Interaction of the fusogenic peptide B18 in its amyloid-state with lipid membranes studied by solid state NMR

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Available online 6 October 2004

Abstract

The interaction of the fusogenic polypeptide segment "B18" from the fertilization protein bindin with lipid membranes was investigated by solid state \textsuperscript{2}H and \textsuperscript{31}P NMR, and by differential scanning calorimetry. B18 is known to adopt different conformations depending on peptide concentration, ionic conditions, pH and lipid environment. Here, the peptide was studied in its \(\beta\)-H\textsubscript{9252}\textsuperscript{-}stranded amyloid conformation. According to \textsuperscript{31}P NMR, the lamellar morphology of the DMPC bilayer remains intact in the presence of B18. In going from low (1:90) to high (1:10) peptide/lipid ratios, an increasing effect on several different \textsuperscript{2}H-labeled lipid segments was observed, reflecting changes in phase behavior and local dynamics. The strongest influence of B18 was detected at the acyl-chains, while no significant effect on the lipid headgroup conformation was observed. This suggests an insertion of B18 in its fibrillar state into the membrane driven by hydrophobic interactions, rather than a peripheral binding mediated by electrostatics.

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Keywords: Solid state \textsuperscript{2}H NMR and \textsuperscript{31}P NMR; Hydrophobic lipid/peptide interactions; Fusogenic peptide B18; DMPC bilayers; Amyloid fibrils; Membrane fusion

1. Introduction

The interaction of peptides or proteins with membranes plays an important role in many cellular processes. Amphiphilic segments mediate a wide range of biological events, such as signal transduction, transport through the membrane, antimicrobial defense, or membrane fusion. These functions are based on unique structural features, which are often not static but rather responsive to the environment. Hence, the local conformation of a peptide may control its interactions with the lipid bilayer and other binding partners. Understanding this molecular interplay in the membrane-bound state is therefore paramount for interpreting biological function.

The peptide studied here is the fusion-mediating sequence "B18" of the sea urchin fertilization protein bindin. This 25 kDa protein is the major component of the apical acrosomal granule of the sperm cell.
which gets exposed on the cell surface during the fusion process with an oocyte (Vaccquier and Moy, 1977; Hofmann and Glabe, 1994; Lopez et al., 1993; Miraglia and Glabe, 1993).

The minimal sequence required for membrane binding and fusion was identified as an 18 amino acid peptide, B18 (LGILLRLHRHHSNLLANI) that is fully conserved amongst all studied sea urchin species (Ulrich et al., 1998; Zigler and Lessios, 2003a, 2003b). By electron microscopy (EM) and fluorescence fusion assays, B18 was shown to induce fusion of uncharged liposomes in the presence of Zn\(^{2+}\) (Ulrich et al., 1998, 1999). The requirement for this particular metal ion makes sense, as the native parent protein is known to contain one equivalent of zinc coordinated by the histidin-rich sequence of B18 (DeAngelis and Glabe, 1990). Recent electrospray ionization mass spectrometry (ESI-MS) experiments on the affinity of B18 for different divalent cations, as well as data obtained using polarized attenuated total internal reflection infrared spectroscopy (ATR-IR) and functional fluorescence studies, have confirmed a modulation of the peptide’s fusion activity by Zn\(^{2+}\) and Cu\(^{2+}\) (Sinz et al., 2003; 1999; Glaser et al., 1999; Binder et al., 2000).

The B18 sequence is thus postulated as a putative “fusion peptide” in functional analogy to the well-known viral fusion peptides (Pécheur et al., 1999; Martin and Ruysschaert, 2000; Nieva and Agirre, 2003).

Comprehensive studies with circular dichroism (CD), nuclear magnetic resonance spectroscopy (NMR), X-ray diffraction and EM showed that B18 is conformationally flexible and able to adopt several distinct structures, depending on the peptide concentration, pH, the presence of divalent metal ions, detergent micelles, or membrane-mimicking organic solvents like trifluoroethanol (TFE) (Ulrich et al., 1998, 1999; Glaser et al., 1999; Barre et al., 2003). At low concentration and in the presence of Zn\(^{2+}\), B18 adopts a predominantly \(\alpha\)-helical conformation in zwitterionic and negatively charged membranes, which correlates with its fusogenic activity. In this conformation, B18 possesses two \(\alpha\)-helical segments connected by a flexible loop, forming a “boomerang”-like shape. This structure was observed in TFE solutions and detergent micelles by high resolution \(^1\)H NMR (Glaser et al., 1999), as well as in solid state \(^{13}\)C NMR investigations of B18 using macroscopically oriented flat bilayers (Afonin et al., 2004). The hydrophobic N-terminal \(\alpha\)-helix of the peptide was found to be immersed obliquely into the lipid bilayer, whereas the amphiphilic C-terminal helix is aligned peripherally. This structure and mode of membrane binding resembles the behavior of viral fusion peptides (Han et al., 2001; Tamm et al., 2002).

