Influence of Membrane-Spanning α-Helical Peptides on the Phase Behavior of the Dioleoylphosphatidylcholine/Water System

Sven Morein,* Erik Strandberg,* J. Antoinette Killian,# Stefan Persson,* Gösta Arvidson,§ Roger E. Koeppe II,¶ and Göran Lindblom*

*Department of Physical Chemistry, Umeå University, S-901 87 Umeå, Sweden, #Department of Biochemistry of Membranes, University of Utrecht, Padualaan 8, 3584 CH, Utrecht, The Netherlands, §Department of Physiological Chemistry, University of Uppsala, Biomedical Center, S-751 23 Uppsala, Sweden, and ¶Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, Arkansas 72701 USA

ABSTRACT The effect of solubilized hydrophobic peptides on the phase behavior of dioleoylphosphatidylcholine (DOPC)/water system was studied by ^{2}H- and ^{31}P-NMR spectroscopy and by x-ray diffraction, and partial phase diagrams were constructed. The utilized peptides were HCO-AWW(LA)$_5$WWA-NHCH$_2$CH$_2$OH (WALP16), which is an artificial peptide designed to resemble a transmembrane part of a membrane protein; and VEYAGIALFFVAAVLTLWSMLQYLSAAR (Pgs peptide E), a peptide that is identical to one of the putative transmembrane segments of the membrane-associated protein phosphatidylglycerophosphate synthase (Pgs) in *Escherichia coli*. Circular dichroism spectroscopy suggests that both peptides are mostly α-helical in DOPC vesicles. The most striking features in the phase diagram of the WALP16/DOPC/water system are 1) a single lamellar liquid crystalline (L_α) phase forms only at very low peptide concentrations. 2) At low water content and above a peptide/lipid molar ratio of ~1:75 a reversed hexagonal liquid crystalline (H_{II}) phase coexists with an L_α phase, while in excess water this phase forms at a peptide/lipid molar ratio of ~1:25. 3) At peptide/lipid ratios $\geq$1:6 a single H_{II} phase is stable. Also, the Pgs peptide E strongly affects the phase behavior, and a single L_α phase is only found at low peptide concentrations (peptide/lipid molar ratios <1:50), and water concentrations <45% (w/w). Higher peptide content results in coexistence of L_α and isotropic phases. Generally, the fraction of the isotropic phase increases with increasing temperature and water concentration, and at 80% (w/w) water content only a single isotropic phase is stable at 55°C. Thus, both peptides were found to be able to induce nonlamellar phases, although different in structure, in the DOPC/water system. The phase transitions, the extensions of the one-phase regions, and the phase structures observed for the two systems are discussed in terms of the molecular structure of the two peptides and the matching between the hydrophobic lengths of the peptides and the bilayer thickness of DOPC.

INTRODUCTION

Several fundamental processes in biological cells involve the interplay between lipids and membrane proteins and/or amphiphilic peptides. One example of this is the problem of partitioning of proteins into membranes and their subsequent folding (Chung and Thompson, 1996; Baumgärtner, 1996; Engelman et al., 1986; Popot et al., 1994; White, 1994; von Heijne, 1994). Another, perhaps even more intriguing example, where protein/lipid interactions are supposed to be involved, originates from the observation that most organisms have a tight regulation of their membrane lipid composition to maintain the membrane integrity [Andersson et al., 1996; Morein et al., 1996a; Rietveld et al., 1993; (for reviews see Hazel and Williams, 1990; Rilfors et al., 1993; Suutari and Laakso, 1994)]. While rather extensive knowledge about the physicochemical properties of membrane lipids has been obtained over the last decades, as for instance their phase behavior in water and buffers or how they take part in maintaining a permeability barrier function of the cell membrane (see, e.g., Lindblom and Rilfors, 1992), much less is known about how proteins and hydrophobic peptides interact with their lipid environment.

Support for the view that membrane proteins can affect the physical state of the surrounding membrane lipids has been published (Brown, 1994; Killian, 1992; Killian et al., 1996; Orädd et al., 1995; Marsh, 1996; Morrow and Davis, 1988; Peschke et al., 1987; Piknová et al., 1993). It has also been shown that the structure and function of integral membrane proteins can be modulated by the lipid composition of the plasma membrane (Cornea and Thomas, 1994; Jensen and Schutzbach, 1988; Johansson et al., 1981; Montecucco et al., 1982). However, very little is known about the molecular mechanism behind this modulation/regulation.

To get a detailed understanding of protein/lipid interactions and how lipids possibly affect the activity of integral membrane proteins, we have begun to investigate, mainly by NMR spectroscopic techniques, the behavior of very hydrophobic peptides mimicking transmembrane segments of integral membrane proteins (Killian et al., 1996; Morein

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Address reprint requests to Dr. Göran Lindblom, Department of Physical Chemistry, Umeå University, S-901 87 Umeå, Sweden. Tel.: +46-90-7865228; Fax: +46-90-7867779; E-mail: goran.lindblom@chem.umu.se.

Abbreviations used: NMR, nuclear magnetic resonance; CD, circular dichroism; CSA, chemical shift anisotropy; DOPC, dioleoylphosphatidylcholine; FID, free induction decay; H_{II}, reversed hexagonal liquid crystalline; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; L_α, lamellar liquid crystalline; Pgs, phosphatidylglycerophosphate synthase; Pgs peptide E, VEYAGIALFFVAAVLTLWSMLQYLSAAR; SDS, sodium dodecylsulphate; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; TLC, thin layer chromatography; WALP16, HCOAWW(LA)$_5$WWANHCH$_2$CH$_2$OH

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et al., 1996b). These studies were performed on aqueous systems containing detergent micelles or phospholipid liposomes. Besides getting information about the liquid crystalline structure of the peptide/lipid/water system and the three-dimensional structure of the peptide itself, we are interested in how peptide-peptide and peptide-lipid interactions occur within the membrane. This is still largely unknown, despite several previous works on transmembrane hydrophobic peptides incorporated in lipid membranes (Davis et al., 1982; Epand et al., 1995; Greathouse et al., 1994; Huschilt et al., 1985; Killian et al., 1989, 1996; Pauls et al., 1985; Roux et al., 1989; van Echteld et al., 1982; Watnick et al., 1990; Xing and Scott, 1992; Zhang et al., 1992, 1995a, b).

