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Spontaneous vesicularization of myelin lipids is counteracted by myelin basic protein

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Hand-vortexed dispersions of several lipids (cerebrosides, sulfatides, PC, PE, PS and sphingomyelin), mixed in the ratios found for these categories of lipids in myelin, exhibit ^{31}P-NMR spectra which have contributions from both isotropic and lamellar resonances. Investigation of this system by freeze-fracture electron microscopy and X-ray diffraction revealed that this lipid mixture has spontaneously formed small unilamellar vesicles (SUVs) (diam. ≈ 400 Å) and large highly convoluted unilamellar vesicles (LUVs) (diam. ≈ 1000 Å), the latter possibly resulting from aggregation and fusion of the SUV structures. This vesicularization of the myelin lipids was reversed by the addition of myelin basic protein: only large multilamellar aggregates were formed in the presence of protein, as shown by all three experimental methods. Although no rigorous physical-chemical explanation for these phenomena is yet available, the possibility is suggested that the high concentration of cerebrosides and/or phosphatidylethanolamine in this particular mixture of myelin lipids play pivotal roles in the formation of these unusual vesicles. Spontaneous vesicularization of myelin lipids is discussed as a potential pathway toward destabilization of the myelin sheath.

Introduction

Myelin membranes contain a diverse collectior of glycolipids (cerebrosides, sulfatides) and phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin) as well as a significant amount of cholesterol [1,2]. Investigation into the morphological behaviour of these various lipids (for a review, see Ref. 3), both as pure and mixed systems, have prompted speculation as to the exact contribution of each component in defining myelin membrane structure.

The native myelin superstructure consists of a series of compacted, concentric bilayer phase membranes (for a review, see Ref. 4). In demyelinating diseases such as multiple sclerosis, these membranes degenerate, leading to subsequent disruption of axonal transmissions. Myelin contains an extrinsic membrane protein, myelin basic protein (MBP), in its interstitial cytoplasmic spaces, which has been suggested to aid in the

Abbreviations: NMR, nuclear magnetic resonance; EM, electron microscopy; H_{II}, hexagonal phase; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PA, phosphatidic acid; CER, unfractionated cerebrosides; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle.

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initial compaction of the bilayers [5]. MBP contains 31 positively-charged sites in its 170-residue sequence which presumably supply the adhesion necessary to hold two bilayers in close apposition by binding to the negatively-charged phosphate headgroups of myelin lipids [6,7]. The incomplete compaction of shiverer mouse myelin has been attributed to the absence of basic protein from the central nervous system (CNS) of this mutant [8].

Using freeze-fracture electron microscopy (EM), X-ray diffraction and phosphorus (^{31}P) nuclear magnetic resonance (NMR), we now report an unusual characteristic of several combined myelin-type lipids (excluding cholesterol) in that they exhibit a tendency to form isolated and highly convoluted clusters of unilamellar vesicles spontaneously when gently dispersed in aqueous buffer. The possibility is discussed that the high concentration of cerebroside (CER) and/or phosphatidylethanolamine (PE) in myelin is responsible for the vesicularization.

Materials and Methods

Bovine brain cerebrosides (CER), phosphatidylserine (PS), phosphatidylethanolamine (PE), and egg phosphatidylcholine (PC) were purchased from Avanti Lipids (Birmingham, AL); bovine brain sphingomyelin was obtained from Sigma (St. Louis, MO); sulfatides were obtained from Supelco (Bellefonte, PA). Lipids were stored in lyophilized form and used without further purification. Basic protein was extracted from normal human brain as described [9] and stored in lyophilized form.

Lipid mixtures characteristic of myelin membranes were prepared (10 mg total lipids) by combining the individual lipids in the following ratios: cerebrosides (2.9 mg); cerebroside sulfate (0.8 mg); PC (2.0 mg); PE (2.5 mg); PS (0.9 mg) and sphingomyelin (0.9 mg). This combination of glyco- and phospholipids represents the major components of the myelin sheath in their in vivo ratios [1], excluding the contributions of cholesterol and minor ($< 5\%$) lipid species.

Lipids were prepared for freeze-fracture electron microscopy (EM), X-ray diffraction and ^{31}P-NMR by dissolving them in chloroform/methanol (9 : 1, v/v), followed by evaporation of the solvent to dryness under nitrogen. Residual $CHCl_3$/MeOH was removed by treatment under vacuum for approx. 1 h. Dried lipids were dispersed under nitrogen by vortexing (10–15 min) at room temperature in 1.5 ml of buffer (^2H_2O used for NMR experiments) containing 10 mM Hepes and 1.0 mM EDTA (adjusted with NaOH to pH 7.0). Required quantities of protein solution were added in 0.2 ml aliquots to the dispersed lipids; additions were followed by vortexing (approx. 5 min) to ensure complete mixing. Protein and lipid concentrations were as given in the figure legends.

Although all NMR experiments were run under dry nitrogen, some samples had to be maintained for several hours at relatively high temperatures which may cause lipid oxidation and/or hydrolysis; small quantities of the resulting degradative products are suspected of inducing significant changes in the overall lipid morphology [10]. Therefore, after each set of spectra was accumulated, thin-layer chromatography (TLC) was performed on silica-gel plates (Fisher) using the solvent system $CHCl_3$/MeOH/H_2O(70 : 30 : 5, v/v); in all cases no measurable decomposition was detected.

Lipid suspensions for freeze-fracture were rapidly frozen without cryoprotectant using a Balzers spray-freezer. Subsequent freeze-fracturing, shadowing and replicating were performed on a Balzers 400T. Replicas were viewed on a Philips 300 electron microscope.

X-ray diffraction experiments on the centrifuged lipid (35 000 rpm for 1 h) were carried out by standard procedures [11]. Diffraction patterns, recorded on film, yield the crystalline nature of the sample, i.e. whether it is amorphous, multilamellar or H_{II}, as well as the dimensions of the latter two.

Proton-decoupled ^{31}P-NMR spectra were obtained on a Bruker WP80 spectrometer operating at 32.42 MHz. Spectra were uniformly collected in 25 K scans over 16K data points with a sweep with of 6000 Hz and a pulse width of 8.0 μs. Each scan was obtained with an acquisition time of 1.36 s followed by a 0.3 relaxation delay. All spectra were recorded at 28°C (ambient probehead temperature) unless specified otherwise. Low temperature experiments utilized the Bruker liquid nitrogen evaporation system. Fourier transformation of free induction decays employed an exponential

multiplication with 50 Hz line broadening.

Density gradient centrifugation of the lipid dispersion was performed on a discontinuous glycerol/water gradient (50%–20%–10%). Samples were centrifuged for 45 min at 10000 rpm in a Beckman SW50.1 swinging bucket rotor.

Results

^{31}P-NMR spectra of myelin lipid mixtures

Phosphorus-31 nuclear magnetic resonance is sensitive to the particular motional characteristics of the lipid headgroups of membranes, and accordingly reflects the structure of a given lipid aggregate (for a review, see Ref. 12). For example, the bilayer form of multilamellar vesicles is represented by a characteristic low-field 'shoulder' accompanied by a relatively sharp higher field peak; this disperse signal results from the orientation dependence of the phosphorus nucleus with respect to the external magnetic field (i.e., the chemical shift anisotropy (CSA)). Myelin lipids which generally fall into this lamellar-forming class (under physiological conditions) are cerebroside [13,14], cerebroside sulfate [13], PS [15], PC [16] and sphingomyelin [14,17]. Since cerebrosides do not contain phosphorus atoms, they do not contribute to the observed ^{31}P spectral pattern. A second distinct ^{31}P-NMR signal is associated with the hexagonal (H_{II}) arrangement of lipids which exhibits a narrower linewidth with reversed spectral asymmetry. PE is the only myelin lipid which, under physiological conditions, has regularly been shown to adopt the H_{II} phase [18,19]. The present myelin lipid mixture does contain a large quantity of PE (25%) which may be sufficient to establish a (population of) H_{II} structure. Such a result would not be an unprecedented finding; for example, the extracted lipids of the photoreceptor membrane [20] which normally form lamellar membranes, have been reported to undergo transitions to the H_{II} and inverted micelle phase when reconstituted in the absence of the integral protein rhodopsin. Finally, smaller lipid particles such as small unilamellar vesicles and micelles, where phosphorus reorientation is sufficiently rapid such that the CSA effect is negligible, give a relatively narrow isotropic ^{31}P signal.

