Reconstitution of proteolipid protein: some properties and its role in interlamellar attachment

Martin B. A. TER BEEST,* Karin HOEKSTRA,* Arjen SEIN† and Dick HOEKSTRA*‡

*Laboratory of Physiological Chemistry, University of Groningen, Bloemsingel 10, 9712 KZ Groningen, The Netherlands, and †Department of Organic Chemistry, University of Groningen, Nijenborgh 16, 9747 AG Groningen, The Netherlands

Proteolipid apoprotein (PLP) isolated from human brain was reconstituted in dioleoylphosphatidylcholine vesicles by dialysis from 2-chloroethanol, using a dialysis buffer of pH 5.0. Under these conditions, and in contrast with dialysis carried out at pH 7.4, well-defined unilamellar vesicles containing the protein were formed. As judged by electron microscopy and quasi-elastic light scattering, the size of the vesicles was determined by the initial protein/lipid ratio used for reconstitution. When the vesicles were incubated in a buffer at neutral pH, aggregation of the vesicles was observed, but their structure remained intact. Asymmetric aggregation occurred when the reconstituted vesicles were incubated with large unilamellar vesicles (LUVs) devoid of protein. This aggregation was accompanied by loss of membrane integrity, as revealed by extensive leakage of the LUVs, and by membrane lipid dilution, indicative of the occurrence of mem-

brane fusion. Destabilization of the vesicles depended on the presence of negatively charged phosphatidylserine in the membrane of the LUVs. Similar effects, but to a lesser extent, were seen when the LUVs contained sulphatide, a negatively charged lipid prominently present in myelin. DM 20, a natural mutant of PLP, appeared to be far less potent in causing membrane lipid dilution than PLP. This could suggest that a distinct protein sequence of PLP, which is absent from DM 20, may be involved in triggering the observed membrane destabilization. Temperature-dependent experiments indicate that this sequence in PLP displays dynamic properties, its exposure being affected by conformational criteria. Exposure of this particular domain, in conjunction with its affinity for negatively charged lipid, could be related to a perturbation of the integrity of the myelin sheath, as will be discussed.

INTRODUCTION

Proteolipid protein (PLP) is the most abundant protein present in myelin of the central nervous system. It forms about 40 % of the total protein content of the myelin. The primary structure has been elucidated and it appears to be highly conserved among species [1,2]. This suggests that the protein fulfils an essential function in myelin. Indeed, mutations in the PLP gene of rodents and human result in severe structural aberrations [3–5].

The exact function of the protein in myelin is still largely unknown. Although its high content may suggest a structural role, a functional one cannot be excluded [6–8].

Regarding its structure, PLP is a highly hydrophobic protein, protruding through the membrane [9]. The interaction of this integral membrane protein with lipids has been thoroughly investigated. It interacts readily with several lipid species via hydrophobic interactions, but, as revealed by several approaches [10–12], it has a preference for negatively charged lipids.

Most reports on PLP–lipid interaction have focused on the direct lateral interaction [13]. However, it is not clear whether the membrane-embedded protein can also interact with lipids in the apposed membrane bilayer, thus forming bridges that could stabilize membrane–membrane interaction within the myelin sheath. To examine this possibility, we have reconstituted the protein in lipid vesicles. For this purpose, a procedure was developed, based on a previously described technique [14], which results in an efficient and highly reproducible reconstitution of native PLP in artificial membranes.

The reconstituted system was characterized, and intermembrane interactions were studied, using vesicles devoid of PLP. PLP reconstituted in phosphatidylcholine vesicles avidly interacts with target membranes of various composition, but displays a particular preference for negatively charged lipids, the presence of which is a prerequisite for PLP-mediated membrane–membrane interaction.