Phase diagrams are often of great help in the interpretation of data obtained by, e.g., NMR spectroscopy in physicochemical studies of membrane peptides (Lindblom, 1996a). In this work we have investigated the effect of two hydrophobic peptides on the phase behavior of the system DOPC/^2H_2O. The amino acid sequences of the two utilized peptides are WALP16 and Pgs-[(149–176)-peptide; Pgs peptide E)]. WALP16 is an artificial peptide designed to be a model for transmembrane segments of integral membrane proteins. It consists of 16 amino acid residues, contains no charges, and is anchored to the polar/nonpolar interface of phospholipid bilayers by two tryptophans at each end of the peptide. WALP16 has a strong ability to induce nonbilayer structures in phosphatidylcholine/water mixtures, provided that the hydrophobic thickness of the lipid aggregates is sufficiently large (Killian et al., 1996). The Pgs peptide E is identical in sequence with a putative transmembrane segment from the Pgs, which takes part in the phospholipid synthesis in *Escherichia coli* (Dowhan, 1992). This peptide consists of 28 amino acid residues, two of which are charged, Glu-2 and Arg-28, while the middle part (residues 4 through 17) is mainly hydrophobic. The C-terminal part has an amphipathic character with both hydrophobic and relatively polar amino acids. The Pgs peptide E was recently shown by two-dimensional ^{1}H-NMR and CD spectroscopy to adopt a major α-helical conformation in SDS micelles (Morein et al., 1996b). In this work we have investigated the phase behavior of two systems, namely WALP16 and Pgs peptide E, in DOPC/water. The studies were performed at 25°C and 55°C and at water concentrations between 20 and 80% by weight. It is shown that both peptides are able to induce nonlamellar structures in a DOPC/water system, depending on both the peptide and the water concentration as well as on the temperature. However, the two peptides show large differences in their ability to induce nonlamellar phases. These results are discussed in terms of differences in the structure and the amino acid composition of the peptides.

MATERIALS AND METHODS

Materials

The Pgs peptide E was synthesized by Dr. Åke Engström at the Department of Medical and Physiological Chemistry, University of Uppsala, Sweden. After analysis by plasma desorption mass spectrometry and analysis of the amino acid composition, a decision was made to use the peptide without further purification. WALP16 was synthesized as described by Killian et al. (1996).

DOPC was purchased from Avanti Polar Lipids Inc. (Alabaster, AL) The purity was checked by TLC and the lipids were used without further purification. The TFA was purchased from Merck (Darmstadt, Germany), and the TFE was obtained from Sigma (St. Louis, MO). The water was deionized and filtered with a Milli-Q water purification system (Millipore, Bedford, MA) and the deuterium oxide (^2H_2O) was purchased from Glaser AG (Basel, Switzerland).

Sample preparation

The peptide/DOPC samples were prepared by a modified version of the two-step method described previously (Killian et al., 1994). The peptide was dissolved in TFA and dried to a film under a stream of nitrogen and subsequently dissolved in 2 ml TFE and again dried to a film in a rotavapor (this second "TFE-wash" takes away some of the residual TFA). Next, the peptide was dissolved in TFE to a concentration of 5–20 mM. The peptide/TFE solution was then added to a concentrated phospholipid/water dispersion buffered to pH 7.0 by 100 mM HEPES. TFE and residual TFA were then removed by a washing procedure described as follows. Additional water was added up to 25 ml and the samples were centrifuged three times at 20°C for 1.5 h at 50,000 rpm (257,000 g_{max}) in a Ti-70 rotor with a Beckman LE-70 ultracentrifuge. Between the centrifugation steps the supernatant was removed and the peptide-lipid pellet was again dispersed in fresh milli-Q-water. The presence of TFE and/or TFA in the supernatant was checked by ^{19}F-NMR. Normally, no fluorine signal could be detected in the supernatant after the third wash. After the washing procedure the resulting peptide/lipid pellet was dispersed in 4 ml milli-Q water and lyophilized. The lyophilized material was then transferred to an 8-mm glass tube and dried to constant weight under high vacuum. Finally, the samples were hydrated with 10 mM HEPES, pH 7.0 in ^2H_2O, and then flame-sealed. The samples were buffered at pH 7.0, since otherwise the aqueous peptide/lipid samples showed discoloring and degradation of the lipids with time. Before the NMR measurements were performed the samples were homogenized by centrifuging the samples back and forth in sealed glass tubes. The samples were then left for a few days in a dark place at room temperature in order to allow them to equilibrate. Typically ^{31}P-NMR spectra of freshly prepared samples exhibited line broadening and high shoulders, which, however, vanished with time (≈3–7 days). Each sample was investigated by NMR spectroscopy at least twice with approximately one week between measurements to ensure that the samples had reached equilibrium. Some samples were also made in duplicate to check the reproducibility of the phase behavior. Degradation of the samples was checked by TLC.

NMR spectroscopy

All the NMR measurements were carried out on a Bruker ACP-250 NMR spectrometer. ^{31}P-NMR measurements were performed at 101.27 MHz. A phase-cycled Hahn echo sequence (Rance and Byrd, 1983) with high-power proton decoupling was used. The 90° pulse was 10 μs, the spectral width was 50 kHz, and a relaxation delay of 1 s was used. External H_3PO_4 was used as chemical shift reference in ^{31}P-NMR. ^{2}H-NMR spectroscopy on ^2H_2O was performed at 38.97 MHz using a quadrupole echo sequence (Davis et al., 1976) with the phase cycling scheme developed by Vold and Vold (1981). The 90° pulse of ^{2}H was 23 μs, the spectral width 100 kHz, and the relaxation delay was 4 s. ^{19}F-NMR spectroscopy was performed at 235.34 MHz with a 90° pulse of 12.5 μs, and a relaxation delay of 1 s.

A variable temperature unit was used to control the air flow around the sample in the magnet, and the temperature in the probe was measured with a calibrated thermistor. During the measurements of the temperature dependence, samples were allowed to equilibrate for ~30 min at each temperature before the spectra were recorded. All experimental FIDs were

transformed from Bruker to Felix format by the routines in Felix 2.0, which was used to process all the NMR spectra. For the ^{31}P-NMR FIDs a line broadening of 50 Hz was applied before Fourier transformation.

Low-angle x-ray diffraction

X-ray diffraction investigations were performed at Station 8.2 at the Daresbury Laboratory (Cheshire, UK) using a monochromatic beam of wavelength 0.15 nm. The samples were allowed to equilibrate for 10 min at each temperature before measurements.

Circular dichroism

These measurements were carried out on a JASCO 600 spectropolarimeter using cells with 0.5-mm path length, a bandwidth of 1 nm, a resolution of 0.1 nm, a response time of 1 s, and a scan speed of 20 nm/min. A baseline correction was achieved using spectra from control samples prepared without the peptide. Before measurements the samples were sonicated for 20 min with a MSE Soniprepp 150 sonifier at an effect of 8 mW. During sonication the sample tube was immersed in a water bath. Titanium particles were removed by centrifuging the samples for 20 min in a swing-out rotor at 4700 rpm in a Hettich Universal 30 F centrifuge.