At high concentration, on the other hand, B18 takes on an entirely different conformation. Here, the peptide forms macroscopic assemblies looking like fibrils by EM, and X-ray diffraction confirmed that they consist of cross-\(\beta\)-strands characteristic of amyloid fibrils (Ulrich et al., 1999). At a molecular level, the \(\beta\)-sheet conformation of B18 was supported by IR (Binder et al., 2000), and recently it was shown to extend over the whole length of the peptide by solid state \(^{13}\)C NMR (Barre et al., 2003). In this fibrillar or at least oligomeric \(\beta\)-sheet state, however, the interaction of the peptide with membranes is less well characterized and its functional relevance needs to be discussed. In this study, we used solid state \(^1\)H and \(^{31}\)P NMR as well as differential scanning calorimetry (DSC) to characterize changes in dimyristoylphosphatidylcholine (DMPC) membranes induced by B18 under conditions where amyloid fibrils prevail.

A pronounced structural plasticity, i.e. the propensity to adopt different conformations, is frequently found in both fusion peptides and amyloidogenic peptides (Soto et al., 1994; Coles et al., 1998; Chang et al., 2000; Saecz-Cirion and Nieva, 2002; Kim and Lee, 2004). Many different peptides are able to assume different conformations upon interaction with lipid membranes, including \(\beta\)-sheets, \(\beta\)-turns, \(\alpha\)- and \(\pi\)-helices (Temussi et al., 2003). In the case of fusogenic peptides, this structural flexibility might be a prerequisite for their function (Pécheur et al., 1999; Saecz-Cirion and Nieva, 2002). In this case, it is intriguing to ask which of the multiple possible conformations is relevant for the actual fusion event, and whether a peptide may assume different structures before and after membrane fusion has proceeded.

2. Experimental

2.1. Sample preparation

The B18 peptide was synthesized as described previously by standard solid phase Fmoc chemistry.
The crude product was purified by reverse-phase high-performance liquid chromatography (HPLC) using a linear water/acetonitrile gradient containing 0.1% trifluoroacetic acid. Purity and mass were confirmed by analytical HPLC and electrospray mass spectrometry. Deuterated lipids, 1,2-myristoyl-sn-3-phosphatidyl-trimethyl-deuteroethylamine (DMPC-d4), 1,2-dimyristoyl-sn-3-phosphatidyl-trideuteromethyl-ethanolamine (DMPC-d9) and 1-deutero-myristoyl-2-myristoyl-sn-3-phosphatidylcholine (DMPC-d27) were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

Mixed peptide/lipid samples for NMR and DSC were prepared by co-dissolving B18 and the phospholipids at molar ratios between 1:10 and 1:90 in TFE, removing the solvent under vacuum overnight, and hydrating the sample with 1:1 (w/w) deuterium-depleted water. All samples were homogenized by repeated freeze/thawing and vortexing.

2.2. DSC

The differential scanning calorimetry measurements were performed with a Pyris 1 DSC calorimeter (Perkin-Elmer, Überlingen, Germany). About 10 mg of hydrated material were used for each experiment. The samples were cooled down from room temperature to 0°C and equilibrated for 5 min before each heating scan. DSC heating curves from 0°C to 90°C were recorded at a rate of 5°C/min.

