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Differential scanning calorimetry and ^{2}H nuclear magnetic resonance and Fourier transform infrared spectroscopy studies of the effects of transmembrane α-helical peptides on the organization of phosphatidylcholine bilayers

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Abstract

We have studied the effects of the incorporation of the α-helical transmembrane peptides Ac-K_2-L_{24}-K_2-amide (L_{24}) and Ac-K_2-(L-A)$_{12}$-K_2-amide ((LA)$_{12}$) on the thermotropic phase behavior of 1,2-dipalmitoyl-d_{62}-*sn*-glycero-3-phosphocholine (DPPC-d_{62}) and 1-palmitoyl-d_{31}-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC-d_{31}) lipid bilayer model membranes by differential scanning calorimetry (DSC) and the conformational and orientational order of the phospholipid chains by Fourier transform infrared (FTIR) spectroscopy and ^{2}H nuclear magnetic resonance (^{2}H-NMR) spectroscopy, respectively. Our DSC and FTIR spectroscopic studies indicate that the peptides L_{24} and (LA)$_{12}$ both decrease the temperature and enthalpy of the gel/liquid-crystalline phase transition of DPPC-d_{62} bilayers, with (LA)$_{12}$ having the greater effect in this regard. An examination of the frequencies of the CH_2 and CD_2 symmetric stretching bands of the infrared spectra of liquid-crystalline states of the peptide-free and peptide-containing DPPC-d_{62} and POPC-d_{31} samples, and a comparison with the orientational order as measured by ^{2}H-NMR spectroscopy as well as with the chain order as measured by electron spin resonance spectroscopy, lead us to conclude that the CH_2 (or CD_2) stretching frequencies of lipid hydrocarbon chains are not a reliable measure of chain conformational order in lipid bilayers containing significant amounts of peptides or other lipophilic inclusions. In contrast, the results of our ^{2}H-NMR spectroscopic studies present a consistent picture in which both L_{24} and (LA)$_{12}$ increased in a similar way the time-averaged orientational order of the lipid chains of their liquid-crystalline lipid bilayer hosts. The comparison of the effects L_{24} and (LA)$_{12}$ on phosphatidylcholine bilayers indicates that the gel-to-liquid-crystalline phase transition appears to be more sensitive to small changes in transmembrane peptide surface topology

Abbreviations: CD, circular dichroism; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPC-d_{62}, 1,2-dipalmitoyl-d_{62}-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; ESR, electron spin resonance; FTIR, Fourier transform infrared; Hepes, *N*-[2-hydroxyethyl]piperazine-*N*′[2-ethanesulfonic acid]; ^{2}H-NMR, ^{2}H nuclear magnetic resonance; L_{24}, Ac-K_2-L_{24}-K_2-amide; (LA)$_{12}$, Ac-K_2-(L-A)$_{12}$-K_2-amide; MLV, multilamellar vesicles; P_{24}, Ac-K_2-G-L_{24}-K_2-A-amide; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPC-d_{31}, 1-palmitoyl-d_{31}-2-oleoyl-*sn*-glycero-3-phosphocholine; $\nu_{C-D}s$, symmetric CD_2 stretching band; $\nu_{C-H}s$, symmetric CH_2 stretching band

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than hydrocarbon carbon chain orientational order in the liquid-crystalline state.

Keywords: Transmembrane peptide; Lipid; IR spectroscopy; NMR spectroscopy; Lipid chain order

1. Introduction

The mutual interactions of lipids and proteins are of fundamental importance for both the structure and the function of all biological membranes (see [1,2]). In particular, the chemical composition and physical properties of the host lipid bilayer can markedly influence the activity and thermal stability of a large number of integral membrane proteins in both model and biological membrane systems (see [1–5]). For this reason there have been many studies of the interactions of membrane proteins with their host lipid bilayers, in both biological and reconstituted model membrane systems, employing a wide range of different physical techniques (see [6–10]). However, our understanding of the physical principles underlying lipid-protein interactions remains incomplete and the actual molecular mechanisms whereby associated lipids actually alter the activity, and presumably also the structure and dynamics, of integral membrane proteins are largely unknown. This situation is due in part to the fact that most transmembrane proteins are relatively large, multidomain macromolecules of complex and often unknown three dimensional structure and topology, that can interact with lipid bilayers in complex, multifaceted ways (see [1–10]). To overcome this problem, a number of workers have designed and synthesized peptide models of specific regions of natural membrane proteins and have studied their interactions with model lipid membranes of defined composition (see [11–13]). Physical studies of such relatively tractable model membrane systems have already significantly advanced our understanding of the molecular basis of lipid–protein interactions.

The synthetic peptide Ac-K_2-G-L_{24}-K_2-A-Amide (P_{24}) and its analogs have been successfully utilized as a model of the hydrophobic transmembrane α-helical segments of integral membrane proteins (see [12,13]). These peptides contain a long sequence of hydrophobic and strongly α-helical promoting leucine residues capped at both the N- and C-termini with two positively charged, relatively polar lysine residues. Moreover, the normally positively charged N-terminus and the negatively charged C-terminus have both been blocked in order to provide a symmetrical tetracationic peptide which will more faithfully mimic the transbilayer region of natural membrane proteins. The central polyleucine region of these peptides was designed to form a maximally stable α-helix, particularly in the hydrophobic environment of the lipid bilayer core, while the dilysine caps were designed to anchor the ends of these peptides to the polar surface of the lipid bilayer and to inhibit the lateral aggregation of these peptides. In fact, circular dichroism (CD) [13] and Fourier transformed infrared (FTIR) [14–16] spectroscopic studies of P_{24} have shown that it adopts a very stable α-helical conformation both in solution and in lipid bilayers, and X-ray diffraction [17], fluorescence quenching [18] and FTIR spectroscopic [14–16] studies have confirmed that P_{24} and its analogs assume a transbilayer orientation with the N- and C-termini exposed to the aqueous environment and the hydrophobic polyleucine core embedded in hydrocarbon core of the lipid bilayer when reconstituted with various phosphatidylcholines. Differential scanning calorimetry (DSC) [13,15,19,20] and ^{2}H nuclear magnetic resonance (^{2}H-NMR) spectroscopic [13,19,20] studies have shown that P_{24} broadens the gel/liquid-crystalline phase transition and reduces its enthalpy. The phase transition temperature is shifted either upward or downward, depending on the degree of mismatch between the hydrophobic length of the peptide and the hydrophobic thickness of phosphatidylcholine lipid bilayers [15]. As well, small distortions of the α-helical conformation of P_{24} are also observed in response to peptide–lipid hydrophobic mismatch [15]. ^{2}H-NMR [21] and electron spin resonance (ESR) [22] spectroscopic studies have shown that the rotational diffusion of P_{24} about its long axis perpendicular to the membrane plane is rapid in the liquid-crystalline state of the bilayer and that a closely related peptide, Ac-K_2-L_{24}-K_2-amide (L_{24}),

exists at least primarily as a monomer in liquid-crystalline 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayers, even at relatively high peptide concentrations.

A related peptide, Ac-K_2-(L-A)$_{12}$-K_2-amide $(LA)_{12}$, in which the polyleucine core of P_{24} is replaced by alternating Leu and Ala residues, has also been investigated to examine whether the replacement of one half of the leucine residues by smaller and less hydrophobic alanine residues would influence the stability of the helical form of the peptide and if the surface topology of transmembrane peptides would affect its influence on lipid bilayers. The application of a variety of physical techniques has revealed that the behavior of $(LA)_{12}$ in solution or in lipid micelles or bilayers is generally similar to that of P_{24} [23,24]. However, $(LA)_{12}$ perturbs the gel/liquid-crystalline phase transition of phosphatidylcholine (PC) bilayers to a greater extent than does P_{24} at comparable concentrations, as inferred from the greater decrease of the temperature and enthalpy of the gel-to-liquid-crystalline phase transition, possibly due to its rougher surface topology. However, the influence of the hydrophobic mismatch between the peptide and the host lipid bilayer on the shift in the phase transition temperature is less pronounced for $(LA)_{12}$ than for L_{24}, possibly due in part to the greater conformational plasticity of $(LA)_{12}$ in response to alterations of the bilayer thickness [24].

In general, the results from the various studies of P_{24} and related peptides obtained by different physical techniques agree rather well with one another. However, the results of several spectroscopic studies of the effects of the incorporation of these transmembrane α-helical peptides on the order of the hydrocarbon chains of the host PC bilayer do not. For example, ^{2}H-NMR studies indicate that the incorporation of P_{24} into 1,2-dipalmitoyl-d_{62}-*sn*-glycero-3-phosphocholine (DPPC-d_{62}) bilayers substantially decreases the time-averaged orientational order of the hydrocarbon chains in the gel and increases slightly their orientational order in the liquid-crystalline state in a manner proportional to the peptide concentration [13,19]. Similarly, a ^{2}H-NMR study reported an ordering of the hydrocarbon chains by P_{24} in 1-palmitoyl-d_{31}-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC-d_{31}) bilayers in the liquid-crystalline state [25]. Moreover, a subsequent ESR study of the effect of the closely related peptide L_{24} on POPC bilayers also indicated a substantial increase in hydrocarbon chain order and a decrease in chain motional rates in proportion to the amount of peptide incorporated into the bilayer [22]. However, FTIR spectroscopic studies of the effects of P_{24} on DPPC-d_{62} bilayers indicate only a slight disordering of the lipid hydrocarbon chains in the gel state and essentially no effect on conformational order in the liquid-crystalline state [15]. Moreover, a complementary FTIR spectroscopic study indicated that the peptide $(LA)_{12}$ significantly decreases the conformational order of the lipid acyl chains in both the gel and the liquid-crystalline phases PCs [24]. However, ESR studies of the peptide $(LA)_{12}$ in POPC bilayers suggests that this peptide is slightly more effective than is L_{24} in increasing the orientational order and dampening the motion of POPC hydrocarbon chains in the liquid-crystalline state (W.K. Subczynski, A. Kusumi, personal communication).

In order to resolve these discrepancies in the literature, we have undertaken a combined ^{2}H-NMR and FTIR spectroscopic study of the effects of L_{24} and $(LA)_{12}$ on the acyl chain order of both DPPC-d_{62} and POPC-d_{31} bilayers over a range of temperatures in their liquid-crystalline states, and have supplemented such studies with a high-sensitivity DSC analysis of these binary peptide-lipid systems. The use of lipids bearing one or two fully deuterated palmitoyl chains was indeed required to perform the ^{2}H-NMR spectroscopy but also has the advantage, in FTIR spectroscopy, of having the methylene stretching bands shifted to a region where the transmembrane peptides do not contribute, preventing potential spectral interference. In order to insure maximum reliability and reproducibility, all three techniques were applied to the same samples, which contained a relatively high concentration of peptide (a lipid/peptide molar ratio of 20:1). This approach allows us to directly compare the effect of the two peptides on the chain order of liquid-crystalline PC bilayers under exactly the same conditions, which was not the case in previous studies. Our study indicates that both the ESR and ^{2}H-NMR techniques provide reliable and almost comparable results indicating that both peptides order liquid-crystalline PC bilayers. On the other hand, the relative frequencies of the methylene symmetric stretching band moni-

tored by FTIR spectroscopy suggests variable effects of these peptides on hydrocarbon chain conformational order. Thus the CH_2 and CD_2 symmetric stretching frequencies do not appear to provide a reliable indication of relative hydrocarbon chain orientational order in these peptide–lipid systems.

2. Materials and methods

2.1. Materials

The peptides L_{24} and $(LA)_{12}$ were synthesized exactly as previously described [23]. POPC-d_{31} and DPPC-d_{62} were purchased from Avanti Polar Lipids (Alabaster, AL). Deuterium-depleted water and *N*-[2-hydroxyethyl]piperazine-*N*′[2-ethanesulfonic acid] (Hepes) were obtained from Aldrich (Milwaukee, WI) and Sigma Chemical Co. (St. Louis, MO), respectively.

2.2. Preparation of peptide-containing membranes

The lipid–peptide mixtures used were prepared by co-solubilizing 30 mg of lipid and the appropriate amount of peptide in methanol and subsequently evaporating the solvent with a gentle stream of nitrogen gas. The samples were placed under vacuum for about 16 h to remove residual traces of solvent. The film was subsequently hydrated with a Hepes buffer (20 mM Hepes, with 100 mM NaCl, 2 mM EDTA (pH 7.0) in deuterium-depleted water) to obtain a final concentration of about 15% (w/w) in total lipid. The procedure involved vigorous agitation of the sample at temperatures some 10°C above the gel/liquid-crystalline phase transition temperature of the pure lipid followed by at least five freeze/thaw cycles between liquid-nitrogen temperatures and about 10°C above the gel-to-liquid-crystalline phase transition of the pure phospholipid.

2.3. Differential scanning calorimetry

Samples were prepared for DSC by diluting the NMR samples with buffer to obtain lipid concentrations near 0.5 mg/ml. The various samples were analyzed using a MicroCal VP-DSC instrument (Microcal, Northampton, MA) operating at heating and cooling scan rates of 20°C h^{-1}. The data acquired were analyzed and plotted using the Origin software package (MicroCal Software, Northampton, MA).

2.4. ^2H nuclear magnetic resonance spectroscopy

The NMR samples were transferred into a home-made Teflon sample holder of 5 mm of diameter and the ^{2}H-NMR spectra were recorded on a Bruker DSX-300 spectrometer operating at 46 MHz for ^2H and equipped with a static probe with a 5-mm coil. Data acquisition involved the acquisition of 20 000 transients using a quadrupolar echo pulse sequence in which the 90° and refocusing pulses were separated by an interpulse delay of 35 μs, and with a recycle delay of 0.5 s. A total of 8192 points were collected in the quadrature mode with a dwell time of 0.5 μs. The dePaking of the spectra obtained and interpretation of the dePaked spectra in terms of smoothed segmental order profiles were achieved using previously published procedures [26]. Sample temperature was controlled with a Bruker variable temperature unit.

2.5. Fourier transform infrared spectroscopy

For FTIR spectroscopy experiments, an aliquot of the sample freshly hydrated or an aliquot of the NMR sample which had been lyophilized and subsequently rehydrated was squeezed between the CaF_2 windows of a heatable, demountable liquid cell equipped with a 5-μm Teflon spacer. Spectra were recorded at a resolution of 2 cm^{-1} using previously published data acquisition protocols [27] with a Bio-Rad FTS-25 spectrometer (BioRad, Cambridge, MA) equipped with an MCT detector. Sample temperature was controlled with thermopumps using a home-made controller [28]. The contribution of water was subtracted using a least-square fitted polynomial simulating the edge of the water O–H stretching band around 3400 cm^{-1} in the C–H stretching region or by subtracting the buffer spectrum recorded at the same temperature to eliminate the contribution of the association band of water at 2070 cm^{-1} in the C–D stretching region. The band maxima reported in this paper correspond to the centers of gravity calculated on the top 5% of the bands.

3. Results

3.1. Differential scanning calorimetry studies

DSC heating thermograms of aqueous dispersions of DPPC-d_{62} alone, or of L_{24}- or $(LA)_{12}$-DPPC-d_{62} mixtures at a lipid–peptide ratio of 20:1, are presented in Fig. 1. The heating and cooling (not shown) scans of DPPC-d_{62} alone reveal two phase transitions, a lower temperature, less energetic pretransition and a higher temperature main phase transition. The pretransition, which occurs at about 30°C on heating and which exhibits a transition enthalpy of 0.9 kcal/mol on heating or cooling, corresponds to the transition from the planar gel (L_{β}') to the rippled gel (P_{β}') phase and exhibits appreciable hysteresis on cooling. The main phase transition, which occurs at 37.0°C on heating and which exhibits a transition enthalpy of 8.4 kcal/mol on heating or cooling, corresponds to the transition from the P_{β}' to the lamellar liquid-crystalline (L_{α}) phases and exhibits only a small degree of hysteresis. These results agree well with those of most previous studies of DPPC-d_{62} large multilamellar vesicles (MLVs) (see Lipid Data Base). A subtransition was not observed here as these samples were not incubated at low temperatures for the long periods of time required for subgel phase formation (see [29]).

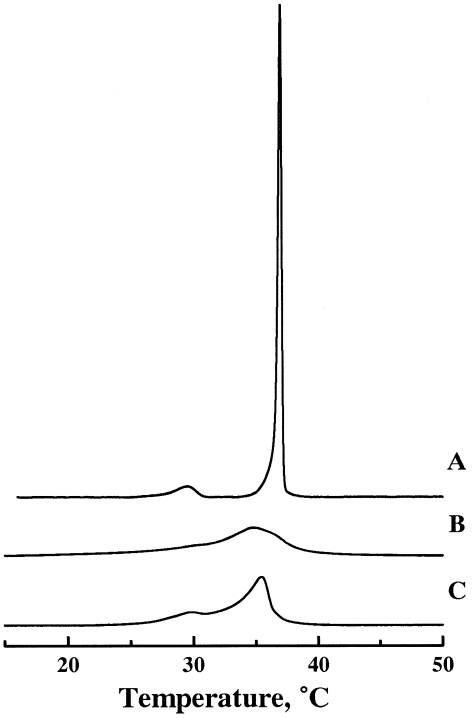

Fig. 1. DSC heating thermograms illustrating the thermotropic phase behavior of (A) DPPC-d_{62} and its mixtures with the peptides (B) L_{24} and (C) $(LA)_{12}$.

The incorporation of either peptide into the DPPC-d_{62} MLVs generally results in a decrease of the enthalpy but not in the temperature of the pretransition, which nevertheless persists at the peptide concentration studied here, and a reduction in the temperature, enthalpy and cooperativity of the main phase transition. When the contribution of the pretransition, present as a lower temperature, hysteresis-exhibiting shoulder on the heating and cooling endotherms, is subtracted, we find that the temperature corresponding to the mid-point of the main phase transition and enthalpy of the L_{24}- and $(LA)_{12}$-containing samples are reduced to about 34°C and 33°C and 7.7 and 7.1 kcal/mol, respectively. The more pronounced reduction in the temperature and enthalpy of the gel/liquid-crystalline phase transition temperatures of DPPC-d_{62} MLVs by $(LA)_{12}$ as compared to L_{24} agrees well with previous DSC studies of $(LA)_{12}$ and P_{24} in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) MLVs [15, 24], and with the results of the FTIR studies to be discussed below.

DSC studies were also attempted with pure and peptide-containing POPC-d_{31} MLVs. By employing cooling scans to supercool the aqueous phase and thus to delay ice formation, we were able to detect a sharp exotherm for POPC-d_{31} at −7.5°C prior to the onset of the large ice formation exotherm. However, using the same approach, we could not detect discrete exotherms for the peptide-containing POPC-d_{31} samples. A reduction in the temperature, enthalpy and cooperativity of the POPC-d_{31} MLVs exotherm by the presence of these peptides apparently precluded an accurate determination of their thermotropic phase behavior by DSC.

3.2. FTIR spectroscopy

The thermotropic phase behavior of DPPC-d_{62} and of its mixtures with the peptides L_{24} and $(LA)_{12}$ were examined by monitoring the temperature-dependent changes in the symmetric CD_2

stretching band ($\nu_{C-D}s$) of the phospholipid hydrocarbon chains which is centered near 2090 cm^{-1}. The frequency of this methylene symmetric stretching vibrational mode is known to be sensitive to changes in the conformational order of lipid hydrocarbon chains and it can therefore be used to monitor the progress of lipid gel/liquid-crystalline phase transitions [30]. Fig. 2A shows that DPPC-d_{62} undergoes a highly cooperative hydrocarbon chain-melting phase transition, as indicated by an abrupt increase in the $\nu_{C-D}s$ frequency from values near 2089 cm^{-1} at temperatures below the transition temperature to values near 2094 cm^{-1} at higher temperatures, respectively. These frequency changes are also accompanied by the significant broadening of the overall band envelope (data not shown). Such changes are typical of hydrocarbon chain-melting phase transitions such as occurs at the gel/liquid-crystalline phase transitions of hydrated lipid bilayers [30]. Fig. 2A also shows that comparable temperature-induced changes in $\nu_{C-D}s$ band frequency also occur with the L_{24}- and $(LA)_{12}$-containing DPPC-d_{62} mixtures, but these changes are observed at a lower temperatures and span a broader temperature range than with the pure lipid, observations in qualitative agreement with the DSC data presented above. Moreover, an examination of the first derivatives of these temperature-induced frequency changes (Fig. 2B) shows that the estimated midpoint temperatures of the gel/liquid-crystalline phase transitions of the pure lipid, L_{24}-containing and $(LA)_{12}$-containing MLVs occur at temperatures near 38°C, 37°C and 34°C, respectively, values in reasonable but not exact agreement with the calorimetrically derived values. Comparable studies of the thermotropic phase behavior of POPC-d_{31} and its peptide-containing mixtures were not performed because of problems arising from the freezing of the aqueous phase at the low temperatures at which POPC-d_{31} exhibits its gel/liquid-crystalline phase transition.

The IR data also display the relative effects of the incorporated peptide on the symmetric stretching frequencies of the CD_2 or CH_2 bands exhibited by MLVs. The data shown in Fig. 2A indicate that at low temperatures, the $\nu_{C-D}s$ frequencies exhibited by the L_{24}- and $(LA)_{12}$-containing DPPC-d_{62} preparations are comparable and are both higher than those of the pure lipid. These results are consistent with the

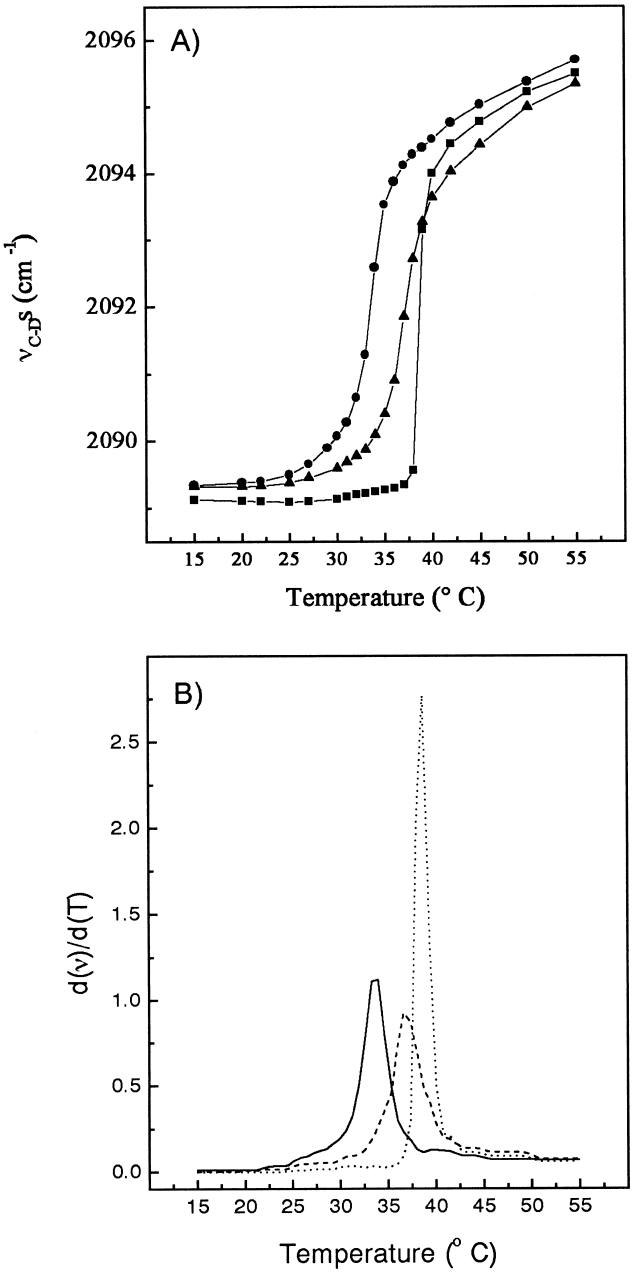

Fig. 2. (A) Thermotropic phase behavior of pure DPPC-d_{62} (■), and DPPC-d_{62}/L_{24} (▲) and DPPC-d_{62}/$(LA)_{12}$ (●) mixtures (lipid/peptide molar ratio of 20:1), as probed by the position of the C–D methylene symmetric stretching band. (B) First derivatives of the curves shown in A. (···) Pure DPPC-d_{62}; (- - -) DPPC-d_{62}/L_{24} mixture; (———) DPPC-d_{62}/$(LA)_{12}$ mixture.

increased frequency of the symmetric CH_2 stretching band ($\nu_{C-H}s$) observed in gel-phase DPPC bilayers upon incorporation of $(LA)_{12}$ [24] and the analog P_{24} [15], an experimental observation that was interpreted as a slight disorder of the all-*trans* hydrocar-

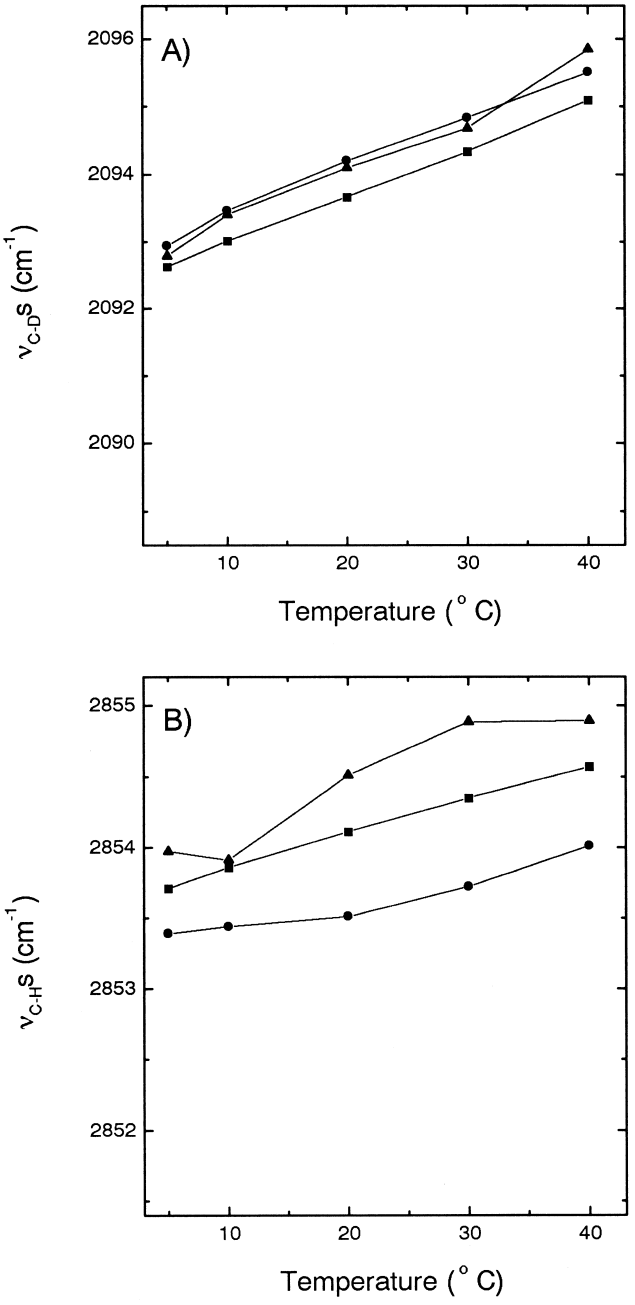

Fig. 3. (A) Position of the CD_2 symmetric stretching ($\nu_{C-D}s$) band of pure POPC-d_{31} (■), and POPC-d_{31}/L_{24} (▲) and POPC-d_{31}/$(LA)_{12}$ (●) mixtures (lipid/peptide molar ratio of 20:1). (B) