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Bilayer-stabilizing properties of myelin basic protein in dioleoylphosphatidylethanolamine systems

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^{31}P-NMR and X-ray diffraction techniques are used to study the comparative ability of myelin basic protein (MBP) vs. other basic proteins to convert hexagonal (H_{II}) phases to stable lamellar (L_α) structures. Pure dioleoylphosphatidylethanolamine (DOPE) at pH 9 and 7, and mixtures of DOPE/phosphatidylserine (PS) (95:5 and 80:20% w/w) at pH 7 were employed for this investigation. The polymorphic behavior of the lipid suspensions was evaluated in the presence and absence of several basic proteins (MBP, calf thymus histone, lysozyme, melittin) and the cationic polypeptide, polylysine (PL). Each of the proteins and PL was capable of binding the pure DOPE H_{II} phase at pH 9 but with varying morphological consequences, i.e., lamellar stabilization (MBP, histone, PL), formation of new protein-DOPE H_{II} phases (lysozyme) or lipid disordering/vesiculation (melittin). Reduction to pH 7 resulted in the dissociation of protein from DOPE – with the exception of melittin – and the reformation of a pure lipid H_{II} phase. Additions of PS to DOPE at pH 7 facilitated protein binding, but among the proteins examined, only MBP was capable of converting the lipid suspension into a stable multilamellar form. Differences in the lipid morphology produced by each protein are discussed in terms of protein physicochemical characteristics. In addition, a possible relationship between MBP–lipid interactions and the stability of myelin sheath lipid multilayers is inferred from the significant bilayer-stabilizing capacity of MBP.

Introduction

Lipids in cellular membranes generally exist as a bimolecular layer. However, interest has heightened in alternative morphological forms when, following the initial observations of Luzzati and co-workers [1–3], some types of lipids were shown to adopt non-bilayer hexagonally-packed cylinders (H_{II} structures) under certain conditions of hydration, pH and temperature. These developments have prompted speculation concerning H_{II}-related non-bilayer structures in the formation of epithelial tight-junctions [4], their involvement in the action of local anesthetics [5], and their possible role in fusion processes such as endo/exocytosis [6]. In the latter process, it is postulated that the tendency to form H_{II} structures can promote fusion between membranes by creating an intermediate fusion state in the form of inverted micelles [7]. While several studies have indicated bilayer-inducing capabilities of integral membrane proteins, such as rhodopsin [8] and glycophorin [9], other peptides (gramicidin [10]) and proteins (cytochrome *c* [11]) have been shown to induce hexagonal structures when associated with a variety of lipids.

The ability of proteins to alter lipid morphology [12–14] may have direct relevance to the myelin sheath, which consists of tightly compacted multilamellar arrays of lipid-protein membranes [15] that provide the essential electrical insulation necessary for efficient nerve conduction. Myelin contains an extrinsic membrane protein, MBP, in its aqueous interstitial cytoplasmic spaces, which has been suggested to aid – through electrostatic and hydrophobic contact sites between

Abbreviations: DOPE, dioleoylphosphatidylethanolamine; H2a, calf thymus histone 2a; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MBP, myelin basic protein; DSC, differential-scanning calorimetry; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, polylysine; PS, phosphatidylserine; TLC, thin-layer chromatography; L_α, lamellar phase; H_{II}, hexagonal phase.

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MBP and lipid – in the initial compaction of the myelin multilamellae [16]. MBP normally constitutes 30% of total myelin protein and its 170-residue sequence contains 31 rather evenly distributed, positively charged sites. Several neurological dysfunctions have been shown to be associated with a reduced myelination due to a significant reduction in MBP synthesis [17]. A consequence of the breakdown of myelin membranes – as is observed in a number of degenerative neurological disorders such as multiple sclerosis – is the onset of uncontrolled muscle tremor and convulsions. One possible mechanism through which this destabilization could progress involves the formation of non-bilayer or similar high curvature (e.g., vesicular) structures within these normally lamellar membranes.