As show in Fig. 1A, the vortexed dispersion of the myelin lipids (see Materials and Methods) yields an asymmetric ^{31}P-NMR signal. Measurement of the chemical shift of the low-field component of Fig. 1A (chemical shift referenced to sonicated PC unilamellar vesicles at zero) indicates that it corresponds to the position of an isotropic signal. Since all of the myelin lipids (with the exception of PE) normally form large multilamellar vesicles when dispersed by vortexing, a multilamellar spectrum should have been observed for this system. Thus, the observed broad spectrum in Fig. 1A could result from the superposition of an isotropic resonance with a broad lamellar signal.

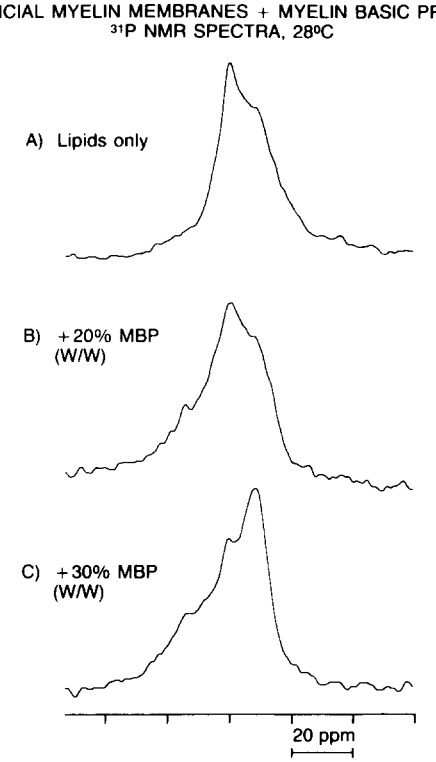

Fig. 1. ^{31}P-NMR spectra (32.42 MHz) of mixtures of myelin lipids (20 mg total lipid dry weight) dispersed in 1.2 ml of $^{2}H_2O$ buffer, pH 7, in the presence of varying quantities of myelin basic protein (MBP). (A) membranes free in solution, $T = 28°C$; (B) same as (A) + MBP (4.0 mg/0.1 ml $^{2}H_2O$ buffer) added; (C) same as (B) + a further 4.0 mg MBP (total 8 mg protein). Lipids present: PC (4.0 mg), PE (5.0 mg), sphingomyelin (1.8 mg), PS (1.8 mg), cerebrosides (5.8 mg.), and cerebroside sulfate (1.6 mg). See Materials and Methods for further details of spectral parameters.

Effect of myelin basic protein on ^{31}P-NMR spectra

Interest in the ^{31}P-NMR signal of the myelin lipid mixture (and the structure(s) it might corresponds to) was heightened by the finding that addition of myelin basic protein (MBP) converted the ^{31}P signal to a characteristic multilamellar pattern. Thus, the interaction of the myelin lipids (Fig. 1B) with MBP (approx. 20% w/w, a protein/lipid mixture similar to the protein's ratio to these lipids in vivo and containing sufficient negatively-charged lipids to bind the protein fully) produced a lineshape which appeared to correspond to an increase in the multilamellar component of the original spectrum. Addition of excess protein (approx. 30% w/w total) eventually gave the characteristic multilamellar spectrum (Fig. 1C). These ^{31}P-NMR results therefore support the contention that MBP promotes a defined multilamellar structure from a phase of apparently mixed morphology in the absence of protein.

Preliminary ^{31}P-NMR studies on the myelin lipids mixture using poly-(L-lysine) as a model 'basic protein' suggest a similar, albeit less effective, propensity (vs. MBP) to promote multilamellar structure.

Freeze-fracture electron microscopy and X-ray diffraction studies on myelin lipid mixtures

To obtain an independent assessment of the structures formed by mixed myelin lipids, with and without MBP, freeze-fracture EM and X-ray diffraction were performed, where possible on aliquots of the same samples used for NMR studies. Freeze-fracture of hand-vortexed dispersions of these lipid mixtures showed unusual vesicularization (Fig. 2A). All lipid appeared in bilayer form, but no multilamellar aggregates were found. This result was confirmed by X-ray diffraction of

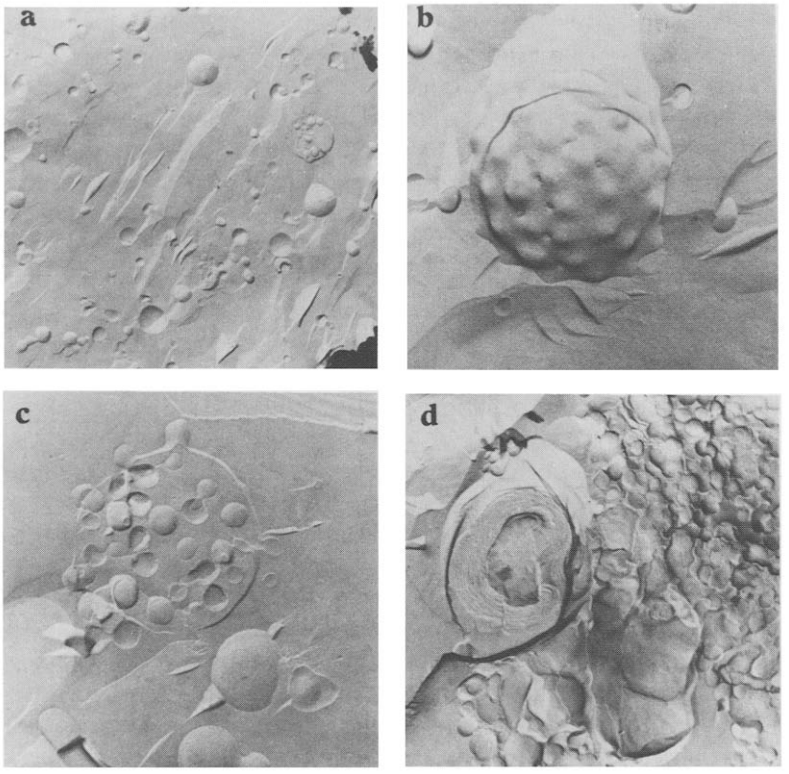

Fig. 2. Freeze-fracture electron microscopy of hand-vortexed dispersions of myelin lipids in an aqueous buffer indicating (a) isolated unilamellar vesicles (magnification 18000×); (b) clusters of vesicles (magnification 53000×); (c) a cross-fracture of the clustered vesicles showing the evaginations (magnification 53000×) and (d) myelin lipids plus 40% myelin basic protein (magnification 35000×). See Materials and Methods for further details of sample preparation.

these samples which showed only central scattering and a complete absence of coherent reflection. The vesicles were either small unilamellar vesicles (approx. 400 Å diameter) or very large (approx. 1000 Å) unilamellar vesicles. The latter were highly convoluted with deep invaginations and evaginations of the size of the unilamellar vesicles, thus giving the appearance of aggregated or possibly fused vesicles (Figs. 2b,c). Cross fractures (Fig. 2c) indicated that the large vesicles contained a multitude of such invaginated and convoluted bilayers. Hence the ^{31}P-NMR line shape of Fig. 1A is correctly accounted for not by H_{II} phase but by the superposition of lamellar phase lineshape and an isotropic signal. The latter signal would arise from the small unilamellar vesicles in the suspension.

When myelin basic protein was added to this dispersion of vesicles, freeze-fracture EM showed almost all the lipid in the form of large multilamellar structures (Fig. 2d). X-ray diffraction showed sharp diffraction lines of multilamellar phase with almost complete absence of central scattering; the lamellar repeat distance was 75 Å. This attribute of MBP to promote multilamellar phases (principally in pure lipid systems) has been previously observed [21,22]. Although only approx. 20% (w/w) of the myelin lipids mixture consists of net negative lipids (i.e., PS and cerebroside sulfate), MBP is still capable of converting all the lipid of the preformed vesicles into the multilamellar structures. This result supports the notion of dual roles of MBP, viz, in the formation and stabilization of the native myelin sheath.

