subtraction yields a fluid spectrum as end point. The effect of binding of MBP on the selectivity of interaction of the different spin-labeled phospholipid species with PLP, determined in this way, is given in Table II. The selectivity pattern is significantly different from that in the PLP–DMPG single recombinants, and the strength of the selectivity relative to phosphatidylcholine is considerably reduced for all lipids.

The results of lipid–protein titration with the 14-PGSL spin-label in the double recombinants are given in Figure 4.

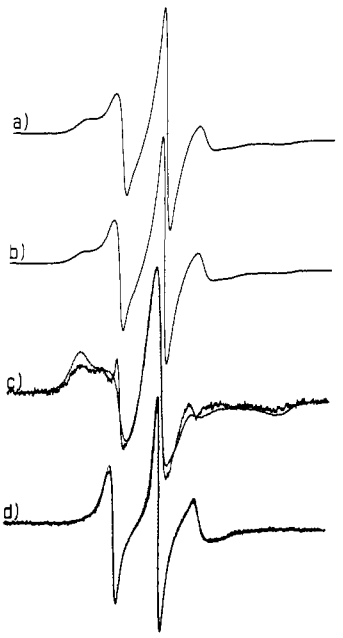

FIGURE 5: Spectral subtractions for the 14-PGSL spin-label in the double- and single-recombinant systems. (a) Spectrum for DMPG–PLP single recombinant (DMPG/PLP = 13:1 mol/mol); (b) spectrum for DMPG–PLP–MBP double recombinant (DMPG/PLP = 13:1 mol/mol); (c) (—) motionally restricted difference spectrum end point obtained by subtracting spectrum b from spectrum a, (---) spectrum of (delipidated) PLP alone; (d) (—) fluid end point difference spectrum obtained by subtracting spectrum a from spectrum b, (---) spectrum of DMPG alone. Spectra recorded at 30 °C. Total scan width = 100 G.

There is a considerable amount of scatter in the data for the double recombinants, but they consistently indicate a smaller population of motionally restricted lipid than in the single recombinants of comparable DMPG:PLP ratio. It is not possible to determine a stoichiometry from these data; the straight line drawn in the figure serves solely as a point of reference.

Discussion

The present work addresses the question of both lipid–protein and protein–protein interactions in the myelin sheath, for both the integral proteolipid apoprotein and the peripheral basic protein. These results will be discussed first in terms of the binding stoichiometries of MBP to the PLP recombinants, and then the ESR results obtained from the 5-position and 14-position phosphatidylglycerol spin-labels will be discussed separately.

MBP Binding in Double Recombinants. The saturation binding stoichiometry of MBP to the single recombinants with PLP remains constant at the value found for the pure lipid (in the absence of PLP) until PLP contents corresponding to a PLP:DMPG ratio of 1:1 w/w in the single recombinants. At values beyond this critical level, the MBP binding stoichiometry then progressively decreases with increasing PLP content in the recombinants. This behavior strongly suggests that at low PLP:DMPG ratios the MBP does not interact directly to any great extent with the PLP but binds preferentially only to the lipids. These conclusions are consistent with the inability of the major fraction of mature MBP to aggregate PC vesicles containing PLP (Woods & Moscarello, 1989). A limited binding of delipidated PLP in Triton X-100 to immobilized MBP has been found (Edwards et al., 1989), but clearly any such association is insignificant relative to the binding of MBP to negatively charged lipid in PLP–DMPG complexes. In the present work, appreciable PLP–MBP in-

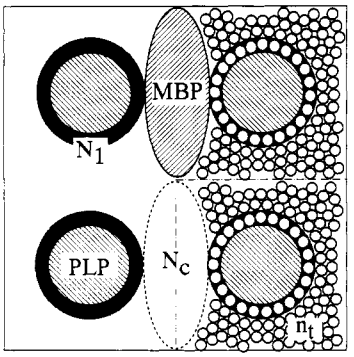

FIGURE 6: Schematic indication of the steric exclusion between the myelin proteolipid protein and the myelin basic protein in double recombinants with a DMPG:PLP ratio of n_t. A critical lower DMPG:PLP ratio, N_c, is required such that the mean PLP–PLP separation is sufficiently large that the binding of MBP to the lipid is undisturbed by the presence of the PLP. At DMPG:PLP ratios below this critical PLP packing density, N_c, the MBP is unable to bind to the first shell of lipids, N_1, surrounding the PLP. If it is assumed that the extent of MBP binding is then proportional to the ratio of the fraction of lipid molecules available for binding, relative to the fraction available at the critical limiting stoichiometry, the dependence of the MBP binding on n_t is given by eq 6.