While multilamellar compaction of lipids by MBP has been well-documented [18], only a limited number of investigations [19] have addressed the question of whether MBP has any selective effect on bilayer stabilization. To assess the involvement of the myelin basic protein in the myelin sheath lipid morphology, we have performed a comparative study of the relative abilities of several basic proteins to convert an hexagonal to a lamellar structure. The hexagonal structure is taken as an extreme representation of the conditions indicative of bilayer destabilization. Experimental differentiation between bilayer and H_{II} phases [20] is often possible using X-ray diffraction, freeze-fracture electron microscopy, DSC and/or ^{31}P-NMR spectroscopy. In the present work, we describe the polymorphic behavior – as determined by X-ray diffraction and ^{31}P-NMR – of pure DOPE and mixtures of DOPE/PS in the presence and absence of MBP. We have compared the effects of MBP with several other basic (positively charged) proteins (histone H2a, lysozyme and melittin) and with the cationic polypeptide, PL. The results presented here suggest that MBP may indeed be responsible, in part, for the stability of the densely packed multilamellar membranes of the myelin sheath.

Materials and Methods

DOPE and bovine brain PS were purchased from Avanti Polar Lipids (Birmingham, AL) and shown to be chromatographically pure as determined by TLC. Lipids were stored under nitrogen at −20 °C in stock chloroform solutions until required. Lipid concentrations of these stock solutions were determined by total phosphate analysis, using the method of Bartlett [21]. MBP was extracted from normal human white matter by standard procedures [22] and stored at −20 °C as a lyophilized powder. MBP used in this study was kindly supplied by Dr. M.A. Moscarello and his associates at The Hospital for Sick Children. PL (free base form, $M_r = 41\,000$), egg lysozyme and H2a were obtained from Sigma (St. Louis, MO). Lipase-free bee venom melittin was purchased from Fluka (Ronkonkoma, NY).

The desired quantities of lipids were prepared for X-ray diffraction and ^{31}P-NMR by mixing the appropriate chloroform solutions and evaporating the solvent to dryness under nitrogen. Samples were then placed under vacuum for 2–3 h to remove any residual chloroform. The dry lipid films were dispersed by vortexing (15 min) at room temperature in 1.0 ml of buffer (lipid suspensions were typically 10 mg/ml). Suspensions containing pure DOPE were hydrated in buffer containing 20 mM borate/1.0 mM EDTA at pH 9. Subsequent adjustment to pH 7 was made by titration of the particular sample with 1.0 M HCl. Mixtures of DOPE and PS were dispersed in buffer containing 10 mM Hepes/250 mM NaCl/1.0 mM EDTA at pH 7. Proteins (MBP, lysozyme, H2a, melittin), and polypeptides (PL) were added to the hydrated lipid in 0.5 ml of buffer. Lipid/protein mixtures were then further vortexed to ensure complete mixing. Specific protein-to-lipid ratios are given in the appropriate figure legends. Assays for the concentrations of protein bound to lipid were obtained from centrifuged (30000 rpm for 1 h) lipid-protein samples using the Amido black method of Kaplan and Pedersen [23]. Protein concentrations were assayed both in the supernatant and in the lipid pellet. The absence of lipid in supernatants of all samples confirmed that protein was in excess.

X-ray diffraction studies on centrifuged pellets (30000 rpm for 1 h) were carried out as described previously [24]. Film-recorded diffraction patterns (as reported in Table I) reflected both the symmetry of any packing lattices, lamellar or H_{II}, and their repeat dimensions.

^{31}P-NMR spectra were obtained on a Bruker WP80 spectrometer operating at 32.42 MHz. Lipid-protein samples were suspended in buffer containing 50% (by vol.) D_2O (Merck, Sharp and Dohme). NMR spectra were obtained from the total lipid-protein suspension and not from a centrifuged pellet, as was the case with X-ray diffraction studies. Typically, spectra were accumulated in 20K scans over 16K data points with a sweep width of 10000 Hz and a transmitter pulse width of 8.0 μs. An acquisition time of 0.819 s was used followed by a 0.5 s relaxation delay. Prior to Fourier transformation, the free induction decays were subjected to exponential multiplication with 50 Hz line broadening. All spectra were referenced to the isotropic peak obtained from a sonicated (small unilamellar vesicle) dispersion of egg PC (arbitrarily set to 0 ppm).

In accordance with the observation that small quantities of lipid degradative products can affect the overall lipid morphology [25], the purity of the lipids from X-ray diffraction and NMR studies was evaluated by TLC (solvent system, $CHCl_3/MeOH/H_2O$, 70:30:5, v/v). In all instances, no measurable decomposition was observed.