EXPERIMENTAL

Materials

Dioleoylphosphatidylcholine (PC) and dioleoylphosphatidylserine (PS) were from Avanti Polar Lipids Inc. (Birmingham, AL, U.S.A.). All fluorescent probes were obtained from Molecular Probes Inc. (Eugene, OR, U.S.A.), except dansylphosphatidylethanolamine (Dns-PE) which was from Avanti Polar Lipids Inc. Hepes was from Sigma Chemical Company (St. Louis, MO, U.S.A.) and 2-chloroethanol was from Fluka (Buchs, Switzerland). All other reagents were the highest quality available. PLP was isolated from human brain (human brain material was provided by The Netherlands Brain Bank, Amsterdam), essentially as described by Bizzozero et al. [15]. DM 20 and DM 20-free PLP were isolated as described by Schindler et al. [16]. To exclude the possible contamination of PLP with myelin basic protein (MBP), specific monoclonal antibodies against the latter were employed in conjunction with Western blotting. In addition, the purity of both PLP and DM-20 was further established by Western blotting with the respective antibodies and by amino

Abbreviations used: PC, dioleoylglycerophosphatidylcholine; PS, dioleoylglycerophosphatidylserine; LUV, large unilamellar vesicle; PLP, proteolipid (apo)protein; Dns-PE, dansylphosphatidylethanolamine; RET, resonance energy transfer; ANTS, 8-aminonaphthalene-1,3,6-trisulphonic acid; DPX, *p*-xylenebispicridinium bromide; QELS, quasi-elastic light scattering; DTT, dithiothreitol; MBP, myelin basic protein; NBD, 7-nitrobenz-2-oxa-1,3-diazole.

‡ To whom correspondence should be addressed.

acid analysis. No impurities were found in the protein fractions isolated as described (results not shown).

Reconstitution

A given amount of PLP, dissolved in 2-chloroethanol, was thoroughly mixed with a certain amount of PC, also dissolved in 2-chloroethanol, and in a dialysis bag (cut-off approximately 12 kDa). Protein/lipid ratios were as indicated in Table 1 and the total concentration of either never exceeded 1 mg/ml. For reconstitution, the protein/lipid suspension was dialysed at 4 °C for 24 h against 3 litres of reconstitution buffer (5 mM Hepes, 10 mM NaCl and 1 mM EDTA, pH 5.0). The buffer was changed three times. After dialysis, the suspension was centrifuged in an Eppendorf bench centrifuge for 15 min to remove aggregates of non-reconstituted protein. The reconstituted material remaining in the supernatant was analysed for protein and lipid content, and used for the experiments described in this work.

Analysis of the reconstituted protein–lipid complex

The protein–lipid complex was analysed on a discontinuous sucrose-density gradient [10–50 % (w/v) in reconstitution buffer, 10 % per step]. The gradients were centrifuged (12 h; 4 °C) at 150 000 *g* in an SW50.1 rotor (Beckman Instruments, Palo Alto, CA, U.S.A.). Fractions were collected for protein and phosphate determination.

The reconstituted product was further examined by transmission electron microscopy. The preparations were stained with a fresh filtered solution of 1 % (w/v) uranyl acetate, after a droplet of the sample had been placed on carbon-coated Formvar grids, pretreated by glow discharge in *n*-pentylamine. Samples were examined using a Philips EM400 electron microscope, operating at 80 kV.

Quasi-elastic light scattering (QELS) experiments were performed with a Nicomp model 370 submicron-particle-sizing system [17].

Preparation of large unilamellar vesicles (LUVs)

Vesicles of different lipid composition were prepared by reversed-phase evaporation as previously described [18]. The vesicle lipid concentration was assessed by phosphorus determination [19]. The buffer used throughout the experiments consisted of 10 mM NaCl, 5 mM Hepes and 2 mM sodium acetate. The pH of the buffer is specified in the legends of the Figures.

Aggregation of vesicles

Aggregation properties of the reconstituted vesicles *per se* were monitored by turbidity, measured as absorbance at 450 nm, as previously described [17]. For this purpose, a Lambda 2 Perkin–Elmer spectrophotometer equipped with a stirring device was used. The temperature was controlled by a thermostatically controlled water-bath. All measurements were carried out at 37 °C unless indicated otherwise. Alternatively, aggregation between PLP/PC vesicles and LUVs of various composition ('asymmetric aggregation') was determined by measuring resonance energy transfer (RET) between the tryptophan residues of the protein and Dns-PE incorporated into the LUV membranes. The occurrence of RET is reflected by an increase in the dansyl fluorescence ($\lambda_{em} = 340$ nm) when tryptophan is

excited at 280 nm. The increase in fluorescence is taken as a measure of asymmetric aggregation [17].