RESULTS

Conformation of the peptide WALP16 and the Pgs peptide E

CD measurements were performed on the WALP16 peptide and the Pgs peptide E incorporated in sonicated vesicles of DOPC. Fig. 1 shows typical CD spectra of these peptides in single-bilayer vesicles at a peptide/lipid molar ratio of 1:25. The spectra of both peptides exhibit significant α-helical characteristics, i.e., minima at 208 and 222 nm, and a cross-over close to 200 nm (Greenfield and Fasman, 1969). Variations of the peptide/lipid ratio in the range of 1:75 to 1:20 or a change in the temperature in the range of 25–55°C did not result in any significant change in the lineshape.

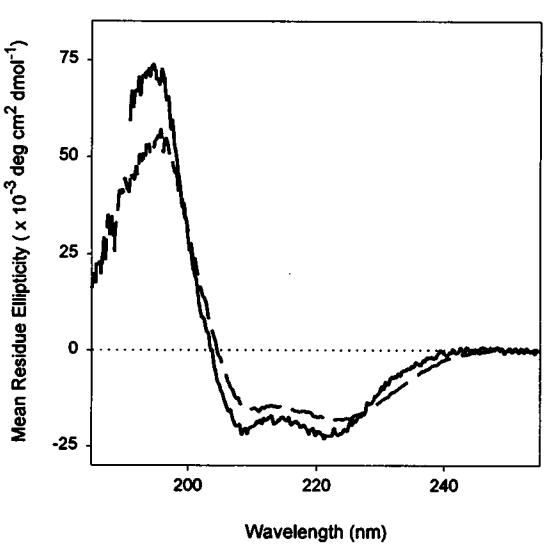

FIGURE 1 CD spectra of the Pgs peptide E (*dashed line*) and the WALP16 peptide (*solid line*) in DOPC vesicles. The peptide/lipid molar ratio was equal to 1:25 and the concentrations of the Pgs peptide E and the WALP16 peptide were 0.02 mM, respectively. Both the spectra were recorded at 25°C.

Phase behavior of WALP16/DOPC/^2H_2O (10 mM HEPES, pH 7.0)

The effect of the WALP16 peptide on the lipid phase behavior was investigated by ^{2}H, ^{31}P-NMR, and by some preliminary x-ray diffraction measurements. The compositions of the samples, utilized to construct the partial triangular phase diagrams at 25 and 55°C, are indicated by the different symbols in Fig. 2, *A* and *B*. It should be pointed out, however, that these phase diagrams are not strictly three-component systems, since the solvent used is not pure water, but instead a buffer containing several components in a thermodynamical sense. Thus, the phase diagram may better be considered as a pseudoternary one; but, for reasons of convenience, may be represented by the common triangular diagram used for a system with three components at constant temperature. This simplification will in most cases not create any problems as long as one is aware of the restrictions applicable. The procedure on how to construct a

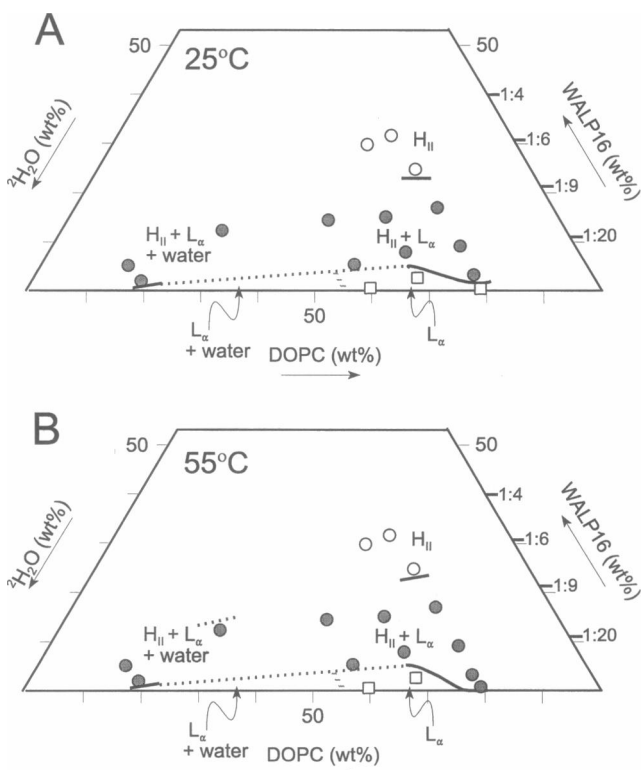

FIGURE 2 Partial pseudoternary phase diagrams of the system containing WALP16/DOPC/^2H_2O (10 mM HEPES pH 7.0). All symbols represent individual samples; (□) L_α phase, (●) L_α and H_{II} phases, (○) H_{II} phase. The solid and dashed lines indicate firm and tentative phase boundaries, respectively. The hatched border lines denote the boundary of the L_α phase toward excess water. (*A*) Phase diagram at 25°C, (*B*) Phase diagram at 55°C. The compositions are denoted by weight percentages on the axes of the triangular diagram. For convenience also peptide/lipid molar ratios are indicated on the peptide/lipid axis.

phase diagram from NMR spectra, exhibiting either quadrupole splittings (^2H) or chemical shift anisotropies (^{31}P) has recently been reviewed and described in detail by Lindblom (1996a). From the experimental data, phase diagrams were constructed at 25°C and 55°C. As a help for the reader, the peptide concentrations are given in both weight percentage and molar ratios in all the phase diagrams. For the DOPC/WALP16 system two liquid crystalline phases could be identified; namely, a lamellar liquid crystalline (L_α) phase and a reversed hexagonal liquid crystalline (H_{II}) phase. An L_α one-phase region is present only at low peptide/lipid molar ratios (WALP16:DOPC < 1:75 at 25°C). For all samples containing an H_{II} phase, keeping the peptide/lipid ratio constant, an increase in the water content results in a decrease of the fraction of the H_{II} phase. For a peptide/lipid molar ratio of 1:23 there is still a detectable fraction of an H_{II} phase at a ^2H_2O content as high as 80% (w/w) (results not shown). Thus, a three-phase area with H_{II}, L_α, and H_2O phases in equilibrium is present in this region of the phase diagram. Furthermore, the temperature dependence shows that an increase in the temperature leads to an increase in the fraction of the nonlamellar phase.

Fig. 3 illustrates a typical sequence of ^2H- and ^{31}P-NMR spectra measured at 25°C as a function of increasing peptide concentration at a constant water concentration of 20% (w/w). It can be inferred from Fig. 3 that a transition from an L_α phase to an H_{II} phase occurs with increasing peptide content [for a description of the characteristic lineshapes observed by ^{31}P and ^2H-NMR spectroscopy for different liquid crystalline phases see, e.g., the review on NMR spectroscopy of lipid phase behavior by Lindblom (1996a)]. From the ^{31}P-NMR spectra it can be concluded that the transition from an L_α phase to an H_{II} phase starts at a peptide/lipid molar ratio somewhere between 1:150 and 1:25 at 25°C and 20% water (w/w). The presence of an H_{II} phase at a peptide/lipid molar ratio of 1:25 is also confirmed by x-ray diffraction measurements, and the lattice parameter was determined to be equal to 4.9 nm (results not shown). At a peptide/lipid molar ratio of 1:6 a single H_{II} phase is observed (Fig. 3, *A* and *B*). Surprisingly, at the peptide/lipid ratio of 1:4, the lineshape of the ^{31}P-NMR spectrum resembles that usually obtained from an L_α phase, although with a severe line broadening (Fig. 3 *A, top spectrum*). In analogy with previous results on gramicidin (Tournois et al., 1987; Gasset et al., 1988) it is believed, however, that this spectrum represents an H_{II} phase, in which the lateral diffusion of the lipids around the tubes of the H_{II} phase is decreased due to the presence of the peptide (see Discussion). X-ray diffraction measurements of a sample with a peptide/lipid ratio of 1:4 confirmed that the structure is that of an H_{II} phase, and the lattice parameter was determined to be equal to 5.6 nm, resulting in a tube diameter of 6.5 nm.

As depicted in Fig. 3, the phase behavior can also be followed by ^2H-NMR on heavy water. This is possible because most often the L_α and H_{II} phases give rise to different ^2H-NMR water quadrupole splittings, and the H_{II} phase usually, but not always, gives a smaller splitting than

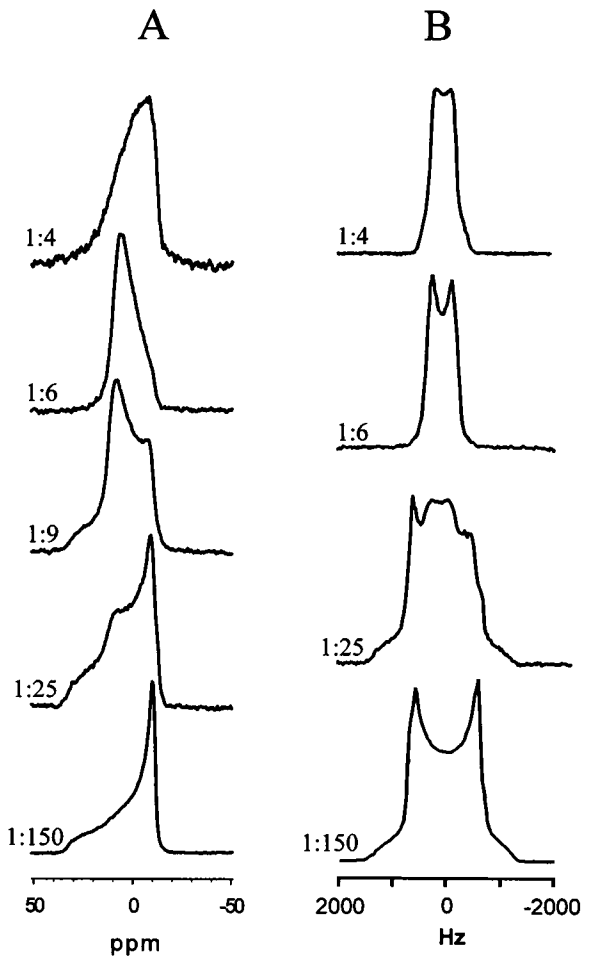

FIGURE 3 ^{31}P-NMR (*A*) and ^2H-NMR (*B*) spectra of the system containing WALP16/DOPC/^2H_2O (10 mM HEPES pH 7.0). The water concentration was equal to 20% (w/w) and the temperature was 25°C. The peptide/lipid molar ratios are indicated on the left hand side of every spectrum.

the L_α phase (Lindblom, 1996a). It can be inferred from Fig. 3 that the single quadrupole splitting observed at a peptide/lipid ratio of 1:150 originates from an L_α phase and the two splittings at a peptide/lipid ratio of 1:25 emanate from L_α and H_{II} phases. Note that the water splitting in the respective phase changes only slightly upon a change in the peptide concentration, e.g., the splitting for the H_{II} phase is equal to 320 Hz for the peptide/lipid molar ratios 1:25 and 1:6, and it is ~300 Hz for a peptide/lipid ratio of 1:4. Although the interpretation of a quadrupole splitting from water in terms of the molecular ordering in a liquid crystalline phase is rather complex, because there are many sites where the water molecules can reside, the results indicate that the chemical exchange between the fraction of water in the different sites is not very dependent on the peptide concentration. This is consistent with the peptide being buried in the hydrophobic region of the lipids. Thus, the anisotropy experienced by the water molecule is primarily determined by the geometry of the phase, and for the peptide/lipid ratios

of 1:6 and 1:4, the ^{2}H-NMR quadrupole splittings of heavy water are very similar and well-resolved. Therefore, from the ^{2}H- and ^{31}P-NMR spectra in Fig. 3 it can be concluded that there is only an H_{II} phase present for the peptide/lipid molar ratios of 1:4 and 1:6 and a water content of 20% (w/w), in agreement with x-ray diffraction measurements (Fig. 4).

Finally, an increase in the temperature was found to lead to an increase in the fraction of the nonlamellar phase. This is illustrated in Fig. 5, which also shows that the temperature dependence is fully reversible.

Phase behavior of Pgs peptide E/DOPC/^2H_2O (10 mM HEPES, pH 7.0)

The phase behavior of the Pgs peptide E/DOPC/^2H_2O system was similarly investigated by ^{2}H- and ^{31}P-NMR for a relatively large number of samples with compositions indicated by the small circles in the triangular phase diagrams in Fig. 6, *A* and *B*.

It is generally observed that, for peptide/lipid molar ratios lower than 1:50 for this system, the only liquid crystalline phase formed is an L_α phase at all temperatures and water contents studied. It is interesting to note that at 25°C the L_α phase area in the diagram in Fig. 6 *A* is relatively large, extending to a peptide/lipid molar ratio as high as 1:4 and to a ^2H_2O concentration of ~33% (w/w). At lower peptide/lipid ratios the L_α phase boundary stretches out to a water concentration of ~45% (w/w), which is the same extension of this phase region as for the binary DOPC/water system, i.e., in the absence of the peptide (Gutman et al., 1984).