teractions do not occur until higher PLP:DMPG ratios, at which point the interaction appears to be one of steric exclusion, rather than an energetically favorable interaction between the two proteins, since at this point the binding stoichiometry of the MBP decreases. In support of this suggestion is the fact that the critical PLP content at which the break occurs in the binding curve of Figure 1 corresponds to a DMPG:PLP molar ratio of approximately 37:1. This value is very close to the DMPG/MBP molar stoichiometry at saturation binding in the absence of PLP [cf. Figure 1 and Sankaram et al. (1989a)], and therefore is consistent with the idea that a minimum critical area of lipid surface between PLP complexes is required for binding of the MBP (cf. Figure 6).

Above the critical value, the decrease in MBP binding stoichiometry is approximately linear with increasing PLP content of the recombinants. Significantly, the MBP binding stoichiometry extrapolates to zero at a DMPG:PLP ratio close to that for the N_1 boundary lipids interacting with the intramembranous surface of the PLP [cf. above and Brophy et al. (1984)]. This dependence can be predicted by a simple model (Figure 6), if it is assumed that the extent of MBP binding is proportional to the ratio of the fraction of lipid molecules available for surface binding, relative to the fraction available at the average critical limiting stoichiometry, N_c, required for binding. If it is assumed that, beyond the critical value, N_c, the PLP boundary lipids are not available for binding of MBP, the total fraction of lipids available is $(n_t - N_1)/n_t$, where n_t is the total DMPG:PLP molar ratio in the recombinant. The fraction of lipids available at the limiting stoichiometry, N_c, is similarly $(N_c - N_1)/N_c$. For the above model, the dependence of the MBP binding stoichiometry at saturation on n_t is therefore given by

$$(\mathrm{MBP/L}) = (\mathrm{MBP/L})_0[1 - (1/n_t - 1/N_c)/(1/N_1 - 1/N_c)] \quad (6)$$

where $(\mathrm{MPB/L})_0$ is the MBP stoichiometry for binding to the lipid alone. Equation 6 is valid for $n_t \leq N_c$, and as can be seen from Figure 1, predicts rather well the decrease in MBP binding stoichiometry with decreasing n_t, with the values N_1 = 10.7 mol/mol and N_c = 37.4 mol/mol. The former is essentially identical with the stoichiometry of the motionally restricted boundary lipid component found in PLP single recombinants with both DMPG (cf. above) and DMPC (Brophy et al., 1984), and the latter is close to the molar stoichiometry of MBP with DMPG alone (Sankaram et al., 1989a), as discussed above.

5-PGSL Hyperfine Splittings. The effects of the reduced binding stoichiometry of MBP in the presence of PLP on the increases, ΔA_{max}, induced in the outer hyperfine splitting of the 5-PGSL spin-label can be predicted by eq 6 if it is assumed that the spectra of the 5-PGSL label correspond to fast exchange on the nitroxide ESR timescale [cf. Sankaram et al. (1989b)]:

$$\Delta A_{max} = \Delta A_{max}'(1 - N_1/n_t)/(1 - N_1/N_c) \quad (7)$$

where $\Delta A_{max}'$ is the value of ΔA_{max} at saturation binding to DMPG alone. For the DMPG–PLP recombinant of lipid:protein ratio 25:1 mol/mol, the value of $\Delta A_{max}/\Delta A_{max}'$ is 0.7 (see Table I), which is in fair agreement with the value of 0.80 predicted by eq 7. For the recombinant with lower lipid:protein ratio (17:1 mol/mol), it is clear that the reduction in binding stoichiometry of MBP is outweighed by mutually reinforcing perturbations of the lipid by both the PLP and the MBP, since the value of A_{max} for the double recombinant is very similar to that for DMPG alone at maximum binding of MBP. Although the binding of the MBP appears to be consistent with an association with independent lipid domains (see Figure 1), the mutual interactions of both proteins with the lipid are more complex and take place in a concerted fashion at low DMPG:PLP ratios.

Lipid Stoichiometry and Selectivity for PLP Single Recombinants. The data obtained with the C-14 position spin-label relate mainly to the lipid interaction with the intramembranous surface of the PLP. The data for the PLP–DMPG single recombinants are consistent with those obtained previously from PLP–DMPC recombinants (Brophy et al., 1984). The difference between the two sets of experiments is that the reconstituting lipid is negatively charged in the present case whereas it was zwitterionic in the previous studies. The phosphatidylglycerol spin-label displays no selectivity relative to the background DMPG (K_r = 1, cf. Figure 4), indicating very little effect of the spin-label group on the thermodynamics of the interaction. The stoichiometry of the first shell of lipids (10 lipids/PLP monomer) is very similar to that found previously in DMPC (Brophy et al., 1984), indicating that the oligomeric state of the PLP is most probably the same in the two different lipids. It was previously shown that the stoichiometry was consistent with a hexameric state of the PLP, in agreement with hydrodynamic measurements in nonionic detergents (Smith et al., 1984).