Results

DOPE phases at pH 7 and 9

Initial investigations were performed using a pure DOPE system which is known to exhibit an L_α-to-H_{II} transition at approx. 12°C [26]. Phosphatidylethanolamines (PE) – one of the few naturally occurring categories of lipids capable of forming hexagonal arrays [27] – have a headgroup conformation which allows for the formation of intermolecular hydrogen bonds [28,29]. This has been suggested to account for increased resistance to headgroup hydration [30]. To circumvent this problem, pure DOPE was dispersed in buffer at pH 9, which deprotonates the ethanolamine ($pK_a = 6.5$) [31] to produce a net negative headgroup charge, and thereby allow the lipid to be dispersed in aqueous media.

To evaluate the effects of pH on phase behavior, the DOPE [pH 9] suspension was examined by X-ray diffraction and ^{31}P-NMR spectroscopy. The diffraction pattern of DOPE (pH 9) (Table I) indicated – from the observation of characteristic $1:\sqrt{3}:2:\sqrt{7}$ reflections – that the bulk lipid structure was hexagonal (H_{II}) at high pH. Previous reports [32] of egg PE preparations at high pH have demonstrated the formation of stable vesicles but the less stringent conditions in our case (i.e., gentle vortexing as opposed to sonication) appeared to have less effect on the DOPE H_{II} structure. Examination of DOPE (pH 9) by ^{31}P-NMR presents the possibility of a slight deviation from an exclusively hexagonal phase; as shown in Fig. 1A, the otherwise characteristic H_{II} signal shows a minor increase in isotropic and lamellar components. While similar increases in the isotropic signal of egg PE at high pH have been reported by Cullis and De Kruijff [26], the minor spectral changes observed for DOPE in this case suggest that the phase of the lipid is essentially hexagonal at pH 9. Titration of the DOPE suspension to pH 7 resulted in the formation of a more sharply defined hexagonal NMR signal (Fig. 1B).

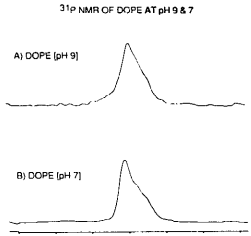

Fig. 1. ^{31}P-NMR (32.42 MHz) spectra of DOPE in aqueous buffer (20 mM borate/1.0 mM EDTA) (A) at pH 9 and (B) titrated to pH 7 with 1.0 M HCl.

DOPE phases in the presence of basic proteins

The addition of basic proteins to DOPE (pH 9) resulted in significant changes in the lipid morphology. MBP (40% w/w; protein net charge +20), histone H2a (40% w/w; protein net charge +17) and PL (20% w/w; fully charged) each produced precipitation of protein-lipid complexes in the form of lamellar phases of varying dimensions (Table I). In agreement with X-ray diffraction results, each of these protein/lipid mixtures exhibited a characteristic multilamellar ^{31}P-NMR signal, as illustrated for the MBP experiment in Fig. 2A.

On the other hand, the addition of lysozyme (40% w/w; protein net charge +8) also resulted in precipitation but the DOPE-lysozyme complex, as determined by X-ray diffraction, maintained its hexagonal packing. An additional positive indication of incorporation of lysozyme is in the increased dimensions of the H_{II} packing (from 67.1 Å in pure DOPE to 71 Å) (Table I). In agreement with the X-ray results, ^{31}P-NMR spectra of the lysozyme-DOPE complex (Fig. 2B) indicated the presence of H_{II} structures. As the initial DOPE structure appeared unchanged, further examination of the

TABLE I

X-ray diffraction measurements of lipid-protein structures indicating the H_{II} and L_α organizations and their repeat dimensions in angstroms (Å)

Experiments were performed with fully hydrated samples at 25°C. Excess protein was used in each sample. See Materials and Methods for further details.