Fig. 7 illustrates a typical sequence of ^{31}P-NMR spectra measured at 55°C as a function of increasing peptide concentration at a constant water concentration of 20% (w/w). This figure shows that a transition from an L_α phase to an isotropic phase occurs with increasing peptide content.

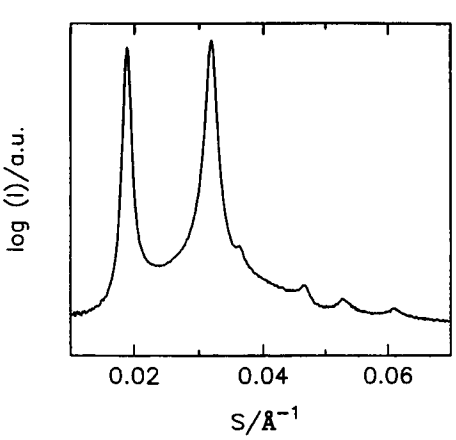

FIGURE 4 Powder pattern of a small-angle x-ray diffraction measurement obtained from the system WALP16/DOPC/^2H_2O (10 mM HEPES, pH 7.0). The peptide/lipid molar ratio was equal to 1:4, the ^2H_2O concentration was 25% (w/w), and the spectrum was recorded at 25°C. The positions of the diffraction peaks all originate from an H_{II} phase.

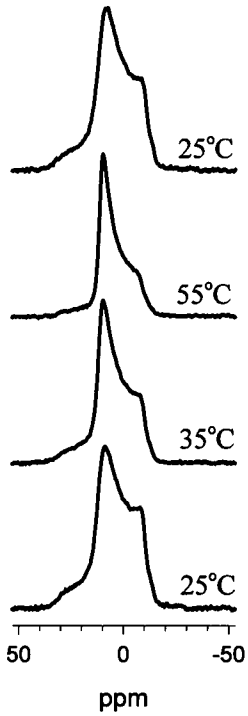

FIGURE 5 ^{31}P-NMR spectra of the system WALP16DOPC/^2H_2O (10 mM HEPES pH 7.0) at different temperatures. The peptide/lipid molar ratio is equal to 1:9 and the water concentration is equal to 20% (w/w). The temperature is indicated at the right hand side of the NMR spectra.

Even at the highest peptide/lipid ratio studied (1:4) the isotropic phase is in equilibrium with an L_α phase. An isotropic phase was generally observed at 55°C, when the peptide/lipid molar ratio was higher than 1:50 at a water concentration of 20% (w/w).

In contrast, at 25°C, the ^{31}P-NMR spectra of these samples showed characteristics indicative of a single L_α phase. On the right-hand side in Fig. 8 the temperature dependence is pictured for a sample with a peptide/lipid ratio of 1:7 and a water content of 20% (w/w). At 25°C a single L_α phase is stable. At 45°C an isotropic phase is affecting the spectra, and at 55°C the fraction of this phase has further increased, resulting in a higher intensity of the narrow peak in the ^{31}P-NMR spectrum. After lowering the temperature to 25°C the isotropic component can still be observed. However, after elongated incubation at 25°C, the isotropic signal disappears completely (not shown). This reversible temperature dependence is observed for all peptide/lipid molar ratios studied. At higher water contents an isotropic phase is again detected by ^{31}P-NMR (Fig. 8, *bottom*). By increasing the water content, keeping the peptide/lipid ratio constant, the fraction of the isotropic phase (the narrow peak in the ^{31}P-NMR spectrum) increases and eventually dominates the ^{31}P-NMR spectrum. Thus, at a water concentration of 80% (w/w) only a small fraction of L_α phase is formed. At 55°C this behavior is even more pronounced and at this temperature also other interesting features are demonstrated (Fig. 8, *top*). At a water concentration of 20% (w/w) a large

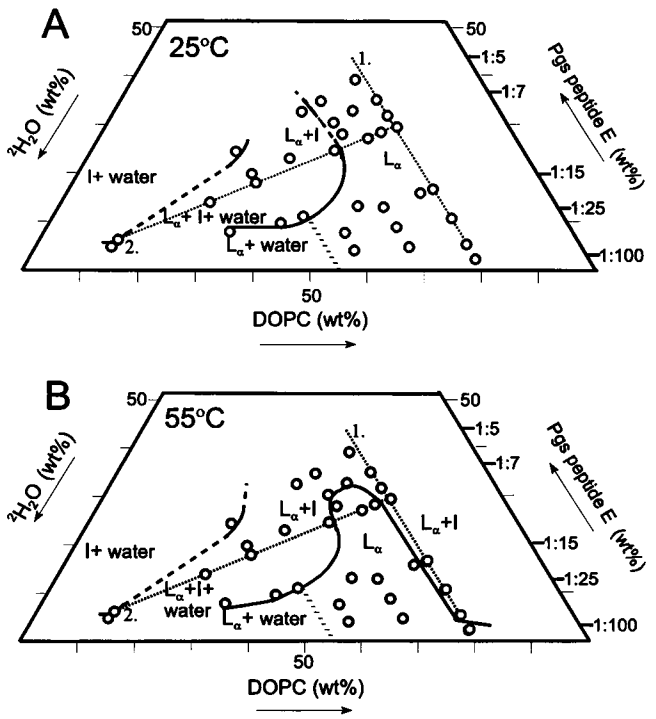

FIGURE 6 Partial pseudoternary phase diagrams of the system containing Pgs peptide E/DOPC/^2H_2O (10 mM HEPES pH 7.0). The circles represent individual samples. The solid and dashed lines indicate firm and tentative phase boundaries, respectively. The hatched border lines denote the boundary of the L_α phase toward excess water.(*A*) Phase diagram at 25°C, (*B*) Phase diagram at 55°C. The compositions are denoted by weight percentages on the axes of the triangular diagram. For convenience also peptide/lipid molar ratios are indicated on the peptide/lipid axis. The two dotted lines denoted by 1 and 2 correspond to the spectra in Figs. 7 and 8, respectively.

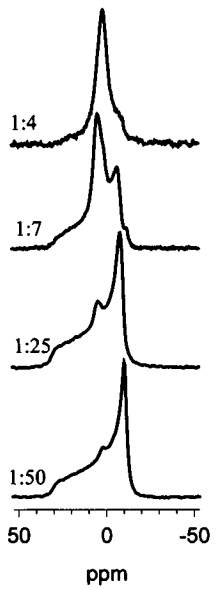

FIGURE 7 ^{31}P-NMR spectra of the system containing Pgs peptide E/DOPC/^2H_2O (10 mM HEPES pH 7.0) as a function of the peptide content. The water concentration was equal to 20% (w/w) and the temperature was 55°C. The peptide/lipid molar ratios are indicated on the left hand side of every spectrum.

fraction of the sample forms an isotropic phase (upper right hand corner in Fig. 8) that disappears at 23% (w/w). Between water contents of 23 and 33% (w/w) the ^{31}P-NMR spectra clearly reveal that only an L_α phase is thermodynamically stable, while above 33% (w/w) a transition to an isotropic phase occurs once again. The fraction of this "second" isotropic phase increases with increasing water content in resemblance with the behavior at 25°C. At a water concentration of 80% (w/w) a single isotropic phase is present (upper left corner in Fig. 8). The reproducibility of this phase behavior was confirmed by measurements on duplicate samples and by measuring several times on the same sample with approximately one week's delay between measurements to avoid nonequilibrium effects. For a peptide/lipid molar ratio of 1:4 a similar phase behavior was observed (not shown). However, no single L_α phase was observed at any water content studied at such a high peptide content. By integration and simulation of the spectra containing several components (Eriksson et al., 1985), it was found that at 55°C and a peptide/lipid molar ratio of 1:4, the isotropic component accounts for the following fractions: 45%, 5%, 12%, and 100% at water concentrations of 20, 30, 35 and 50% (w/w), respectively. Finally, an increase in the temperature always results in an increase of the fraction of the isotropic phase, independent of whether this phase is formed at "high" or "low" water contents.

^{2}H-NMR quadrupole splittings of heavy water were recorded also for all the samples of this system. In most cases the NMR spectrum exhibited a water quadrupole splitting of a rather small magnitude (~1–2 kHz), normally observed for an anisotropic liquid crystalline phase, like the L_α phase (Lindblom, 1996a), but also a relatively broad superimposed central peak (Fig. 9 *B*). This broad peak in the ^{2}H-NMR spectrum makes it rather difficult to accurately determine the phase boundary of excess water. However, information about the macroscopic gross structure of the liquid crystalline sample may be obtained. This will be discussed later.

DISCUSSION

Until now almost nothing has been known about how α-helical transmembrane peptides can influence lipid phase behavior. Yet, a knowledge of the phase diagram of different systems is important as a basis for conclusive structural and physico-chemical investigations of membrane peptides, such as studies on the intermolecular interactions between peptides, and between peptides and lipids (Lindblom, 1996a). We have constructed phase diagrams of two different systems containing hydrophobic peptides solubilized in DOPC and ^2H_2O. The WALP16 peptide has previously been shown to have an α-helical conformation and adopt a transmembrane orientation in bilayers of dimyristoylphosphatidylcholine (Killian et al., 1996). The Pgs peptide E has

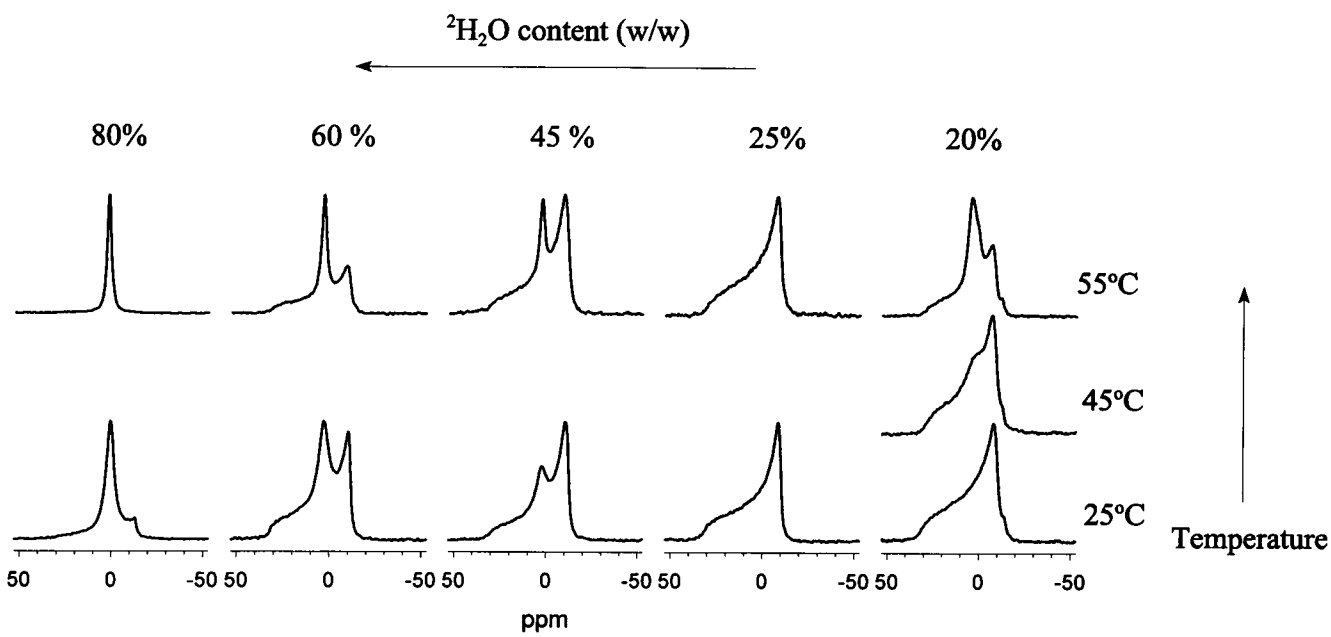

FIGURE 8 ^{31}P-NMR spectra of the system Pgs peptide E/DOPC/^2H_2O (10 mM HEPES pH 7.0) as a function of the temperature and water content. The peptide/lipid molar ratio was equal to 1:7.

been shown to adopt a major α-helical conformation in SDS-micelles (Morein et al., 1996b) and studies on the orientation of this peptide in lipid bilayers are currently being undertaken. In the present study the WALP16 peptide and the Pgs peptide E both were found to adopt an α-helical configuration in sonicated DOPC vesicles. These peptides were both found to have a large effect on the phase behavior of the DOPC/^2H_2O system, which in the absence of these peptides exhibits only an L_α phase at all temperatures and water contents studied here (Gutman et al., 1984). However, the two peptide/lipid systems studied demonstrate many differences both concerning the properties of their respective phase diagrams as well as in features observed in their NMR spectra, which will be considered in more detail below.

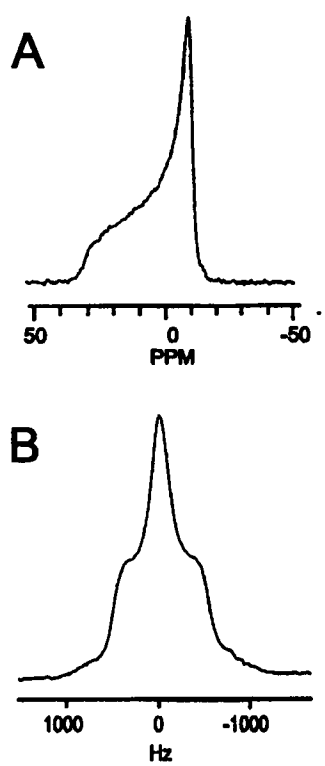

FIGURE 9 (*A*) ^{31}P-NMR and (*B*) ^{2}H-NMR spectra of the system containing Pgs peptide E/DOPC/^2H_2O (10 mM HEPES pH 7.0). The spectra were recorded at 25°C, the water concentration was 25% (w/w) and the peptide/lipid molar ratio was equal to 1:7.

The WALP16 peptide

A dominating feature of the phase diagram of the WALP16/DOPC/^2H_2O system in Fig. 2 is the large extension of the two- and three-phase regions, where an H_{II} phase is present. The fact that the fraction of the H_{II} phase increases with increasing temperature or decreasing water concentration is consistent with the general observation that an H_{II} phase in lipid/water systems preferably forms at high temperatures, and low water contents (Seddon, 1990). It is striking that in the phase diagram there is no bicontinuous cubic liquid crystalline phase, which often occurs in between L_α and H_{II} phases (Lindblom and Rilfors, 1989).

The induction of an H_{II} phase by hydrophobic transmembrane peptides in DOPC at high water concentrations is itself very unusual, and has previously only been observed for gramicidin (Killian, 1992). This peptide is similar to WALP16 in that it is completely hydrophobic, has about the same length, and contains interfacially localized tryptophan

residues. Aromatic amino acids, in particular tryptophan, have a high affinity for the lipid/water interface (Jacobs and White, 1989; Wimley and White, 1996) and it has been suggested that they are involved in the anchoring of peptides and membrane proteins in this region.

Previous experiments (Killian et al., 1996) showed that the effect of WALP16 and homologous peptides on lipid phase behavior, all in excess water, depended on the precise extent of (mis)match between the length of the peptide and the hydrophobic thickness of the phospholipid bilayer. A negative mismatch (the peptide being shorter than the hydrophobic thickness of the bilayer) generated an H_{II} phase, and a slightly longer peptide resulted in the formation of an aggregate structure that might be a bicontinuous cubic phase. When the length of the peptide increased further an L_α phase was formed. Recent experiments with WALP analogs containing only one tryptophan residue at each end of the peptide suggested that it was not the total length of these hydrophobic peptides that determined the accurate extent of mismatch, but rather the length of the stretch between the tryptophan residues (De Planque, M. M. R., Van der Wel, P., Greathouse, D. V., Koeppe II, R. E. and Killian, J. A., manuscript in preparation). Thus it appears that two factors are important for the induction of nonlamellar phases by hydrophobic transmembrane peptides: 1) a mismatch between the hydrophobic length of the peptide and the hydrophobic thickness of the bilayer, and 2) the presence of interfacial aromatic amino acids. The results in the present work on both the WALP peptide and the Pgs peptide E support this hypothesis.

A model was proposed for the formation of an H_{II} phase in the WALP16/DOPC/water system (Killian et al., 1996), in which the peptide molecules span the distance between two adjacent tubes in the H_{II} phase, where the intertube distance is the shortest, so that the tryptophans are located at the lipid/water interface and the peptide molecules can aggregate linearly along the tubes. Such linear aggregates could act as a translational diffusion barrier for the lipids. The H_{II} phase will be favored because the monolayer thickness in an H_{II} phase is much less than in a bilayer. This explains the requirement of a negative mismatch and the requirement of a high peptide/lipid molar ratio (1:6 or higher as found by Killian et al., 1996, and in the present study). The results obtained in this work strongly support this model. The crucial argument is provided by the ^{31}P-NMR spectrum at a peptide/lipid ratio of 1:4, where the line shape exhibits a low-field shoulder, strongly indicating that the peptide located in the lipid aggregates is hindering the translational diffusion of the lipid molecules around the water cylinders in the H_{II} phase. Finally, it seems reasonable to assume that the driving force behind the formation of an H_{II} phase in the DOPC/peptide system depends on the spontaneous curvature of the lipid monolayer. Previous studies have shown that the DOPC monolayer has a negative spontaneous radius of curvature (Sjölund et al., 1987; 1989). Since solubilized short peptides will tend to curve the monolayer toward a positive radius in the close vicinity of the peptides, the lipid bilayer will be locally "frustrated" eventually resulting in an L_α to H_{II} phase transition with increasing peptide concentration, i. e. a release of curvature energy upon the transition to the more favorable concave lipid monolayer in the H_{II} phase.

The Pgs peptide E

The Pgs peptide E is also a hydrophobic peptide but, in contrast to WALP16, it contains both charged and polar amino acid side chains. Furthermore, the Pgs peptide E is longer than WALP16. Therefore, it can be expected that the Pgs peptide E influences the phase behavior differently than WALP16. It was found that also the Pgs peptide E induces nonlamellar structures in the DOPC/water system. However, these structures give rise to a narrow signal at about zero ppm in the ^{31}P-NMR spectrum, indicating the presence of an isotropic phase instead of an H_{II} phase as found for WALP16.

The phase diagram of the system Pgs peptide E/DOPC/^2H_2O at 25°C exhibits a relatively large one-phase area consisting of an L_α phase (Fig. 6 *A*). At water concentrations higher than ~30% (w/w) and a peptide/lipid molar ratio higher than 1:15 an isotropic phase is formed. At 55°C the extension of the L_α one-phase area diminishes and an isotropic phase, that is not present at 25°C, appears in the phase diagram at ^2H_2O contents lower than 23% (w/w) (Fig. 6 *B*). Thus, the propensity of the L_α phase to solubilize the Pgs peptide E decreases strongly with increasing temperature. There are several possible structures that may give rise to the narrow ^{31}P-NMR peaks observed, e. g. isotropic phases like small vesicles, micelles or cubic liquid crystalline phases. For the isotropic structures formed at high water content the presence of small aggregates can be excluded because the isotropic component is still present in the pellet formed after centrifugation of the samples. The hysteretic behavior of the isotropic phase formed at low ^2H_2O contents is similar as found for bicontinuous cubic liquid crystalline phase in pure lipid systems (Lindblom and Rilfors, 1989). To resolve the structure of the isotropic phase(s), and if cubic liquid crystalline structures are involved, will require extensive further investigations by e. g. NMR diffusion measurements (Lindblom and Orädd, 1994) and x-ray diffraction. Such studies are in progress in our laboratories.

