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Selectivity of lipid–protein interaction with myelin proteolipids PLP and DM-20. A fluorescence anisotropy study

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The two main myelin proteolipids, PLP (30 kDa) and DM-20 (25 kDa), differ by an internal deletion in DM-20. The deleted fragment, of 35 amino acids (116–150), corresponds to the major hydrophilic domain of PLP. Fluorescence anisotropy experiments using diphenylhexatriene as a fluorescent probe were performed to detect the phase separation induced by these two proteolipids in multilamellar vesicles of binary composition. We found that in vesicles composed of 30% L-α-PS and 70% DPPC, the PLP boundary layer contained about 18 motionally restricted phospholipids, almost exclusively L-α-PS. On the contrary, the DM-20 boundary layer contained only 14 to 15 phospholipids, with a composition no different from that of the bulk vesicle. In mixtures of DMPG and DPPC, the selectivity of PLP for the acidic phospholipid DMPG was maintained, but was lower than that observed for L-α-PS. We assume that this selectivity of PLP stems mainly from electrostatic interactions between the charged residues of the 116–150 fragment, deleted in DM-20, and the acidic phospholipids. These results suggest that fragment 116–150 may play a specific role in the interaction of PLP with the lipid bilayer of the myelin membrane.

Introduction

Central-nervous-system myelin has a relatively simple composition: 30% proteins, 70% lipids. More than 50% of the total myelin proteins are intrinsic membrane proteins known as proteolipids [1]: a knowledge of the effect of these hydrophobic proteins on lipid organization in the membrane helps to clarify the formation and structure of the myelin sheath.

Most of the studies devoted to proteolipid–lipid interactions have been run on total myelin proteolipids extracts, known as Folch-Pi apoprotein or proteolipid apoprotein or lipophilin (in the case of human myelin).

Using vesicles composed of well-defined phospholipids as model membranes, the perturbation induced in the lipid organization by the insertion of the proteolipid apoprotein has been explored by physical techniques such as freeze-fracture electron microscopy [2–5], X-ray diffraction [6,7] differential scanning calorimetry [4,6] and various spectroscopic techniques: electron-spin resonance [8–10], Raman spectroscopy [5], nuclear magnetic resonance [11–13], circular dichroism [14], intrinsic fluorescence [15] and fluorescence anisotropy [13]. However, in most of these studies, little or no attention has been paid to the heterogeneity of the proteolipid apoprotein, considered as a single entity.

Recently, we were able to separate and purify to homogeneity the two major myelin proteolipids, PLP (molecular mass 30 kDa) and DM-20 (molecular mass 25 kDa) [16]. We have shown that these two proteolopids differ by an internal deletion in DM-20 [17,18] and the fragment deleted was found to be fragment 116–150 [19–21]. This sequence, particularly rich in charged amino acid residues [22], is assumed to be involved in the compaction of myelin [23]. It is, therefore, particularly interesting to study and compare the interactions of PLP and DM-20 with lipids.

In this paper, we report the effects of the incorporation of PLP ad DM-20 into the phospholipid bilayers, measured by fluorescence anisotropy, using diphenylhexatriene as a fluorescent probe. This technique appraised the lipid chains organization both below and above the phase-transition temperature and the results

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can be analyzed by assuming the coexistence of two phospholipid populations, i.e. bulk and boundary lipids, the latter varying in composition according to the proteolipid species incorporated.

Materials and Methods

Reagents

1,6-Diphenyl-1,3,5-hexatriene (DPH), chromatographically purified, was from Koch-Light. The phospholipids: dimyristoyl-L-α-phosphatidylcholine (DMPC), dipalmitoyl-L-α-phosphatidylcholine (DPPC), dimyristoyl-L-α-phosphatidyl-DL-glycerol, ammonium salt (DMPG), dipalmitoyl-L-α-phosphatidyl-DL-glycerol, ammonium salt (DPPG), dipalmitoyl-DL-α-phosphatidyl-L-serine (DPPS) and L-α-phosphatidyl-L-serine from bovine brain (L-α-PS) were obtained from Sigma. All the solvents were of spectroscopic grade.

Proteolipids

PLP and DM-20 were purified from a chloroform-methanol extract of bovine brain by a combination of gel permeation and ion-exchange chromatography as previously described [24].

Preparation of vesicles with incorporated proteolipids

The method was first recommended by Papahadjopoulos et al. [2]. Phospholipids and protein were dissolved together in chloroform/ methanol/ water (10 : 5 : 1, by vol.) in the case of PLP, or in chloroform/ methanol (10 : 5, v/v) in the case of DM-20. The mixture was then dried as a thin film by rotary evaporation (the vacuum being maintained for 30 min after complete evaporation to remove any residual solvent) and finally suspended in the buffer (Hepes 2 mM, L-histidine 2 mM, NaCl 100 mM, EDTA 1 mM, pH 7.4) by mechanical shaking for 10 min to give a suspension of multilamellar vesicles. This step and the equilibration (1 h) were performed at 10°C above the transition temperature of the high-melting phospholipid. Each mixture contained approximately 3 μmoles of lipids, 0.5 to 1 mg of protein and was suspended in 3 to 5 ml of buffer. This method of incorporation, chosen for its convenience and relative rapidity, was shown to give identical results to the more often used 2-chloroethanol dialysis method [25]. The fluorescent labelling of the vesicles, at a lipid/DPH ratio = 400, was performed by dispersing, under stirring, a few μl of a DPH stock solution (10^{-3} M in tetrahydrofuran) in the buffer, just before it was added to the dry film. The vesicle suspension obtained appeared homogeneous, but it was nevertheless chromatographed on Sephadex G-75 and the eluate was analyzed for phosphate and protein. Phosphate was titrated according to Bartlett [26] and protein according to Lees and Paxman [27] for protein in the organic stock solution or to Schacterle and Pollack [28] when it was incorporated into the vesicles.

Fluorescence anisotropy measurements

An SLM-8000 spectrofluorometer, in the T-format, was used to measure the steady-state fluorescence anisotropy $r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$. The excitation wavelength was 360 nm, and 435 nm Schott interference filters were used for the emitted light. A home-built device ensured automatic rotation of the excitation polarizer, allowing continuous measurement of r. Cuvette temperature was maintained with a circulating water bath (Haake F3), itself piloted by a temperature programmer (Haake PG10). The heating rate was 1 C°/min and the temperature was continuously monitored with a thermocouple inserted into the cuvette. The temperature profiles of fluorescence anisotropy were thus obtained directly on an XY recorder. Just before the heating scan, the sample underwent a new equilibration for 10 min at the initial temperature (5°C),which was found to be crucial for good reproducibility.

Determination of the number of motionally restricted lipids per protein

If we assume that the fluorescence quantum yield of DPH depends little on the physical state of the lipids in which the probe is embedded and since this probe shows an equipartition between fluid and gel phases of the bilayer [29], the average fluorescence anisotropy of a labelled vesicle can be expressed:

$$\text{at } 48\,^{\circ}\text{C}: \; r = x r_b + (1-x) r_f$$

$$\text{at } 5\,^{\circ}\text{C}: \; r' = x r_b' + (1-x) r_f'$$

which gives

$$r' - r = x(r_b' - r_b) + (1-x)(r_f' - r_f) \qquad (1)$$

where r_b, r_b' and r_f, r_f' refer to the fluorescence anisotropies characterizing, respectively, the motionally restricted lipids associated with the protein (boundary lipids, L_B) and the remaining bulk lipids not affected by the protein (free lipids, L_F), while x is the fraction of motionally restricted lipids ($L_B/(L_F + L_B)$).

r and r' are directly determined on the curve for the protein-containing vesicle, and r_f and r_f' are estimated, by interpolation (see below), using the set of curves for the vesicle devoid of proteins. An estimated value of $(r_b' - r_b)$ is 0.04, assuming a temperature effect for the fluorescence anisotropy characterizing the motionally restricted phospholipids (remaining in a gel phase at all temperatures). The ratio x can then be expressed as:

$$x = (r' - r - r_f' + r_f)/(0.04 - r_f' + r_f) \qquad (2)$$

giving L_B, and then L_F.

Owing to the binary composition (L^1, L^2) of the vesicles, the ratio for the two species of the remaining bulk undergoing phase transition (L^1_F/L^2_F) can be obtained from the transition temperature of the fluorescence anisotropy curve (the inflexion point) for the protein-containing vesicle and comparing it, by interpolation, with those of a set of curves for vesicles of varying composition, but devoid of proteins, the basic idea being that a given transition temperature corresponds to a given composition of free lipids. L^1_F and L^2_F are then deduced from total L_F. As the total number of each lipid per protein ($L^1_B + L^1_F$, $L^2_B + L^2_F$) is initially known by titration, the number of each motionally restricted lipid (L^1_B, L^2_B) is directly obtained by difference.

Results

The influence of the incorporation of the organo-soluble forms of DM-20 and PLP proteolipid proteins was investigated on multilamellar vesicles of binary composition. Vesicles composed of phosphatidylserine (L-α-PS) and dipalmitoylphosphatidylcholine (DPPC) gave the clearest results. The temperature profiles of the fluorescence anisotropy curves for vesicles with various L-α-PS/DPPC ratios,with or without PLP or DM-20 incorporated, are shown in Fig. 1.

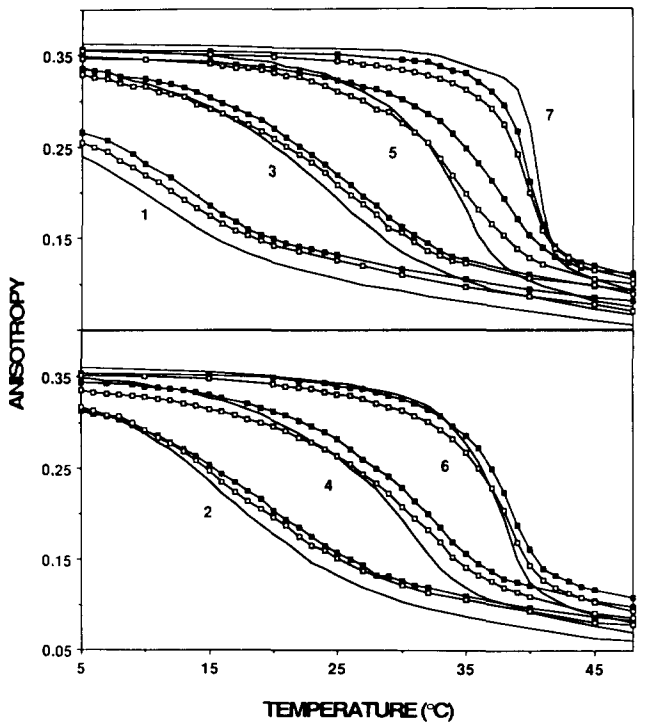

Fig. 1. Fluorescence anisotropy data obtained with L-α-PS/DPPC vesicles of varying compositions (full lines) and their complexes with proteolipids (■: PLP; □: DM-20). The incorporation ratio for the proteolipids (weight%) was about 31. The L-α-PS : DPPC molar ratios were: 1, 100 : 0; 2, 80 : 20; 3, 60 : 40; 4, 40 : 60; 5, 20 : 80; 6, 10 : 90; 7, 0 : 100. The set of curves was repeated twice.

By first considering the effects of both proteolipids on 100% DPPC vesicles (Fig. 1, curve 7), we see that PLP and PM-20 induce a decrease in the gel-to-liquid crystal phase transition temperature (T_c^0, corresponding to the inflexion point). The negative shift in T_c^0 observed with DM-20 is larger than that observed with PLP.

Above T_c^0, a small increase in fluorescence anisotropy is observed with both proteolipids, corresponding to a decrease in the average bilayer fluidity. In this case, the effect caused by PLP has a scarcely more pronounced effect than DM-20. On the contrary, below T_c^0, the bilayer in the gel phase is destabilized by the incorporation of PLP and DM-20, as shown by the decrease in fluorescence anisotropy, DM-20 being the more destabilizing protein. The same behaviour was observed when PLP and DM-20 were incorporated into dipalmitoylphosphatidylserine or dimyristoylphosphatidylglycerol vesicles (data not shown).

In contrast with pure DPPC vesicles, when L-α-PS is present, even at low ratio, the incorporation of the proteolipids no longer induces a decrease in the phase-transition temperature. On the contrary, PLP incorporation clearly induces an increase in the phase-transition temperature. Apart from this single, but important, difference, the effects of PLP and DM-20 on the fluorescence anisotropy for binary vesicles are of the same nature as those observed for 100% DPPC vesicles (Fig. 1, curves 1 to 6):

For vesicles presenting a clearly cooperative phase transition (L-α-PS ratio $\leq 30\%$, see also Fig. 2) the incorporation of both proteolipids always results in rigidification of the liquid-crystal phase of the bilayer and in destabiliztion of its gel phase.

For vesicles without cooperative transition (L-α-PS ratio $\geq 80\%$), which can be considered as being in the liquid crystal phase for the whole temperature scale considered, the incorporation results in rigidification of the bilayer, as indicated by the overall increase in fluorescence anisotropy (except in the 5–10°C range for the 20% L-α-PS vesicles).

For intermediate vesicles (L-α-PS ratio = 40% or 60%), the same rigidification is observed for vesicles in the liquid crystal phase. But, at low temperature, a decrease in fluorescence anisotropy is only observed for DM-20 containing-vesicles. In these cases, however, it is difficult to define at what temperature the vesicle is in a gel phase.

To summarize the effects of PLP and DM-20 on binary vesicles, we can say that, as in 100% DPPC vesicles, PLP always shows a greater rigidifying effect than DM-20 for vesicles in a fluid phase, whereas, for vesicles in gel phase, the destabilizing effects of DM-20 is always greater than that of PLP.

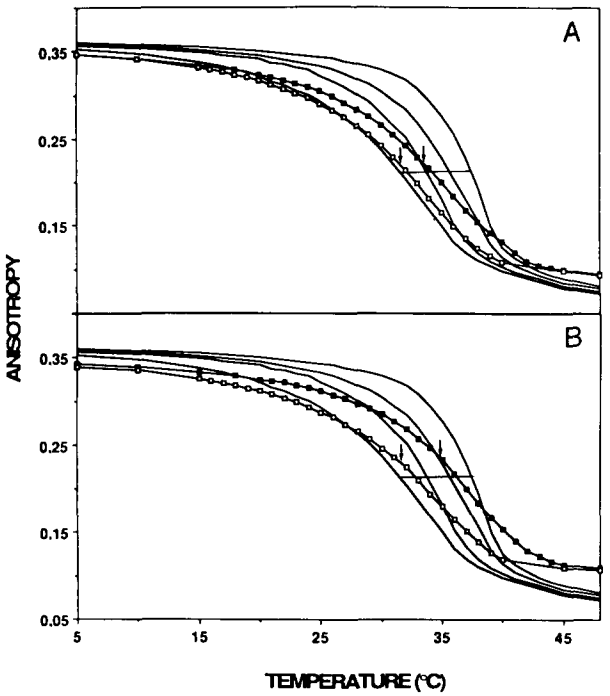

Fig. 2. Fluorescence anisotropy data obtained with increasing amounts of proteolipids incorporated in 30% L-α-PS/70% DPPC vesicles. Symbols as for Fig. 1. The incorporation ratios for the proteolipids (weight %) were: (A) PLP = 21% ($T_c^0 = 33.6 \pm 0.4$° C); DM-20 = 22.8% ($T_c^0 = 31.5 \pm 0.04$° C). (B) PLP = 31.5% ($T_c^0 = 35.0 \pm 0.4$° C); DM-20 = 30.4% ($T_c^0 = 31.5 \pm 0.4$° C). These transition temperatures are identified by arrows. The data for the complexes are compared with the data for vesicles devoid of proteolipids, at different L-α-PS/DPPC molar ratios (30:70, $T_c^0 = 31.5$° C; 20:80, $T_c^0 = 33.8$° C; 15:85, $T_c^0 = 35.6$° C; 10:90, $T_c^0 = 37.4$° C from lower to upper curve, respectively, these temperatures being linked by a straight line). The curves obtained for proteolipids were repeated five times, with similar proteolipid incorporation ratios.

In order to bring out these differences between PLP and DM-20, we studied, for vesicles containing 30% L-α-PS/70% DPPC, the variation in fluorescence anisotropy in function of increasing amounts of proteolipids. Results are shown in Fig. 2. The fluorescence anisotropy curves for the PLP-containing vesicles show an increase in transition temperatures as compared to the pure phospholipid vesicles, the transition temperatures corresponding to L-α-PS/DPPC bulk ratios of 17:83 and 21:79 for the higher and lower protein contents, respectively. These results can be interpreted as the induction, by PLP, of phase separation in L-α-PS/DPPC vesicles, with the formation of a shell of motionally restricted phospholipids at the boundary layer of the proteolipid. An upward shift in the transition temperature means that low-melting lipids such as L-α-PS are shielded from the transition and are the main component of the boundary layer. For the DM-20 containing vesicles, the transition temperatures do not vary, which means that the L-α-PS ratio of the bulk undisturbed lipids remains the same as in the whole vesicle.

Anyhow, whatever the proteolipid considered, we could evaluate the amount of motionally restricted phospholipids molecules of both species according to the procedure outlined in Materials and Methods. The results summarized in Table I show that the boundary layer of motionally restricted lipids around PLP is composed of about 17 to 18 lipids, being almost exclusively L-α-PS, while the boundary layer around DM-20 contains fewer lipids (14 to 15) with no change in composition as compared to the bulk vesicle.

In order to confirm the affinity of PLP for acidic phospholipids, we performed the same fluorescence anisotropy experiments with 30% DMPG/70% DPPC vesicles.

As Fig. 3 shows, PLP again shifts transition to higher temperatures than DM-20, but the difference between the two proteolipids is less pronounced than with L-α-PS/DPPC vesicles. The values of the motionally restricted phospholipids around PLP and DM-20 are reported in Table II. For PLP, the boundary layer is again enriched in acidic phospholipid (DMPG), but this enrichment is not as great as in the case of L-α-PS. DM-20

TABLE I

Number of motionally restricted phospholipids (PhL.) per proteolipid in proteolipid-loaded vesicles composed of 30% L-α-PS/70% DPPC

Results obtained from the experiment shown in Fig. 2, repeated five times for similar lipids/protein ratios, with excellent reproducibilities.

Proteolipid	PhL. [a]	L-α-PS [b]	DPPC [b]	$\frac{\text{L-α-PS}}{\text{PhL.}}$ [b] (%)
PLP				
21 % weight; 152 total lipids/protein	18.3 ± 2.8	17.6 ± 2	$0.7^{+2}_{-0.7}$	89 ± 11
31.5 % weight; 88 total lipids/protein	16.9 ± 1.6	14.6 ± 0.7	2.3 ± 0.7	87 ± 12
DM-20				
22.8 % weight; 114 total lipids/protein	13.7 ± 2.1	4.1 ± 1.5	9.6 ± 1.5	30 ± 13
30.4 % weight; 77 total lipids/protein	15.4 ± 1.5	4.6 ± 1	10.8 ± 0.9	30 ± 7

[a] ΔPhL. was obtained from Eqn. 2 with $\Delta r = 0.001$.

[b] were obtained by cumulating ΔPhL. and ΔT_c^0 for proteolipid-containing vesicles. ΔT_c^0 was estimated to be ± 0.4° C which for the L-α-PS/DPPC ratio of the remaining bulk gives ± 1.5% (most cases) or ± 1% (PLP, higher concentration) depending on the interpolation limits.

TABLE II

Number of motionally restricted phospholipids per proteolipid in proteolipid-loaded vesicles composed of 30% DMPG/70% DPPC

Results obtained from the experiment shown in Fig. 3, repeated four times. See annotations in Table I ($\Delta r = 0.001$; $\Delta T_c^0 = \pm 0.4°C$; Δ(DMPG/DPPC) of the remaining bulk = $\pm 1.5\%$).

Proteolipid	PhL.	DMPG	DPPC	$\frac{\text{DMPG}}{\text{PhL.}}$ (%)
PLP				
32.3 % weight; 87 total lipids/protein	21.5 ± 1.7	10.4 ± 1.4	11.1 ± 2.2	48.3 ± 7.0
DM-20				
31.4 % weight; 76 total lipids/protein	16.3 ± 1.5	6.1 ± 1.3	10.2 ± 2.0	37.4 ± 7.0

also shows a slight selectivity for DMPG and its boundary layer contains fewer lipids (about 16) than PLP (about 21).

We also performed some fluorescence anisotropy measurements with binary vesicles composed of high-melting acidic phospholipids (such as DPPS and DPPG) and a low-melting lecithin (DMPC) to see if PLP is really selective for acidic polar headgroups of phospholipids. In this case, the transition temperatures of the proteolipid-containing vesicles are shifted to lower temperature than those of the reference vesicles (data not shown), indicating that the bulk phospholipids undergoing phase transition are enriched in DMPC, and that the boundary bilayer is enriched in DPPG or DPPS. However, because of the weakness of the effects, no difference could be detected in the selectivity of PLP and DM-20 and no quantization was made. Nevertheless, the inversion of the transition temperature shift, compared to the previous sets of experiments, suggest that PLP does not bind simply to the low-melting lipids, but rather to the acidic polar headgroups containing lipids.

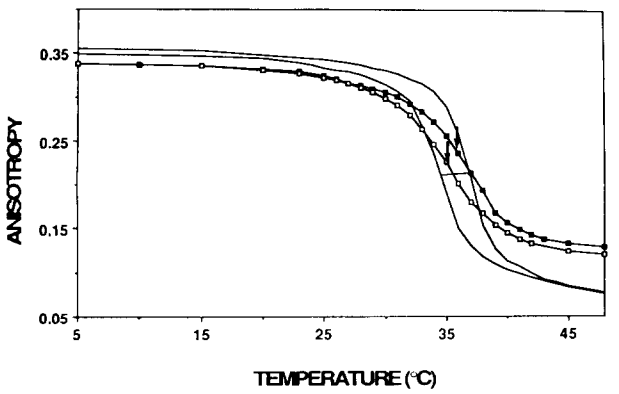

Fig. 3. Fluorescence anisotropy data obtained with proteolipids incorporated in 30% DMPG/70% DPPC vesicles. Symbols as for Fig. 1. The incorporation ratios for the proteolipids (weight %) were: PLP = 32.3% ($T_c^0 = 35.8 \pm 0.4°C$); DM-20 = 31.4% ($T_c^0 = 34.8 \pm 0.4°C$). The data for the vesicles devoid of proteolipids correspond to DMPG/DPPC molar ratios of 30:70 (lower curve, $T_c^0 = 34.3°C$) and 20:80 (upper curve, $T_c^0 = 36.8°C$). The curves obtained for proteolipids were repeated four times.

Discussion

Our results, obtained after the incorporation of the organo-soluble forms of PLP and DM-20 into 100% DPPC vesicles, clearly show that both proteolipids behave as integral membrane proteins. We observed a broadening of the phase transition, corresponding to a destabilization (fluidization) of the gel phase, combined with a small decrease in the transition temperature and a slight stabilization (rigidification) of the fluid phase. The integral membrane protein characteristics [30,31] also appeared more pronounced for DM-20 since it destabilized the gel phase more than PLP with a greater negative shift of the transition temperature.

Similar results were obtained by Goñi et al. [13] with Folch-Pi apoprotein incorporated in its organo-soluble form into DMPC vesicles, using infrared spectroscopy, nuclear magnetic resonance and fluorescence anisotropy. They observed a slight stabilization of the bilayer above T_c^0 and a destabilization below T_c^0.

In the case of lipophilin, Boggs and co-workers [8] showed the existence of motionally restricted lipids, assuming that only boundary and bulk unperturbed lipids are present. Selectivity of acidic phospholipids for the boundary layer of lipophilin was also shown by Boggs et al. [3] using differential scanning calorimetry, and later by Brophy et al. [10] by ESR spectroscopy. The coexistence of boundary and bulk unperturbed lipids was supported both by removing a number of lipids from the cooperative transition in calorimetric experiments and by the appearance of two components in the ESR spectrum of spin-labelled vesicles, one due to immobilized lipids, and the other characteristic of a lamellar phase.

In spite of the fact that a shift in transition temperature may involve protein-induced changes in the lipid-phase behaviour, and that DPH fluorescence anisotropy may reflect effects on fluidity that would be experienced for long distances from the protein, we interpreted our results by the induction, either by PLP or by DM-20, of a shell of motionally restricted phospholipids in the bilayer. From the fluorescence anisotropy curves obtained with 30% L-α-PS/70% DPPC vesicles, we as-

sessed the number of phospholipids in contact with PLP and DM-20 and found that 18 phospholipids were associated with PLP and 14 to 15 with DM-20. It has already been emphasized by Brophy et al. [10] that such a low number of associated lipids per protein indicates that proteolipids oligomerize in the membrane, reducing the number of surface sites available per protein monomer.

Furthermore, we found that PLP and DM-20 exhibited different selectivities for phospholipids: for PLP, the shell of motionally restricted lipids consisted almost exclusively of L-α-PS, while DM-20 showed no particular selectivity, its lipid boundary layer having the same composition as the bulk vesicle. We can reasonably assume that the selectivity of PLP for acidic phospholipids originates from electrostatic interactions between the charged residues of fragment 116–150, which is deleted in DM-20, and which corresponds to the major hydrophilic domain, with seven out of 35 deleted amino-acids carrying net charges, six of them positive [19]. As the hydrophobic core of the two proteolipids remains identical, the difference noted in the number of bound phospholipids could stem from a wider range effect of the electrostatic interactions, and/or from an indirect effect resulting from changes in proteolipid conformation.

Two models for PLP topology in the myelin membrane have been proposed [23,32], and, more recently, a third one [33] for PLP orientation in the plasma membrane of cultured oligodendrocytes. In all these models, fragment 116–150 is outside the lipid bilayer, either in the cytoplasmic space [23] or on the extracellular surface [32,33], and is assumed to interact, via its positively charged groups, with the same or opposite lipid bilayer. In a recent study, we showed that in the developping brain, DM-20 appears before PLP (Schindler et al., submitted). All these results are in agreement with the assumption that PLP and DM-20 may have different functions in the myelin membrane.

In this paper, we have shown that while PLP and DM-20 both interact with phospholipids, only PLP shows a strong specificity for acidic phospholipids, thus suggesting the role of fragment 116–150, deleted in DM-20, in the interaction with the lipid bilayer and its possible involvment in myelin compaction. However, we must be aware that DPH fluorescence anisotropy is not the best technique available to determine such stoichiometries. These stoichiometries (a fortiori those for other phospholipid mixtures) should be confirmed, for example, by ESR spectroscopy using spin-labelled phospholipids, which according to the recent works of Marsh and his collaborators [10,34,35] appears to be the ideal technique. As a confirmation, very recently, this group has studied the lipid specificities of PLP and DM-20 with spin-labelled lipids, after reconstitution into DMPC vesicles [36]. Their results, confirming ours, show that the 116–150 fragment plays a major role in determining lipid selectivity for PLP proteolipid.

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