Stability of reconstitution products: leakage and lipid mixing

The leakage of aqueous content was determined using the 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS)/*p*-xylene-bispyridinium bromide (DPX) assay [20]. As the physiological salt concentration already has an effect on self-aggregation of the vesicles, ANTS/DPX concentrations were kept as low as possible, i.e. 10 and 20 mM respectively in 5 mM Hepes/5 mM sodium acetate, pH 7.4. The vesicles were passed over a Sephadex G-100 column eluted with buffer consisting of 5 mM Hepes, pH 7.4, 5 mM sodium acetate and 40 mM NaCl. Leakage experiments were carried out in the same buffer. All experiments were performed at 37 °C, at a lipid concentration of 25 μ M, unless otherwise stated.

To determine whether vesicle–vesicle interaction led to fusion, a lipid-mixing assay based on RET was used [21]. Target vesicles contained *N*-NBD-PE and *N*-Rh-PE (each 0.8 mol %). The fluorescence scale was calibrated by setting the fluorescence of the labelled phospholipid vesicles, before addition of the reconstituted PLP-containing vesicles, at 0 %. The 100 % level was determined after the addition of 1 % (v/v) Triton X-100. Fluorescence was corrected for dilution and quenching of the 7-nitrobenz-2-oxa-1,3-diazole (NBD) by Triton X-100.

All fluorescence measurements were carried out with a Perkin–Elmer MPF 43 fluorospectrophotometer. The incubation temperature was 37 °C.

Additional procedures

In some cases pyrene/PC (10 mol %) was added to the lipid and protein before dialysis, thereby producing fluorescently tagged reconstituted material [22]. RET between tryptophan present in PLP and pyrene-labelled PC was determined at an excitation wavelength of 280 nm (slit width 4.5 nm), and emission was scanned from 290 to 500 nm (slit width 4.5 nm). Direct excitation of pyrene was at 341 nm and emission was scanned from 350 to 500 nm.

Protein was assayed by a modified Lowry determination [23], and phosphate was determined as described by Bottcher et al. [19].

RESULTS

Reconstitution of PLP in vesicles

PLP, isolated and purified as described in the Experimental section, was mixed with PC in 1 ml of 2-chloroethanol at various protein/lipid ratios. Samples were put in a dialysis bag and dialysed for 24 h at 4 °C against three volume changes (3 litres each) of a reconstitution buffer composed of 5 mM Hepes, pH 5.0, 10 mM NaCl and 1 mM EDTA. After dialysis a clear to opalescent suspension was obtained, the turbidity of which depended on the starting protein/lipid ratio (Table 1). Furthermore, the starting protein/lipid ratio also determined the morphological appearance of the reconstituted vesicles, as seen by electron microscopy (Figure 1) and determined by QELS (Table 1). A relatively high protein content resulted in vesicles with a relatively small diameter (Figure 1a), ranging from 200 to 500 nm. With decreasing protein content the size of the vesicles increased up to 1 μ m in diameter (Figure 1b). Unlike a more spherical structure commonly seen for extruded unilamellar vesicles containing only phospholipid, the reconstituted vesicles show a typical 'doughnut-like' appearance.

Table 1 Effect of PLP/PC ratio on morphology of the reconstituted vesicles

Absorbance was spectrophotometrically determined with reconstitution buffer set at zero. QELS measurements were carried out as described in the Experimental section. Results are means $\pm$ S.D. Electron micrographs were analysed by measuring ± 150 vesicles in five different fields to give vesicle diameter. Values in parentheses are range of size distribution.

Ratio PLP/PC	A_{450}	QELS	Vesicle diameter (nm)
1:1	0.185	135 ± 43	350 ± 150 (200–500)
1:2	0.241	200 ± 45	750 ± 300 (400–1000)
1:3	0.607	258 ± 63	—
1:1 (pH 8.0)*	—	265 ± 50	—
PC-containing LUV	0.120	100 ± 20	145 ± 15

* Vesicles were prepared at pH 5.0; after preparation the pH was adjusted to 8.0.

Figure 1 Reconstituted PLP/PC vesicles, visualized by electron microscopy

Preparations were negatively stained with 1% uranyl acetate. (a) PLP/PC (1:1) vesicles (bar = 2 μ m); (b) PLP/PC (1:2) vesicles (bar = 2 μ m).

The initial protein/lipid ratio appeared to be critical not only with respect to size but also for a reconstituted product of a vesicular nature to be obtained (Table 1). Thus lowering the protein/lipid ratio from 1 to 3 to about 1 to 10 resulted in the formation of large aggregates without a distinctive structure.

Figure 2 Sucrose-density-gradient analysis of PLP reconstituted with PC

The dialysed protein/lipid (1:1) complex was layered on top of a continuous sucrose gradient (10–50% sucrose in reconstitution buffer, pH 5.0). After centrifugation for 12 h, the gradient was analysed by taking samples from the top (fraction 1). Phosphorus (as a measurement of lipid) and protein were determined in each fraction and expressed as a fraction of the total recovery.

Another critical factor appeared to be the pH of the reconstitution buffer. When dialysis was carried out against a buffer of pH 7.4 (instead of pH 5.0), no vesicular structures were obtained. In fact PLP and lipid readily precipitated under such conditions.

To characterize the efficiency with which both protein and lipid participated in forming reconstituted vesicles, the preparations were also analysed on sucrose-density gradients (Figure 2). In the gradient fractions collected neither free protein nor free lipid was detected, indicating that under the given conditions (i.e. a protein/lipid ratio of 1:1) essentially all protein and lipid are in a reconstituted form.

Symmetric and asymmetric aggregation of reconstituted PLP/PC vesicles

In contrast with their ability at pH 5.0, some aggregation, although minor [extent of absorption was 0.04 for 100 nmol of PLP/PC (1:1) vesicles; compare with Table 1], of the reconstituted vesicles occurred when the pH was brought to neutral (results not shown). The symmetric aggregation was partly reversible (25%) when the pH was readjusted to 5.0 by the addition of an aliquot of 1 M HCl.

However, substantial aggregation took place when the reconstituted vesicles were incubated with phospholipid vesicles of various composition. Asymmetric aggregation, as shown in Figure 3, was monitored by RET between tryptophan (PLP/PC vesicles) and Dns-PE (incorporated into the protein-devoid lipid vesicles). At a fixed phospholipid concentration, phospholipid-dependent differences in the rate of asymmetric aggregation became apparent at higher concentrations of PLP/PC vesicles, i.e. above concentrations (expressed as PLP content) of approx. 5 μ g/ml. Thus the rate increases when the mole fraction of negatively charged lipid in the target vesicles increases. However, the final extent of aggregation was not influenced by the presence of PS (Figure 3, inset).

Besides being influenced by vesicle concentration and lipid composition, the initial rate of aggregation is further affected by the density of PLP in the reconstituted vesicles. As shown in Figure 4, although moderate, the initial rate of aggregation

Figure 3 Interaction of reconstituted PLP/PC vesicles with vesicles of different lipid composition

The protein-devoid vesicles were prepared with Dns-PE (10 mol%) in their bilayers. As a measure of interaction, the increase in fluorescence signal of the Dns group caused by RET was monitored (expressed as the amount of protein) in reconstitution buffer, pH 5.0, at 37 °C. The initial rate of asymmetric aggregation (measured as the initial rate of fluorescence increase) as a function of the (PLP) protein concentration is shown. The inset shows the extent of fluorescence increase as a function of protein concentration. The maximal degree of RET obtained was determined after 10 min. At that time, the system had reached equilibrium and no further change occurred. The lipid compositions of the lipid vesicles used were: ○, PC; □, PC/PS (9:1); △, PS.

Figure 4 Aggregation of PLP/PC vesicles with PC/PS (9:1) vesicles as a function of protein density

The lipid concentration was 25 μM. The pH of the incubation buffer was 5.0 and all aggregation experiments were carried out at 37 °C. The initial rate of aggregation was determined as a function of protein concentration. The protein densities in the reconstituted vesicles, expressed as PLP/PC ratios, were 1:1 (○), 1:2 (△) and 1:3 (□).

(monitored by a change in turbidity) between PLP/PC vesicles and protein-devoid PC/PS vesicles increased as the PLP density decreased. The aggregated complexes thus formed appeared to be quite stable, as subsequent treatment with a 2–3-fold excess (relative to PLP) of trypsin (30 min, at room temperature) did not result in any loss of the RET signal or decrease in absorption.

Destabilization of membranes by interaction with PLP/PC vesicles

As shown above, reconstituted PLP/PC vesicles readily engage in asymmetric intermembrane interactions. The extent to which

Figure 5 Leakage induced by PLP/PC vesicles

The kinetics of leakage were determined by following the relief of ANTS fluorescence quenching when ANTS and its quencher DPX, together in the aqueous volume of the vesicles, leak from the vesicles. As a result of extensive dilution in the medium, the fluorescence of ANTS increases. The lipid concentration was 25 μM and all other conditions were as described in the Experimental section. The rate of leakage is given as a function of lipid concentration [●, PC with PC/PLP (1:1); ○, PC/PS (9:1) with PLP/PC (1:1); △, PC/PS (9:1) with PLP/PC (1:2); □, PC/PS (9:1) with PLP/PC (1:3)].

the stability of the target membrane is affected by such interactions was examined next.

When PLP/PC vesicles were added to vesicles containing the ANTS/DPX complex as an aqueous content marker, extensive leakage was observed, as long as the lipid vesicles contained negatively charged lipid (Figure 5). In the absence of negatively charged lipid, no significant leakage was observed (●). Furthermore, with PLP/PC vesicles of different protein/lipid ratios, a marked increase in the rate of leakage occurred with increasing PLP content in the reconstituted bilayer (Figure 5).

PLP penetration and membrane fusion

The observed leakage could be the result of penetration of a part of the PLP and/or occur as an accompanying event, as frequently reported for artificial membranes, during membrane fusion. The latter would be supported by the observation that the aggregated complex cannot be dissociated by treatment with proteolytic enzymes or at high salt concentration, indicating the formation of an irreversible aggregate. To examine this possibility further, we carried out a lipid-mixing assay, based on RET, to monitor lipid dilution during fusion [21]. As shown in Figure 6, a relatively minor degree of lipid mixing occurred when PLP/PC vesicles were mixed with PC vesicles. However, lipid mixing was greatly facilitated when negatively charged lipids were incorporated into the protein-devoid lipid vesicles. The exclusive presence of PS resulted in the highest rates of fluorescence increase (Figure 6b). In this case the final extent of lipid mixing reached the maximum level.

When lipid dilution was monitored as a function of the amount of PLP reconstituted in PC bilayers, a trend became apparent, indicating that the lower the amount of protein (1:1 compared with 1:3 protein/lipid) the faster the rate of lipid mixing (Figure 6a). To examine the specificity of PLP and its structural requirements for induction of lipid mixing, various parameters were evaluated. Neither trypsin treatment of PLP/PC vesicles nor treatment with the reducing agent dithiothreitol (DTT) caused any reduction in the efficiency of lipid mixing

Figure 6 Lipid mixing occurring during interaction of PLP/PC vesicles with target lipid vesicles

Lipid mixing was measured by RET, as described in the Experimental section. Lipid concentration and conditions were the same as in Figure 5. (a) Initial rate of membrane dilution as a function of protein density: ○, PLP/PC (1:1); △, PLP/PC (1:3). Target vesicles consisted of PC/PS (9:1). (b) Lipid dilution between PLP/PC and vesicles of different lipid composition, as a function of PLP/PC ratio. △, PS with PLP/PC (1:1); ○, PC/PS (9:1) with PLP/PC (1:1); □, PC with PLP/PC (1:1).

Table 2 Effect of different parameters on membrane destabilization

Lipid mixing between PLP/PC vesicles and PC/PS (9:1) was followed by an assay based on RET [21].