The lipid specificity established for the different spin-labeled lipid species in Table II, however, is rather different from that found relative to a DMPC host for the same spin-labels. The selectivity in DMPG is in the sequence CLSL ≈ SASL > PASL > PGSL > PESL > PSSL > PCSL, whereas that found previously in DMPC had the sequence SASL > PASL > CLSL ≥ PSSL > PGSL ≈ PCSL > PESL (Brophy et al., 1984). This difference must presumably arise from the different bulk properties of the background host lipid (DMPG in one case and DMPC in the other) to which the relative association constants in Table II are referred as standard state. The energetics of the lipid–lipid interaction differ between the various lipid species depending on the host lipid matrix in which they are incorporated. This points to a possible way in which the complex lipid composition of natural biological membranes may modulate the local lipid environment of the integral proteins, other than by the direct affinity of the different lipid species at the lipid–protein interface. A further way in which the host lipid background may affect the af-

finities is via the high surface charge density of the DMPG bilayers. This will give rise to shifts in the apparent pK_a of the lipids due to the electrostatically enhanced proton concentration at the lipid–water interface. For instance, stearic acid has a pK_a of 6.7 in DMPC bilayers (Horváth et al., 1988), whereas in DMPG bilayers at low ionic strength it is increased to a value of 8.0 (Sankaram et al., 1989c). Phosphatidic acid in DMPC bilayers also has a pK_a in the neutral range (Horváth et al., 1988), whereas that for cardiolipin lies at much lower pH. Therefore, it seems likely that the change in the relative selectivities of these three lipids can be explained by pK_a shifts of 14-SASL and 14-PASL, giving rise to an increased degree of protonation, which is known to reduce the selectivity for the PLP (Horváth et al., 1988).

Stoichiometry and Selectivity for PLP in Double Recombinants. The spectra of the 14-PGSL label in the double recombinants indicate that the proportion of motionally restricted lipids is reduced relative to that in the single recombinants (see Figure 5), due to binding of the MBP. This suggests that the MBP disturbs the interaction of the lipids with the intramembranous portion of the PLP, possibly by means of the membrane-penetrant sections of the MBP. (Note that the motionally restricted component quantitated in Figure 4 comes in the first approximation from interaction with the PLP and not from possible hydrophobic interaction with the MBP, since the data were determined by spectral intersubtraction between double recombinants both containing saturating amounts of MBP but different amounts of PLP.) The reduction in the fraction of motionally restricted lipids associated with the PLP could come either from a reduction in the stoichiometry of association sites or from a reduction in the selectivity of interaction, or from both. Unfortunately, the scatter on the data in Figure 4 does not allow a firm decision on this point. In a sense, the results with the 14-PGSL label given in Figure 4 are the converse of those obtained with the 5-PGSL label which are given in Table I. The presence of the MBP reduces the extent of interaction of the lipids with the PLP and vice-versa, indicating a mutually reciprocal effect of the integral and peripheral protein on the characteristic types of lipid–protein interaction with the corresponding species.

The binding of the MBP also has some influence on the pattern of lipid selectivity for the PLP (cf. Table II). The selectivity sequence of the 14-position labels in the double recombinants follows the order CLSL > SASL > PGSL ≈ PASL > PESL ≥ PSSL ≈ PCSL. The differences from the single recombinant are not so great as those between the DMPG and DMPC single recombinants and lie mainly in that the selectivity of SASL is definitely reduced relative to CLSL, that of PASL becomes comparable to that for PGSL, and the selectivities of PESL, PSSL, and PCSL all become more comparable. These effects can be interpreted as above, in terms of a different standard state to which the selectivities are referred, in the one case DMPG and in the other case DMPG with MBP bound. The latter situation is related directly to the preferential selectivities of the various lipids for interaction with MBP when bound to DMPG. This selectivity sequence has been established previously by using 5-position spin-labels (Sankaram et al., 1989a,b) and is in the order PSSL > $SASL^-$ > CLSL > $SASL^0$ ≥ $PASL^{2-}$ > PGSL > $PASL^-$ > PESL > PCSL, where the values for PASL and SASL are given for the two different protonation states. With the exception of PSSL, this selectivity sequence is mostly in a similar order to that for the PLP in the single recombinants and therefore will have the effect of reducing the relative selectivities for PLP in the double recombinants. For example, all relative association constants for PLP in Table II are reduced relative to that for PCSL on binding MBP. The competing effects between the two proteins can be seen in the reduction of the selectivity for PLP of PESL relative to PCSL and PASL relative to PGSL, and the increase in selectivity of CLSL relative to SASL. The large selectivity of PSSL for MBP (Sankaram et al., 1989a) serves to reduce yet further its already low selectivity for PLP.

In summary, it is found that the MBP does not bind directly to the PLP in double-recombinant DMPG membranes. The interaction between the two proteins is rather one of steric exclusion: the MBP is unable to associate with the first shell of lipids surrounding the PLP (cf. Figure 6). The presence of either protein tends to reduce the extent of lipid perturbation by the other. The lipid selectivity of the PLP, which is found to be different in DMPG from that in DMPC, is reduced by direct competition on binding of MBP. These results are of direct relevance to the molecular interactions taking place in the compaction of nerve myelin and also illustrate some of the principles that may be generally operative in biological membranes.

Acknowledgments

We thank Mrs. S. Chatterjee and Frau B. Angerstein for their skillful technical assistance in the preparation of the proteins and spin-labeled lipids, respectively.

Registry No. DMPG, 61361-72-6; stearic acid, 57-11-4.

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Regulation of CTP:Phosphocholine Cytidylyltransferase by Lipids. 1. Negative Surface Charge Dependence for Activation†

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Received July 6, 1990; Revised Manuscript Received March 18, 1991

ABSTRACT: The activity of phosphocholine cytidylyltransferase (CT), the regulatory enzyme in phosphatidylcholine synthesis, is dependent on lipids. The enzyme, obtained from rat liver cytosol, was purified in the presence of Triton X-100 [Weinhold et al. (1986) *J. Biol. Chem. 261*, 5104]. The ability of lipids to activate CT when added as Triton mixed micelles was limited to anionic lipids. The relative effectiveness of the lipids tested suggested a dependence on the negative surface charge density of the micelles. The mole percent lipid in the Triton mixed micelle required for activation decreased as the net charge of the lipid varied from 0 to −2. Evidence for the physical association of CT with micelles and vesicles containing phosphatidylglycerol was obtained by gel filtration. The activation by micelles containing PG was influenced by the ionic strength of the medium, with a higher surface charge density required for activation at higher ionic strength. The micelle surface potential required for full activation of CT was calculated to be −43 mV. A specificity toward the structure of the polar group of the acidic lipids was not apparent. CT was activated by neutral lipids such as diacylglycerol or oleyl alcohol when included in an egg PC membrane, but the activities were reduced by dilution with as little as 10 mol % Triton. Thus Triton mixed micelles are not suitable for studying the activation of CT by these neutral lipid activators. We conclude that one way that lipid composition can control CT–membrane binding and activity is by changing the surface potential of the membrane. Other distinct mechanisms involved in the activation by neutral lipids are discussed.

Cytidylyltransferase (CT)[1] is a key regulatory enzyme in the synthesis of phosphatidylcholine (PC) in higher eucaryotes. The regulation of CT resembles that of a group of proteins (including protein kinase C and 5-lipoxygenase) which have been referred to as *amphitropic* (Burn, 1988). The enzyme is active in its membrane-bound form and inactive in its lipid-free cytosolic form[2] and appears to interconvert between the two forms in response to a variety of effectors of PC biosynthesis (Sleight & Kent, 1980, 1983a,b; Tercé et al., 1988; Weinhold et al., 1981; Pelech et al., 1981, 1983, 1984a,b; Lim et al., 1983; Cornell & Vance, 1987a; Sanghera & Vance, 1989a). There is disagreement as to which cellular membrane CT binds: the endoplasmic reticulum, Golgi, or the nuclear membrane (Tercé et al., 1988; Vance & Vance, 1988; Morand & Kent, 1989).

† This work was supported by grants from the British Columbia Health Care Research Foundation and the Natural Sciences and Engineering Research Council of Canada.
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[1] Abbreviations: CT, CTP:phosphocholine cytidylyltransferase; PC, phosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; DPPC, dipalmitoylphosphatidylcholine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

[2] A third form is present in some cells—a high molecular weight cytosolic lipid–protein aggregate of undefined physical structure (Weinhold et al., 1989, 1991).

0006-2960/91/0430-5873$02.50/0