It is not surprising considering the difference in length and heterogeneity of the amino acid sequences of the two peptides that they differ in ability to induce nonlamellar phases in a DOPC/water system. This difference could be due to differences in orientation and localization of the peptides in the lipid bilayer. In contrast to the WALP16 peptide, there are no experimental data so far on the orientation of the Pgs peptide E within the lipid bilayer. However, the latter peptide has been shown by 2D H^1-NMR to adopt a major α-helical conformation in SDS micelles, and the CD measurement in the present study suggests an α-helical conformation also in DOPC vesicles. Therefore, it

seems reasonable to assume that also this peptide most probably adopt a transmembrane orientation. It is possible that the aromatic amino acids Tyr-3 and Trp-18 of the Pgs peptide E could function as anchors at the polar/apolar interfaces of the DOPC bilayer, just like the tryptophans of the WALP peptides. The highly hydrophobic sequence between Tyr-3 and Trp-18 may then constitute a transmembrane part of the peptide. The C-terminal part of the peptide, on the other hand, contains polar and charged amino acids (Arg-28 is charged at pH 7.0 and Ser-19, Ser-25 and Gln-22 have hydroxyl groups) as well as some hydrophobic residues. This bulky, relatively polar part of the peptide could interact with the zwitterionic head group of the lipids by either intra- or interlamellar interactions; by bending toward the bilayer or being oriented along the bilayer normal, respectively. Thus, the transmembrane part of the Pgs peptide E would be slightly longer than that part of the WALP16 peptide, implying that the Pgs peptide E should fit better in a DOPC lipid bilayer and therefore be able to induce an isotropic phase instead of an H_{II} phase, similar to the longer WALP analogs (Killian et al., 1996). This is also experimentally observed.

^{2}H-NMR spectra (Fig. 9 *B*) of several Pgs peptide E/DOPC samples showed a broad, central peak. This is a rather puzzling observation, since these samples are expected to consist of anisotropic phases only. Since ^{31}P-NMR (Fig. 9 *A*) clearly demonstrates that there is no isotropic phase present (a possible origin of the central peak), and a ^{31}P-NMR chemical shift anisotropy (CSA) typical for an L_α phase is observed, it has to mean that the central peak observed in the ^{2}H-NMR spectrum cannot be assigned to a particular phase. The most probable explanation for this appearance might emanate from limitations of the technique to observe well resolved quadrupole splittings from a very heterogeneous sample. A probable molecular interpretation of these findings is that the Pgs peptide E has its bulky C-terminus sticking out of the bilayer thereby causing relatively large steric perturbations. This could cause difficulties in the formation of sufficiently large and regular microcrystallites, a necessary condition for obtaining well characterized ^{2}H-NMR spectra (Lindblom, 1996a). Since the water diffusion is rapid, water molecules residing on the "edge or boundary regions" of the microcrystallites will chemically exchange between different sites, giving rise to the observed broad central peak in the spectrum. On the other hand, the ^{2}H-NMR spectra for the WALP16 system do not exhibit broad, central peaks, but well resolved quadrupole splittings (Fig. 3). This is probably due to the fact that this smaller and completely hydrophobic peptide is fully incorporated in the lipid bilayer. Since no part of the peptide is sticking outside the lipid aggregate surface, the perturbation of the growth of the microcrystallites is much less.

Since the C-terminus of the Pgs peptide E contains charged and polar amino acid side chains, one would expect an L_α phase solubilizing this peptide to incorporate large water quantities and to swell to large distances between the lamellae. This would result in a maximum hydration that is higher than the value of 45% (w/w) water reported for the DOPC/water system without a solubilized peptide. This is, however, not observed. Instead of a swelling L_α phase an isotopic phase forms already at a water concentration of 33% (w/w) at a peptide/lipid ratio $>$ 1:15, and this phase behavior gives further support for the concept that this peptide strongly perturbs the molecular packing in the L_α phase. Studies of a possible peptide aggregation in the lipid bilayer are currently being carried out.

CONCLUDING REMARKS

Very little is known about the effects of hydrophobic α-helical transmembrane peptides on the phase behavior of phospholipids in water, and no systematic study has so far been accomplished. Studies have been achieved on the effect of transmembrane peptides on the gel to liquid crystalline phase transition (e.g. Huschilt et al., 1985; Zhang et al., 1995b). Furthermore, several studies have been published on how hydrophobic peptides can promote the formation of nonlamellar phases with lipids which themselves have a tendency to form nonlamellar structures in water (Keller et al., 1996; Killian et al., 1990). However, peptide-induced formation of nonlamellar phases in phosphatidylcholine systems is still a rare phenomenon, observed for only a few peptides, like gramicidin, WALP 16 and the Pgs peptide E, as discussed in this study. Similarly, the induction of nonlamellar phases in phosphatidylcholine systems by hydrophobic molecules, like alkanes, is also of a rather recent date (Sjölund et al., 1987; 1989). Whether the mechanism for the induction of nonlamellar phases by hydrophobic alkanes and peptides are in some part related (e.g. by allowing the monolayer curvature to adopt, or come close to its spontaneous radius of curvature by a release of the interstitial packing constraints at the L_α to H_{II} phase transition) remains to be further explored.

The present work suggests that among the more important factors influencing the effects of transmembrane peptides on the phase behavior of phosphatidylcholine/water systems are the extent of hydrophobic matching and the presence of interfacially localized aromatic amino acid residues. An obvious extension of this study would now be to determine phase diagrams of peptides with different hydrophobic lengths in the absence and the presence of interfacially localized aromatic amino acids.

Furthermore, when information has been extracted about the orientation and the precise secondary or tertiary structure of the peptide in a lipid membrane and about the structure of the isotropic phases formed in the peptide/lipid/water systems, it will be possible to investigate the molecular details of the peptide-peptide and peptide-lipid interactions. Hopefully, in a near future, it will be possible to elucidate the secondary and tertiary structure of the Pgs peptide E and the WALP peptides and other membrane spanning peptides incorporated in a phospholipid bilayer, or in a bicontinuous cubic phase, by the means of for example

magic angle spinning (MAS) NMR (Lindblom, 1996b; Cross, 1996).

Note added in proof: It has been shown by ^{15}N-NMR on a macroscopically aligned L_α phase, where a ^{15}N-labeled amino acid was incorporated in the middle of the Pgs peptide E, that this peptide is oriented perpendicular to the lipid bilayer as assumed in this work (Strandberg, E., F. A. Kovacs, T. A. Cross, and G. Lindblom. Orientation of a hydrophobic α-helical peptide in phospholipid bilayers determined by solid state NMR spectroscopy, to be submitted).

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