	V_i (% per min)	Extent (%)
PLP/PC (1:1)	$150 \pm 10^*$	$72 \pm 5^*$
PLP/PC + DTT†	$155 \pm 5^*$	$70 \pm 3^*$
PLP/PC + trypsin‡	152	71
DM 20/PC (1:1)	80	60
PLP/PC, pH 7.4	122	40

*Mean $\pm$ S.D. of three experiments.

† Before the addition of the LUVs, PLP/PC vesicles were incubated with 50 mM DTT for 1 h at 0 °C.

‡ PLP/PC vesicles were incubated with trypsin (PLP/trypsin, 1:1) at 37 °C for 30 min. The efficiency of the trypsin treatment was checked by SDS/PAGE and proteolysis was found to be complete.

(Table 2), even though proteolytic degradation of PLP by trypsin was complete as judged by SDS/PAGE. When DM 20, a natural PLP mutant, was reconstituted and subsequently examined for

Figure 7 Effect of temperature and specificity of negatively charged lipid on PLP-induced membrane mixing and aggregation

All conditions were as for Figure 6. (a) Initial rate of membrane mixing [PC/PS (9:1) with PLP/PC (1:1) at 25 °C (○) and 37 °C (●); PC/sulphatide (9:1) with PLP/PC (1:1) at 25 °C (□) and 37 °C (■)]. (b) The extent of aggregation of PLP/PC (1:1) vesicles with PC/PS (9:1) vesicles at 25 °C (○) and 37 °C (●).

its ability to induce lipid mixing, a substantially lower activity was observed than that obtained with PLP/PC. At neutral pH, the rate and extent of lipid mixing between PLP/PC and PS/PC (1:9) vesicles were significantly lower than at pH 5.0. Furthermore, the temperature and the type of negatively charged lipid (sulphatide) influenced the rate (Figure 7a) and extent (not shown but similar result to that for the rate) of probe dilution. Interestingly, although lipid mixing was markedly affected at these temperatures, aggregation was only marginally influenced under similar conditions (Figure 7b).

Modulation of PLP/PC bilayers during intermembrane interactions

As demonstrated by the above experiments, reconstituted PLP/PC vesicles readily engage in intermembrane interactions which cause perturbation of the target membranes and extensive lipid mixing, presumably as a result of membrane fusion. During these interactions, the lateral organization of PLP and PC in the reconstituted membranes is also likely to change. To monitor such phenomena, changes in RET between the tryptophan in PLP and pyrene-PC, reconstituted together in PLP/PC bilayers, and changes in the excimer/monomer ratio of pyrene-PC *per se* were measured under various conditions. No changes in energy-transfer efficiency or excimer/monomer ratio were observed when the pyrene-PC-labelled reconstituted PLP/PC vesicles were incubated with unlabelled PLP/PC vesicles, irrespective of the pH of the incubation medium (range pH 5.0–8.0). A decrease in

Figure 8 Lipid mixing occurring on interaction of vesicles with target lipid vesicles, using pyrene-PC as a membrane probe

All conditions were as in Figure 6. In this case PLP/PC (2:1) vesicles containing 10 mol% pyrene/PC were used. (a) Pyrene-PC emission spectra (excitation at 280 nm) recorded for the pyrene/PC-labelled PLP/PC vesicles incubated under various conditions: A, pyrene-labelled PLP/PC vesicles in incubation buffer; B, as for A, but in the presence of PS vesicles (80 μ M); C, as for A, after the addition of Triton X-100 [1% (v/v) final concentration]. (b) Change in excimer/monomer ratio and dequenching tryptophan after addition of unlabelled vesicles of various lipid composition. In this experiment, 4 μ g/ml PLP/PC (2:1) vesicles were used. Target vesicles (at a concentration varying from 0 to 80 μ M) were: $\circ$, PC/PS (9:1), excimer/monomer ratio; $\square$, PS, excimer/monomer ratio; $\bullet$, PC/PS (9:1), tryptophan dequenching; $\blacksquare$, PS, tryptophan dequenching.

energy transfer between tryptophan and pyrene/PC occurred when the PLP/PC vesicles were incubated with both PS and PC/PS vesicles (Figure 8), indicating that the intermolecular distance between the protein and (fluorescent) lipid increased, presumably as a result of membrane fusion. The diluting effect was most pronounced with pure PS bilayers. Intriguingly, the opposite effect became apparent when changes in the pyrene excimer/monomer ratio were monitored under similar conditions. In this case, an apparently higher extent of dilution occurred on interaction with PC/PS vesicles. As will be discussed, it appears likely that this contradictory effect is related to a phase separation of pyrene-PC occurring when the PLP/PC vesicles interact with pure PS vesicles.