Density gradient centrifugation of myelin lipid mixtures

The apparent superimposed ^{31}P-NMR signals in Fig. 1A did raise the question of lipid segregation. To assess this possibility, density gradient centrifugation of a preparation of the myelin lipid mixtures was performed; four bands were obtained, corresponding to different average sizes of vesicular particles. The lipids comprising these bands were then isolated, dehydrated, redissolved in $CHCl_3$/MeOH (9:1, v/v) and analyzed by thin-layer chromatography, from which it was determined that each mass of vesicles contained a homogeneous mixture of all the original lipids. Therefore, the observed superimposed ^{31}P-NMR signals do not result from the segregation of individual lipid species. In addition, these latter results, in conjunction with freeze-fracture data, indicate that the anisotropic component of the ^{31}P signal is Fig. 1A likely corresponds to the population of large, unilamellar vesicles (Figs. 2B, C).

Discussion

Lipid monomers normally form large multilamellar structures when only a minimal amount of energy (i.e., vortexing) is applied; formation of unilamellar vesicles generally requires a much greater expenditure of energy (i.e., sonication [23] or passage through a French press [24]). Spontaneously-forming vesicles have been previously observed but only under controlled circumstances such as (i) a transient increase in pH of phosphatidic acid-containing lipid suspensions [5,6]; (ii) with mixtures of long (acyl chains greater than 14 carbons) and short (6–8 carbons) chain synthetic phosphatidylcholines [27]; or (iii) mixtures of PC and charged single-chain detergents [28]. The highly convoluted vesicles we observed upon vortexing myelin lipid mixtures are apparently unique for natural lipids. While the physical-chemical reasons for the myelin vesicularization remain to be elucidated, the previous instances of vesicularization suggest that the packing properties of the lipids, expressed as a function of their particular effective volumes [29], may be the crucial determinant (i.e. the ionization of phosphatidic acid with the accompanying alteration in effective head group size [25,26], or the relatively reduced volumes of the detergents [28] and short chain phosphatidylcholines [27]).

Bovine brain PE (consisting primarily of ether-linked plasmalogens) exhibits a lamellar-to-hexagonal transition at approx. 18°C [19]. Therefore the high concentrations of this PE (25% of total) in the myelin lipids mixture could sufficiently disrupt lipid packing so that the formation of the unilamellar vesicles represents the lowest energy state at temperatures where pure bovine brain PE is hexagonal. While no H_{II} formation was detected by any of the experimental techniques, the individual PE monomers will still retain their tem-

perature volume dependency even when incorporated into lamellar vesicles. In this context, a preliminary low temperature (4°C) ^{31}P-NMR spectrum was obtained for the myelin lipids mixture to ascertain whether or not a change in effective PE volume, normally associated with the hexagonal-to-lamellar transition, had any effect on the lipid mixture. The resulting spectrum at 4°C (not shown) did display a decrease in the population of the isotropic spectral component, likely corresponding to a temperature-dependent increase in the average particle size in the system. Further investigation by freeze-fracture EM and X-ray diffraction is necessary to ascertain the exact nature of the low temperature morphology of this lipid mixture.

Myelin is one of the few membranes [2,30,31] that contains such a high concentration of cerebrosides (approx. 40% of experimental system); these are present as a mixture of hydroxylated and non-hydroxylated acyl groups [32,33] with a smaller percentage in the sulfated form [2]. A metastable form [34] of these lipids has been observed and is characterized by a non-reversible exothermic transition (approx. 50°C) followed by an abnormally high gel-liquid crystalline transition (approx. 70°C). This thermal behaviour is believed to be a function of head group hydration and the high degree of intermolecular hydrogen-bonding at the lipid head group region [35,36]. Available data concerning the morphological behaviour of pure cerebrosides [13,37], or of mixtures of cerebrosides and phospholipids [38–40] suggest that cerebrosides are essentially lamellar phase lipids. Since the cerebrosides represent a relatively unexplored class of lipids the possibility remains that their unusual properties, such as the tight head group packing arising from the network of hydrogen bonding, may well provide conditions conducive to the formation of the observed vesicles.

It is emphasized that the present investigation utilizes an in vitro model system which excludes cholesterol, a myelin steroidal component which has been shown to exert a variety of effects on lipids morphology [41–43]; cerebroside-cholesterol mixtures retain the lamellar phase but exhibit complex behaviour as a function of their relative concentrations [40]. The morphological interplay between MBP, the myelin lipids, and cholesterol is presently under investigation.

Mixtures of myelin lipids thus exhibit spontaneous vesicularization in the absence of MBP. The reversal of this process and subsequent stabilization of a multilamellar myelin-like structure by MBP may indicate a similar function in vivo for this protein.

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