2.3. Solid state 2H and 31P NMR

The NMR studies were performed using a Varian Inova NMR spectrometer operating at a 2H resonance frequency of 76.7 MHz and a 31P resonance frequency of 202 MHz. 2H NMR spectra were acquired on a home-built wideline probe using a quadrupolar echo sequence with a pulse width of 8 μs and an echo delay of 20 μs. Between 80 and 400 transients for relaxation experiments and ~2500 scans for spectra, respectively (dwell time 4 μs, recycle delay 0.5 s), were averaged for each FID. To facilitate the determination of the quadrupolar splittings, the spectra were deconvoluted (“de-paked”), using a home-written computer program based on the weighted fast Fourier-transform algorithm (McCabe and Wassall, 1997). Quadrupolar splittings were estimated from the intensity maxima of corresponding peaks in the individual 2H doublets. Molecular order parameters S_{mol} (−1/2 < S_{mol} < 1) were determined from the splittings of de-paked spectra as the quotient of the measured splitting and the theoretical maximal value. Longitudinal relaxation times (T_1) were obtained by saturation recovery (Ernst et al., 1990), with recovery times ranging from 1 ms to 2 s, and transverse relaxation times (T_2) were measured using a quadrupolar echo sequence (Bloom et al., 1992) with varied pulse spacing (between 16 μs and 13 ms). Relaxation times were determined by fitting the integrated spectral intensity with a single exponential, using identical sets of recovery times or pulse spacings for all headgroup or chain data sets. The fit to DMPC-d27 using all except for the methyl residues gave slightly different values for T_1 and T_2, but the temperature behaviour and conclusions remain the same. 31P NMR spectra were acquired with the same probe, using a Hahn echo sequence with a pulse spacing of 30 μs and 2H decoupling. 1200 scans with a recycle delay of 5 s and a dwell time of 2 μs were averaged. 31P chemical shift ranges given in the text were estimated from the onset of spectral intensity in the NMR spectra. 2H and 31P NMR experiments were performed at temperatures ranging from 0°C to 45°C (error ~2°C), equilibrating the sample for 30 min at each temperature.

2.4. Electron microscopy

Freeze-fracture electron microscopy (EM) was performed as described previously (Ulrich et al., 1999). Suspensions with the desired DMPC/B18 ratio were incubated for 30 min at 30°C in a water bath prior to fracturing. Samples were sandwiched between copper plates and thermally quenched in liquid propane. The frozen samples were fractured in a BAF400 freeze-etching unit (BAL-TEC, Balzers, Liechtenstein) at ~−150°C and shadowed with Pt/C. The carbon-coated replicas were cleaned with CHCl_3 and examined under a CEM 902A (Zeiss, Germany) electron microscope.

3. Results

The impact of the fusogenic peptide B18 on the structural and dynamic properties of DMPC was examined using 31P and 2H NMR spectroscopy and...
DSC. With both techniques we observed significant changes in the lipid induced by the peptide under conditions where B18 is known to self-assemble into amyloid fibrils.

3.1. Phosphorus NMR

$^{31}$P NMR is a valuable tool to characterize the morphology and molecular organisation of phospholipids (Tilcock, 1986; Tilcock and Cullis, 1987). Changes in the $^{31}$P chemical shift are indicative of either conformational changes in the lipid headgroup, orientational modifications of the lipid molecule as a whole, or both (Burnell et al., 1980; Cullis and de Kruijff, 1979; Smith and Ekiel, 1984; Seelig, 1978). The $^{31}$P NMR signal is only marginally affected by conformational adjustments of the mobile and flexible acyl-chains. To monitor the influence of B18 on DMPC, the $^{31}$P NMR spectra of aqueous lipid dispersions with different amounts of peptide were compared over a temperature range from 15°C to 30°C. Representative spectra at 30°C are shown in Fig. 1. All spectra exhibit an overall similar powder lineshape: a broad anisotropic $^{31}$P NMR signal with a pronounced peak at high field and a less intense shoulder at the low field edge. This shape and width are typical for phospholipids organized in a lamellar phase (Seelig, 1978). The addition of B18 increases the chemical shift anisotropy (CSA) width as a consequence of a decreased overall mobility of the lipids (data not shown). For pure DMPC, the width changes from 33 ppm at 30°C to 48 ppm at 15°C. The increased spectral width of the samples containing B18 is preserved over the whole temperature range, with a similar temperature dependence. Hence, the presence of B18 decreases the motional averaging of the lipid headgroups, and/or induces a slight change in their conformation (see below). At all B18 concentrations and over the entire temperature range, the $^{31}$P NMR spectra did not show any signals characteristic of an inverted hexagonal phase (10-25 ppm) or other phases with an isotropic peak (~0 ppm). Therefore, we conclude that the lipid is maintained as a lamellar phase upon binding of B18, even at high peptide concentrations.

3.2. Deuterium NMR

Headgroup-deuterated DMPC-d4 is a powerful indicator of structural changes in the lipid headgroups, induced by interactions at the membrane surface such as peptide binding, electrostatic effects, or dehydration (Sixl et al., 1984; Seelig et al., 1987; Scherer and Seelig, 1989; Marassi and Macdonald, 1992; Ulrich and Watts, 1994; Franzin and Macdonald, 1996; Crowell and Macdonald, 1999; Hori et al., 2001). The quadrupole splittings of the two pairs of deuterons in the ethylene moiety of the headgroup are related to the choline tilt angle, thus giving a direct measure of any structural influence by a peptide on the lipid headgroup. With B18, only a very modest effect was found (Fig. 2). The outer splitting (assigned to the $\alpha$-deuterons) decreases from 5.6 kHz to 5.4 kHz with increasing peptide concentration, whereas the inner splitting (assigned to the $\beta$-deuterons) stays nearly constant. Overall, the lineshapes are significantly broadened with increasing amounts of B18. These observations support the $^{31}$P
Fig. 2. Deuterium NMR spectra of headgroup labeled lipids (DMPC-d4) in the presence of different amounts of B18 at 35°C. The quadrupolar splittings of the two pake-doublets are related to the zwitterionic lipid headgroup tilt angle. The modest influence of the peptide on the splittings reflects only weak electrostatic interactions between B18 and the lipid headgroups. NMR data in terms of a minor conformational adjustment and a pronounced decrease in the mobility of the lipid headgroups.

The influence of B18 on the lipid mobility in the membrane was studied by varying the temperature. Deuteration of the lipid headgroups (DMPC-d9) and acyl-chains (DMPC-d27) allowed examining different regions of the bilayer. The respective 2H NMR spectra of samples without and with B18 (1:10 mol/mol ratio) are compared in Figs. 3 and 4. In both cases, the phase transition shows up in the pure lipid samples with a distinct change of the 2H NMR spectra at a temperature between 24°C and 28°C. While spectra above the phase transition temperature possess pronounced doublet features, these are broadened in the low temperature gel state. (Note that the deuterium isotope effect might lower the transition temperature of chain deuterated DMPC below 24°C. The deviation in our case is likely due to experimental uncertainty in the absolute temperature calibration.) In the presence of B18, a clear phase transition is no longer discernible (Figs. 3b and 4b), instead a gradual change in the spectra is observed over the entire range of temperatures.

![Fig. 3. Deuterium NMR spectra of DMPC-d9 (a) and B18/DMPC-d9 at a molar ratio of 1:10 (b) at temperatures between 20°C and 43°C as indicated. The distinct phase transition, observed between 24°C and 28°C for the pure lipid, is no longer observed in the presence of B18.](image1)

![Fig. 4. Deuterium NMR spectra of DMPC-d27 without B18 (a) and with B18 at a peptide/lipid ratio of 1:10 (b), at temperatures between 20°C and 43°C as indicated. Spectra of lipids in the presence of B18 lack a sharp phase transition, and the spectral broadening of the gel state persists in the liquid crystalline phase.](image2)
Relaxation measurements were used to describe the influence of the peptide on local and global lipid dynamics. Transverse ($T_2$) and longitudinal ($T_1$) relaxation is caused by processes on a slow (ms) and fast (10 ns) time scale, respectively, thus providing complementary measures of lipid dynamics.

Transverse relaxation as a function of temperature is shown in Fig. 5, following the same trends as already indicated by the $^2$H NMR spectra. Without peptide a significantly higher $T_2$ value (3.5–4.0 ms and 0.50–0.55 ms for DMPC-d9 and DMPC-d27, respectively) was found for temperatures above the phase transition than below (1.6 ms and 0.13 ms for DMPC-d9 and DMPC-d27, respectively). In the presence of B18 (1:10 mol/mol ratio), however, the $T_2$ values especially in the high temperature range decrease significantly, and low $T_2$ values between 1.0 ms and 2.2 ms (DMPC-d9) and between 0.10 ms and 0.23 ms (DMPC-d27) are observed in the whole temperature range. These findings correlate well with the broadening of the $^2$H NMR spectra (Figs. 3 and 4), since short $T_2$ relaxation times induce strong line-broadening. As found for the spectral lineshapes, also the $T_2$ values show the same trend for both headgroup- and chain-deuterated lipids.

In contrast to the similar $T_2$ response and spectral broadening found for both, headgroups (Fig. 5a) and acyl-chains (Fig. 5b), the $T_1$ relaxation differs for these two lipid regions (Fig. 6). No changes are induced in the $T_1$ relaxation of the DMPC-d9 headgroups by the addition of peptide (Fig. 6a), where both series exhibit a steady increase with temperature. On the other hand, the lipid chains experience a change in $T_1$ upon the addition of peptide (Fig. 6b), with decreased values in the presence of peptide above the phase transition temperature.

In order to localize the influence of the peptide on the individual lipid segments in greater detail, the $^2$H NMR spectra of chain-deuterated DMPC-d27 at 35 °C were analyzed in terms of their order parameter profile. A measure for the site-specific order along the lipid chain is the segmental order parameter $S_{mol}$, which is directly proportional to the quadrupole splitting. With the aid of "de-paked" spectra (Bloom et al., 1981), the quadrupole splittings of the individual deuterated groups were resolved and their mobilities...
Fig. 7. Molecular order parameters ($S_{mol}$), obtained from “de-paked” $^1$H NMR spectra of pure DMPC-d27 (open circles) and in the presence of B18 at a peptide/lipid ratio of 1:10 (filled circles). Despite de-paking, individual residues could not be resolved in the order parameter plateau region (positions 2–7 and 2–6 for lipids without and with B18, respectively), and an average value is given here instead. A decrease of $S_{mol}$ by a factor of 0.92 is observed in the presence of B18 for carbon positions 7–14.

Deconvoluted to reveal the effect of the peptide on $S_{mol}$ at different depths in the lipid bilayer (de-paked spectra not shown). The order parameter profiles of samples with and without peptide are compared in Fig. 7. A slightly lower order parameter plateau (by a factor of 0.96) is observed for the carbons close to the glycerol backbone in the presence of B18. However, a significant change is found to be induced by the peptide in the carbon range C7–C14. Here, the order parameter is reduced by a factor of 0.92 ± 0.01, indicating an increase in local mobility or decrease of order in the central-to-lower region of each bilayer leaflet.

3.3. Differential scanning calorimetry

The influence of B18 on the thermotropic phase behavior of DMPC in excess water was examined by DSC, as illustrated in Fig. 8. In the absence of peptide, DMPC alone shows the expected two phase transition events: the less enthalpic lamellar gel-to-rippled gel (pre-transition) and the main gel-to-liquid crystalline acyl-chain melting transition. In the presence of B18, the pre-transition disappears. The width of the main transition is significantly broadened with increasing amounts of peptide, which makes a determination of the transition temperature less accurate. As a general tendency, the main transition is seen to be shifted towards lower temperature, and the enthalpy is significantly reduced especially for the 1:10 B18/DMPC sample.

4. Discussion

4.1. Phase behavior of the B18/DMPC mixture

Model membranes formed by hydrated DMPC exhibit a distinct thermotropic phase behavior, characterized by a low temperature gel state and a high temperature liquid crystalline phase. The acyl-chain melting transition at around 24°C is highly cooperative in pure lipid bilayers, as reflected in the small width of the main transition peak by DSC (Fig. 8) and the distinct change in the $^1$H NMR spectra at the phase transition (Figs. 3a and 4a).
The addition of B18 changes the phase behavior substantially under conditions where amyloid fibrils are formed. The observed broadening of the DSC curves and the loss in cooperativity evident from DSC and $^2$H NMR suggest a close interaction of B18 with the lipids, ruling out a separation of the peptide and lipid components. Also, the fact that only a single phase transition event is observed by DSC up to a peptide/lipid ratio of 1:10 (mol/mol) indicates a uniform behavior of the system, ruling out the formation of peptide-rich domains. The lipid phase behavior in the presence of B18 is similar to that found for membranes consisting of more than one species, or mixtures of lipids and lipid-soluble molecules. Prominent examples are phospholipid/cholesterol systems, which exhibit a comparable broadening of the phase transition at cholesterol concentrations up to 50 mol% (McMullen et al., 2000).

The formation of a single peptide/lipid phase is further supported by electron microscopy (Fig. 9). Preparations of 1:10 B18/DMPC are strongly striated but homogeneous in their overall appearance, showing neither separate lipid vesicles nor discrete peptide fibrils. This suggests the formation of uniform lipid/peptide complexes, confirming the close interaction of B18 with DMPC in full agreement with previous studies (Ulrich et al., 1999; Binder et al., 2000). Another indication for the formation of a homogeneous mixture is provided by the spectral linebroadening in $^2$H and $^{31}$P NMR (Figs. 1, 3 and 4). The addition of B18 leads to a considerable broadening of the spectral features at temperatures above the phase transition. In principle, relaxation (homogeneous linebroadening), but also sample heterogeneity (inhomogeneous linebroadening) can cause such spectral broadening. However, only the homogenous linebroadening $\Delta v$ (full width at half height) is related to the transverse relaxation time $T_2$ by $\Delta v = 1/(\pi T_2)$. The $T_2$ values measured in this study fully account for the observed linebroadening, as judged by a comparison of the experimental data with simulated $^2$H NMR spectra using linebroadening according to the observed $T_2$ values (data not shown). Any further extent of linebroadening due to sample heterogeneity therefore seems unlikely.

4.2. Influence of B18 on collective membrane dynamics

The transverse relaxation time ($T_2$) is a measure of slow collective bilayer motions, such as lateral diffusion or bilayer undulations, thus revealing the effect of B18 on “mesoscopic” membrane dynamics. A significant change in $T_2$ relaxation was noticed in the presence of B18, where both lipid headgroups and chains are affected in a similar way (Fig. 5). The effect of the peptide on slow motions is thus attributed to an alteration of lipid diffusion and collective membrane motions. The gel and liquid crystalline lipid phases are affected by B18 in different ways. Below the main lipid phase transition very little change is seen upon addition of B18, but a significant increase in the relaxation efficiency is noticed in the liquid crystalline state. At high temperature, the $T_2$ values in the presence of B18 (2.2 ms and 0.23 ms for DMPC-d9 and DMPC-d27, respectively) are close to the low values of pure lipids in the gel-state unaffected by B18 (1.6 ms and 0.13 ms, respectively). The slow dynamics of the gel state thus appear to be preserved also at temperatures above the phase transition when B18 is bound to DMPC membranes. It is conceivable that extended peptide fibrils are embedded in the lipid bilayer, where they act as diffusion barriers for the lipid molecules and lead to a stiffening of the membrane, thereby reducing collective motions.
4.3. Influence of B18 on lipid segmental dynamics and structure

The slow motions addressed via T\textsubscript{2} measurements reveal similar effects of B18 on the lipid headgroups and acyl-chains. In contrast to this influence of B18 on the global membrane properties, its direct effect on the order and dynamics of different lipid segments is mostly confined to the acyl-chains. T\textsubscript{1} relaxation (Fig. 6), representing molecular motions on a fast timescale (\sim 10 ns), and the local segmental order apparent from the quadrupole splittings (Fig. 7) are much more sensitive to B18 in the acyl-chain region than the T\textsubscript{1} values and quadrupole splittings measured for the headgroups.

The temperature dependence of T\textsubscript{1} (Fig. 6a versus Fig. 6b) thus suggests a rather weak interaction of B18 with DMPC headgroups, if any at all. Likewise, the tilt angle of the lipid headgroups remains rather unaffected by the presence of B18, as the quadrupole splittings in the DMPC-d\textsubscript{4} spectra changed only slightly with the addition of B18 (Fig. 2). The “molecular volt-meter” DMPC-d\textsubscript{4} had been used in the past to monitor charge-charge interactions of the labeled lipid headgroups with ionic lipids and surface-bound molecules (Sixl et al., 1984; Seelig et al., 1987; Scherer and Seelig, 1989; Marassi and MacDonald, 1992; Franzin and MacDonald, 1996; Crowell and Macdonald, 1999). For many peptides, significant changes in the \textsuperscript{2}H quadrupole splittings have been observed and related to a structural response in the polar/apolar interface due to electrostatic interactions. The lack of similar changes in the \textsuperscript{2}H NMR spectra in the presence of B18 thus indicates the absence of electrostatic interactions.

In contrast to the very slight changes in the headgroup region, B18 exerts a considerable influence on the acyl-chains. Their T\textsubscript{1} relaxation behavior is significantly altered, suggesting a change in fast timescale motions. Most local segmental motions such as trans-gauche isomerization, have contributions in this frequency range. A possible way, how B18 might alter such lipid motions in the chain region, could be a change in the available conformational space in the presence of the peptide fibrils. Further evidence for an interaction of B18 with the lipid acyl-chains is provided by their order parameter profile (Fig. 7). A decrease in local order, and hence an increase in local mobility and/or a change in local lipid alignment, was observed here in the DMPC segments from C7 to C14. Similar indications for an interaction of B18 with the chain region have been found recently in POPC bilayers (Barre et al., 2003), where a reduction of the lipid order parameter due to B18 was observed over the entire length of the chains. At first, it may seem implausible that B18 should influence the lower part of the DMPC chains much more than the upper plateau region. However, the observed order parameter profile can be explained if the change in local order is due to a modification of the accessible conformational space due to the presence of B18. In this case, even an insertion of the peptide only into the upper region of the bilayer leaflet could cause a reduction in lipid packing in the lower regions, which could be consistent with the observed decrease in order parameter.

The most likely mode of interaction between B18 and DMPC therefore seems an insertion of the peptide in an oligomeric or fibrillar state, based on hydrophobic interactions with the lipid membrane. The depth of insertion of B18 or a possibility of a transmembrane immersion, however, remains unclear, also with regard to the alignment of the self-assembled units.

4.4. Fusogenic activity of B18

To explain the fusogenic action of peptides it is important to demonstrate their influence on the bilayer structure, namely their ability to cause transitions from lamellar to non-lamellar lipid arrangements. Typically, the fusogenic effect is related to the appearance of non-bilayer structures, where isotropic lipid motions occur (Burnell et al., 1980; Cullis and de Kruijff, 1979; Smith and Ekiel, 1984; Seelig, 1978). These can be detected by the appearance of a signal around 0 ppm in \textsuperscript{31}P NMR or a central peak in \textsuperscript{2}H NMR, but no such signals were observed with B18.

When different biophysical methods are used to correlate the fusogenic activity (e.g. fluorescence dequenching) of a peptide with its structure (e.g. CD, or
solid state NMR), special attention has to be paid to the exact sample conditions: not only the peptide/lipid ratio has to be considered, but also the absolute peptide concentration in the sample (fluid volume versus liposomes), and the method of sample preparation and its storage history. Previous CD and ATR-IR data (Ulrich et al., 1998; Glaser et al., 1999; Binder et al., 2000) as well as solid state $^{13}$C NMR (Barre et al., 2003) have shown that the total peptide concentration per se is the most critical factor causing it to adopt a β-sheet structure. The β-sheet conformation again correlates with the appearance of amyloid fibrils in X-ray diffraction and striated lipid images in freeze-fracture electron micrographs (Ulrich et al., 1999). Upon checking the appearance of samples by EM following a series of fluorescence fusion assays, it turned out that striated lipid images were observed only under conditions where fusion had been inefficient. On the other hand, under favorable fusogenic pH and ionic conditions (i.e. in the presence of Zn$^{2+}$, or when using negatively charged lipids), EM showed round liposomes with vastly increased size and smooth (if not slightly dotted) surfaces (Ulrich et al., 1999). In our current NMR and previous X-ray samples, the peptide was codissolved with lipid in an organic solvent and dried, before 50% water (w/w) was added. In typical fusion experiments, the liposomes were preformed as a dilute dispersion before the peptide was added externally, but we obtained similar (though not as homogeneous) EM images that way (Ulrich et al., 1999). We note that our previous $^{19}$F NMR determination of the helical B18 structure in oriented DMPC/DMPG (80:20) bilayers had been carried out with a much lower peptide/lipid ratio of 1:150 (Afonin et al., 2004) than the 1:10 peptide/lipid ratio used here. The above findings thus suggest that the B18 peptide is not in its fusogenically active state in the samples investigated here.