DISCUSSION

The occurrence of PLP and DM 20 as a group of highly hydrophobic 'proteolipids' was already established some four decades ago [24]. Yet the precise functions of these two proteins in the biogenesis and stabilization of the myelin sheath are still largely unknown. In order better to define their structural and functional roles, their reconstitution in model membranes would appear to be imperative. In the present work, we have reconstituted native PLP and have characterized several of its properties when inserted in PC bilayers.

Reconstitution is critically dependent on the protein/phospholipid ratio and pH. With 2-chloroethanol, a homogeneously dispersed suspension of lipid vesicles is not obtained on dialysis of the solvent. Only precipitates sedimenting at the bottom of the dialysis bag are found. This experimental coincidence illustrates a corequirement for both lipid and protein for stable lipid vesicle formation under the present reconstitution conditions. Thus under these conditions, PLP 'stabilizes' the reconstituted bilayers by keeping them homogeneously dispersed in bulk solution.

Apart from stabilizing the 'vesicular' phase, PLP-lipid interaction may have another relevance in the present system.

Given the hydrophobic properties of PLP, which become particularly apparent around neutral pH (see below), it is likely that PLP requires interaction with unlike molecules in order to avoid self-aggregation and hence prevent its functional elimination. We have observed that, in its pure form, PLP is most 'stable' at lower pH. Whether pH-induced conformational changes govern the exposure of domains which results in self-aggregation remains to be determined. At any rate, at neutral pH, pure PLP obtained by the procedure of Bizzozero et al. [15] readily aggregates, precluding its reconstitution. However, contamination of PLP preparations with other myelin proteins may stabilize the proteolipid at neutral pH, allowing its reconstitution under such conditions [14]. When the reconstituted vesicles are brought to pH 7.4, some aggregation, as determined by absorbance and QELS measurements, occurs (Table 1). This observation is consistent with the notion that PLP may play a role in intermembrane interaction in the myelin sheath. Given the minor degree of aggregation, it is apparent that simple PLP-PLP interactions do not suffice and that other molecular parameters must be involved. In fact, at low protein density we observed a higher initial rate of aggregation (Figure 4), indicating that protein-protein interactions may interfere with intermembrane attachment and implying the relevance of protein-lipid interaction. Entirely in line with this notion is the observation that the rate of lipid mixing tends to increase when the protein density decreases (Figure 6a). Thus, at high PLP density, the protein acts as a barrier to intermembrane interaction and, although protein-protein interactions can readily occur for PLP, such interactions seem to be too weak to support stable membrane aggregation.

In contrast with the effect of protein density on aggregation and lipid mixing was the effect on membrane permeability. Increased leakage was noted with increasing protein density. This would indicate that the frequency of intermembrane interaction *per se* is not the sole factor involved in leakage-accompanying membrane destabilization. Rather, lateral PLP interactions are also involved, as it seems reasonable to assume

that the frequency of such interactions will probably increase when the density increases, thus presumably leading to pore formation or 'defects' which cause leakage of target vesicles after their insertion into the latter membranes via fusion. This is indicated by the observation that leakage and lipid mixing are seen with PS-containing vesicles only (Figure 5). In this context it has been noted that PLP may display voltage-dependent conductance, which remains after treatment with proteolytic enzymes such as trypsin [25,26]. Similarly, leakage was not inhibited by treatment with trypsin.

Apart from protein-density-dependent leakage, PLP conformational aspects are also probably involved (Figure 7). Membrane dilution diminished substantially when the temperature was decreased. On the other hand, aggregation was not affected by temperature, indicating that the two events are not necessarily related, as further indicated by differences in leakage and lipid mixing, whereas aggregation was similar, when PC vesicles were compared with PS vesicles.