Sample	Pure DOPE		DOPE/PS (% w/w) (pH 7)	
	pH 9	pH 7	95:5	80:20
Lipids only	H_{II} (67.1)	H_{II} (64.3)	H_{II} (66.0)	central scatter
+ MBP (40% w/w)	L_α (74.0)	H_{II} (66.6)	L_α (78.0)/H_{II} (66.0)	L_α (78.0)
+ histone 2a (40%)	L_α (66.4)	H_{II} (65.4)	H_{II} (66.0)	–
+ polylysine (20%)	L_α (58.6)	H_{II} (64.7)	H_{II} (66.6)	H_{II} (66.0)/L_α (62.5)
+ lysozyme (40%)	H_{II} (71.0)	H_{II} (65.2)	–	–
+ melittin (40%)	central scatter	central scatter	–	–

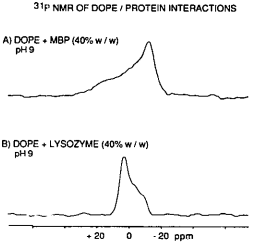

Fig. 2. ^{31}P-NMR (32.42 MHz) spectra of DOPE in aqueous buffer (pH 9) indicating the multilamellar lipid phase when in association with (A) MBP, 40% w/w. H2a (40% w/w) and PL (20% w/w) produced spectra similar to (A). (B) DOPE (pH 9) in association with lysozyme (40% w/w).

DOPE–lysozyme interactions was made through the use of sonicated suspensions of DOPE (pH 9), for which an exclusively isotropic (i.e., vesicular) NMR resonance was observed. Subsequent addition of lysozyme to the DOPE vesicles produced an hexagonal ^{31}P-NMR signal similar to that seen in Fig. 2B (spectrum not shown). Thus, lysozyme was able to convert a completely isotropic suspension of DOPE into a stable hexagonal arrangement. Similar lysozyme-induced hexagonal phases have been reported previously for phosphatidylinositol systems [12].

Titration of the lipid/protein suspensions to pH 7 (and the accompanying loss of protein–lipid electrostatic interactions) resulted in reversion of DOPE/protein mixture to H_{II} (Table I). Assays of lipid-associated protein (at pH 7) indicated that essentially all the H2a, PL and lysozyme had dissociated from the DOPE, although a small fraction (5–7%) of the MBP remained bound to the lipid. In the cases of the DOPE-lysozyme (pH 7) system, which was hexagonal at pH 9, the reduction in pH and loss of protein–lipid interactions resulted in the formation of an H_{II} phase of a different dimension (65.2 Å as compared to 71.0 Å).

Effects of melittin on DOPE morphology

Basic proteins and peptides which contain suitable electrostatic and hydrophobic sites may not necessarily stabilize multilamellar structures, as illustrated by the action of melittin. Extracted from bee venom, melittin is a relatively small protein (26 residues), but contains two distinct domains, one cationic and one largely hydrophobic [33]. Previous investigations [34–36] have demonstrated the tendency of melittin to induce vesiculation/fragmentation of multibilayers. Larger protein molecules, such as cardiotoxin (60 residues and net charge of +10), display similar membrane-disruptive properties [37]. For comparative purposes, it was therefore of interest to examine melittin in the same systems as studied here for other basic proteins.

Addition of melittin (40% w/w; protein net charge +5) to a DOPE (pH 9) suspension resulted in the formation of an isotropic signal normally associated with small rapidly reorienting lipid particles (Fig. 3A). X-ray diffraction of melittin-DOPE (pH 9) combinations showed only diffuse central scattering indicative of disorder (Table I). Reduction of the pH to 7 produced an NMR signal which consisted of both isotropic and lamellar components (Fig. 3B). This indicated that melittin was still capable of binding the net neutral DOPE, whereas all other basic proteins studied dissociated from the lipid at pH 7 with concomitant formation of the hexagonal phase. However, the melittin-DOPE (pH 7) remained in a disordered form as shown by the observation of central scattering in the X-ray diffraction pattern and it is therefore plausible that this complex is in a bilayer (i.e., vesicle) form. This latter possibility is supported by the observation of a significant lamellar resonance in the ^{31}P-NMR spectrum.

Interactions of DOPE/PS mixtures and basic proteins

To examine specifically the lipid binding contributions of uncharged protein residues, a mixture of the net neutral DOPE (95%) and anionic PS (5% w/w) was prepared (pH 7) which produced a homogenous H_{II} (in buffer containing 250 mM NaCl) (Table I). Addition of MBP (40% w/w) demonstrated a preferential ability of MBP to stabilize a significant fraction of the lipid into a multilamellar phase as determined by the approximately equal intensities of lamellar and hexagonal X-ray reflections. As the protein was in excess, the H_{II} content of this sample likely corresponds to a protein-free lipid fraction. Corresponding X-ray diffraction patterns of histone (40%) or PL (20%) plus DOPE/PS (95 : 5) indicated the presence of only an H_{II} phase with no detect-

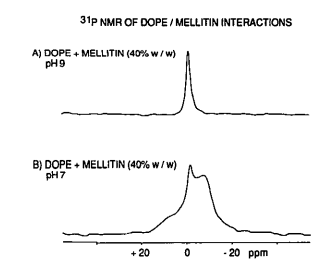

Fig. 3. ^{31}P-NMR (32.42 MHz) spectra of DOPE in aqueous buffer (A) at pH 9 in the presence of melittin (40% w/w) and (B) at pH 7 in the presence of melittin (40% w/w).

able lamellar reflections. Increasing the PS content to 20% (w/w) resulted in the loss of coherent hexagonal X-ray reflections (i.e., disordered lipid structure) for the bulk lipid in the absence of protein. However, the addition of MBP resulted in a complete transformation of the lipid into a stable multilamellar phase which exhibited a repeat dimension similar to that observed for the 5% PS case. In contrast, the addition of PL to the DOPE/PS (80:20) mixture resulted in the formation of mixed L_α and H_{II} presumably due to lateral separation of the two lipids. Thus in both DOPE/PS mixtures, MBP displayed a unique ability to stabilize significant portions of the lipid into a multilamellar phase.

Discussion

That the major basic protein–lipid binding forces involve electrostatic interactions is clearly demonstrated in the pure DOPE study, where the hexagonal-to-lamellar transition was effected by MBP, H2a and PL at pH 9 (DOPE-negative) but not at pH 7 (DOPE-neutral) (Table I). Previous investigations by De Kruijff et al. [38] have shown that prior binding of PL inhibited the calcium-induced L_α-to-H_{II} transition of cardiolipin. These results have led to the suggestion that the PL chains were extended linearly within the aqueous species separating the bilayers and cross-bridging the multilamellar membranes through charge–charge interactions. It is probable that the electrostatic interactions of MBP, H2a and PL with DOPE function in the same fashion as the PL-cardiolipin model to stabilize the DOPE (pH 9) lamellar phase. In this respect, the lipid model used (DOPE (pH 9)) is an artificial case, since, under physiological constraints, the H_{II}-forming PE is zwitterionic and thus not necessarily associated with charged protein sidechains.

Factors affecting protein-induced lipid morphology

While electrostatic interactions of basic proteins with lipid clearly provide the essential 'strong' forces to maintain binding, the proteins examined herein (MBP, H2a, lysozyme, melittin) differ among themselves by only one positive charge when the charge is calculated per unit protein mass. Thus our present results suggest that other factors, such as interactions of lipid with uncharged protein residues, distribution of positive charges within the protein sequence, and/or the protein conformation, must also be important in determining the final lipid structure. For example, proteins generally contain numerous uncharged residues variously capable of binding lipid polar and/or hydrocarbon components. Epand et al. [39] have demonstrated that interactions between MBP and neutral lipids such as PC occur very slowly, even at high MBP concentrations, but could be enhanced with the addition of PE. Similar MBP–PE interactions may also occur in vivo, although, as demonstrated by the DOPE (pH 7) study, hydrophobic interactions between MBP and DOPE alone were not sufficient to effect the hexagonal-to-lamellar transition (Table I). Nevertheless, when a small amount (5% w/w) of the anionic PS is provided, it was shown that MBP was capable of converting a significant proportion of the neutral PE into L_α (as judged from the relative intensities of the X-ray reflections). By increasing the PS content (20% w/w) and thereby providing additional sites of lipid–protein electrostatic interactions, MBP was capable of binding the total lipid mixture and converting it completely into a stable multilamellar structure. It has been shown by X-ray diffraction of oriented monolayers [40] and NMR studies [41] that the hydrophobic/neutral sidechains of MBP reside primarily within the headgroup region of the lipid with little or no penetration into the hydrophobic core. Therefore, these residues would in effect increase the 'size' of the polar portion of the DOPE and allow stable packing of lipid and protein into lamellar arrays. In contrast, even though H2a and PL contained the necessary electrostatic interactions, they appeared incapable of binding the neutral DOPE (in the DOPE/PS mixture) and, as a result, caused lateral phase segregation of the two lipid species.

Conformational effects may be responsible for the differential results obtained with lysozyme vs. the MBP, H2a and PL systems. A critical difference between lysozyme and the other proteins studied here is that it has a more globular conformation due to the constraints of four disulfide bridges [42]. As shown by its H_{II}-promoting capabilities, it is possible that the highly folded lysozyme is accommodated – once bound to DOPE – within the cylindrically packed H_{II} lipids. The expansion of the H_{II} repeat dimension in the presence of lysozyme (from 67 to 71 Å) is sufficient to create enough physical space for the protein, as judged from the crystal structure of this protein, which predicts a radius of gyration of only 13.8 Å [43]. This ability of lysozyme to bind DOPE, apparently without overextending the internal radius of curvature of the lipid, thus actually stabilizes the H_{II}-phase by providing suitable counter charges to reduce DOPE headgroup–headgroup electrostatic repulsion. In this respect, Arvinte et al. [44] have reported a cell-fusing property of lysozyme that is thought to be due in part to the protein's enzymatic activity, but also to its electrostatic and conformational characteristics. The present results suggest that this cell-fusing activity may in fact be related to lysozyme's propensity to form non-bilayer structures.

Addition of melittin to the pure DOPE [pH 9] suspension resulted in an isotropic ^{31}P-NMR signal and X-ray central scattering, which are interpreted in terms of small, rapidly reorienting structures of limited order,

likely of a non-multilamellar type. At pH 7, melittin was able to affect the lipid morphology – unlike the other basic proteins investigated – as indicated by the observed NMR signal containing both isotropic and lamellar resonances (Fig. 3B). The ability of melittin to bind DOPE in the absence of strong electrostatic interactions may be due to a thermodynamic requirement to isolate the hydrophobic portion of the peptide within the lipid core. Thus, in this case, the distinct demarcation of electrostatic and hydrophobic domains appears operative in affecting the nature of protein–lipid binding and consequently the lipid organization.

Lamellar-stabilizing properties of MBP

The observed ability of MBP to induce stable multilamellar arrangements of lipid and protein from the 'bilayer-destabilizing' hexagonal model may have direct relevance to the myelin membrane in vivo. Intrinsic myelin instability has been suggested from the observation that a protein-free collection of myelin lipids exhibits a spontaneous vesicularization [18], which may arise from intrinsic packing properties of PE and/or cerebrosides [45], but which was readily restored to myelin-like multilamellar structure by MBP. Indeed, PE is the most abundant H_{II}-forming lipid in myelin and is actually the single most abundant phospholipid, being third in percent abundance after cholesterol and cerebrosides. Thus, any structural arrangement of myelin lipids that concentrates PE – such as would arise, for example, by membrane asymmetry or lipid segregation – coupled with the inherent high degree of membrane compaction in myelin, may create an unstable situation. The importance of MBP in the formation and compaction of myelin has been well-documented, as shown, for example, in transgenic shiverer mice [46]. However, our observations of the bilayer-stabilizing ability of MBP suggest the possibility that MBP not only compacts the myelin membranes but also helps stabilize the tightly compacted multilayers.

MBP derived from multiple-sclerosis patients has been reported to display a reduced efficiency to bind lipid vesicles [41,47,48]. This may account for the observation that MS-myelin sheaths are reported to be less tightly compacted than in normal individuals [49]. These results have contributed to speculation that MS-MBP has been modified, whether chemically, sequentially, and/or conformationally, to produce a net reduction in its effective cationic character [41]. The extent to which these MS-MBP 'electrostatic' deficiencies affect the stability of the characteristic myelin multilamellar structure remains to be determined.

Conclusions

While the proteins/peptides studied here can all be classed as 'basic', their individual characteristics determine their relative ability to produce a stable myelin-like multilamellar structure. Properties which promote myelin-like structures include: (i) sufficient complementation of protein–lipid electrostatic interactions; (ii) sufficient hydrophobic protein–lipid interactions; (iii) balanced distribution of protein charged and hydrophobic sites (i.e., no appreciable domination of hydrophobic sites within the primary sequence); and (iv) a non-globular protein conformation. Of the proteins/peptides studied, MBP appears to be the best suited in all systems investigated for the promotion of a lamellar phase from a pre-existing hexagonal phase.

Acknowledgements

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