5. Conclusions

The influence of the fusogenic segment B18 from the fertilization protein bindin on the lipid environment was studied by solid state NMR and DSC. The peptide can adopt a variety of conformations depending on concentration, ionic conditions, pH, and lipid environment. The peptide structure and its behavior in the membrane have been previously well characterized for the helical conformation adopted by B18 at low peptide concentration (in the presence of Zn$^{2+}$, or when embedded in negatively charged DMPC/DMPG bilayers). In the current study, we have investigated the peptide/lipid interactions corresponding to a different conformation of B18 at high peptide concentration (and in the absence of Zn$^{2+}$). In this state, the peptide is known to form amyloid fibrils with a characteristic β-sheet structure (Ulrich et al., 1999; Barre et al., 2003).

Clear evidence was found for a close interaction of the B18 peptide with DMPC bilayers at a molecular level. Both $^2$H NMR and DSC showed that the peptide reduces the cooperativity of the lipid phase transition. Furthermore, the pre-transition is lost when B18 is present, and the phase transition temperature is decreased. These findings indicate the formation of a uniform lipid/peptide system, rather than isolated domains of pure lipid and peptide aggregates. The interaction of B18 with the membrane is likely to involve extended peptide fibrils, although it cannot be ruled out that single molecules and oligomers co-exist with the amyloid in a thermodynamic equilibrium. However, a distinction of the smallest peptide unit interacting with and strongly influencing the membrane is not possible on the basis of the solid state NMR and DSC results. The appearance of extended striated patterns in the freeze-fracture electron micrographs (Fig. 9) indeed suggests that long fibrils are embedded in the membranes. Such bundles of parallel aggregates could be responsible for the observed texture and for the absence of vesicles in the electron micrographs. No isolated fibrils were seen in the electron micrographs, underlining again the formation of a single peptide/lipid phase. The presence of long membrane-embedded fibrils is also consistent with the reduced diffusion of lipid molecules (concluded from their $T_2$ relaxation and line broadening) and with the lack of cooperativity in the phase transition.

On the basis of the solid state NMR results, it was possible to gain a differentiated picture of the influence of the peptide on different regions of the bilayer. The headgroup structure and dynamics are influenced only marginally by the addition of peptide. However, significant change of segmental order and dynamics were found in the acyl-chain region of the lipids in the presence of fibrillar B18. Therefore, an insertion of
the peptide at least partially into the membrane, driven by hydrophobic interactions seems more likely than a peripheral binding dominated by electrostatic interactions. The strong influence of the peptide on the order parameters of C7–C14 suggests an insertion of B18 fibrils into the bilayer interior, but does not necessarily imply a transmembrane penetration. Despite B18 being a fusogenic peptide, only lamellar lipid morphologies were observed in this study. The absence of highly curved fusion intermediates suggests, though does not prove, that the peptide conformation studied here is not the structure relevant for the fusogenic activity of B18. Indeed, the formation of β-sheet aggregates has been demonstrated for a large number of peptides typically as a pathogenic rather than a functional feature. Preliminary data from our lab suggest that the actual fusogenic state of B18 cannot be a self-assembled β-sheet, since peptide analogues containing a single sterically obstructive d-enantiomeric side chain exhibit the same fusion activity as their all-l analogues (Afonin et al., 2003). On the other hand, it has been argued that high local B18 concentrations and oligomerization are essential requirements for fusion to occur (Barre et al., 2003). This suggestion would be especially appealing in the case of viral fusion proteins, which are known to undergo a concerted action involving several trimeric units at the same time (Skehel and Wiley, 1998; Tamm et al., 2003). However, with small peptides it may not be possible to orchestrate such mechanism in a spatially and timely coordinated manner, and the risk of non-specific aggregation is much higher than for well-folded proteins. Indeed, the conformation of several small viral fusion peptides has been recently demonstrated to be controlled mainly by the lipid composition of the membrane (Yang et al., 2001; Bodner et al., 2004).

In summary, B18 in its β-sheet conformation was found to interact strongly with DMPC bilayers. The results suggest a hydrophobic insertion of the peptide rather than a peripheral binding due to electrostatic interactions with the headgroups. Presumably, the presence of self-assembled B18 amyloid fibrils alters the lipid chain structure and dynamics and affects the mesoscopic dynamic properties of the bilayer. It will be interesting to find out whether this mode of binding might also resemble that of other membrane-associated amyloid fibrils.

Acknowledgements

At the FSU Jena, we would like to thank Heike Bunjes for her help with DSC measurements, and Helmut Meyer for the electron microscopy. Financial support from the DFG is gratefully acknowledged (SFB 197, TB B13) and the TMWFK for a stipend for SLG.

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