Aggregation, lipid mixing and leakage, although occurring to variable extents when reconstituted PLP/PC vesicles were incubated with uncharged bilayers, were initiated only (leakage) or were strongly facilitated when the target membrane contained PS. Electrostatic interactions appear to play a role therefore. Presumably, these interactions involve a strongly positively charged domain (116–150) of the PLP, which is thought to be exposed at the extracellular face [27,28]. Indeed, with reconstituted DM 20, the PLP isoform in which this domain is largely missing [2], the initial rate of lipid mixing is drastically reduced (Table 2). However, the final extent of lipid mixing is very similar to that observed for PLP, indicating that initial electrostatic interactions are not crucial. It has been reported that the moderately hydrophobic C-terminus readily penetrates into membrane vesicles [29]. This region is well conserved among species as well as among isoforms [30], and hence it is possible that this region may play a role in proteolipid-mediated intermembrane interaction in both PLP and DM 20. Whether exposure of this region is affected by temperature, thus modulating lipid dilution (Figure 7; see above), remains to be determined.

The significance and nature of the hydrophobic and electrostatic interactions in which reconstituted PLP/PC vesicles engage become apparent when comparing their interaction with PC- and PS-containing vesicles. The lipid-dependent interaction shows that, as far as aggregation is concerned, electrostatic interactions facilitate the rate of aggregation, but do not affect the extent of aggregation (Figure 3). Also hydrophobic interactions that in all likelihood primarily mediate the interaction with PC vesicles lead to a similar extent of aggregation (Figure 3). However, neither leakage nor lipid mixing is observed between PLP/PC and PC vesicles, in contrast with events seen with PS-containing vesicles (Figures 6b and 7). Assuming that leakage requires membrane penetration of hydrophobic segments of the protein, as is the case for fusion, of which lipid mixing is a reflection, it would appear that the nature of the hydrophobic interaction of PLP with PC vesicles and that with PS vesicles are fundamentally different. It follows that, in contrast with the interaction with PS-containing vesicles, PLP interacts only in a superficial manner with PC-containing vesicles, without substantially penetrating them. Hence PS itself may govern the mode of PLP interaction to a substantial extent, perhaps giving rise to PLP conformational changes after initial electrostatic interactions that expose hydrophobic regions which subsequently penetrate.

It has been reported previously that PLP displays a high affinity towards negatively charged lipids [10–12]. Our results

suggest that PLP, by interacting with PS, can also cause phase separations in mixed bilayers. When determining lipid dilution using either the NBD-PE/Rh-PE assay or the assay based on RET between pyrene-PC and PLP tryptophan residues, using pure PS or mixed PC/PS vesicles as targets, apparently contrasting results were obtained (Figure 8b), PC/PS vesicles being more susceptible to lipid dilution when monitored by the pyrene assay. This incompatibility is best explained by taking into account the probability that PS associates with PLP as a boundary phase, reducing distance-dependent RET between pyrene-PC and the PLP tryptophan residues. In the lateral plane of a bilayer, lipid-phase separation itself may give rise to membrane destabilization. As demonstrated here, the presence of PS in the apposed membrane also has devastating consequences for the integrity of that membrane when PLP interacts with the lipid. Hence such interactions should be prevented in myelin or they may lead to demyelination. Laterally, such interactions could be prevented by the basic protein, MBP, neutralizing most negatively charged lipid. On the other hand, PLP-MBP interactions, which are thought to occur within the myelin sheath [31,32], may control aberrant interactions of PLP with PS. In this context it is of interest to note that more recent work on the transmembrane topology of PLP has suggested an intracellular location of a distinct polar region for the molecule [33]. Consistent with the present work, such a proposed topology would provide possibilities for a delicate interplay between PLP, MBP and acidic phospholipids such as PS. Along these lines, it could thus be possible that when MBP stabilization is perturbed, e.g. as a result of MBP degradation or when lipid asymmetry is no longer maintained in the myelin sheath, uncontrolled PLP-PS interactions may set the pace for the myelin destruction that occurs during neurological diseases such as multiple sclerosis. In the light of the present observations, it would be of interest to examine the consequences of perturbation of lipid asymmetry for the molecular and morphological integrity of the myelin sheath.

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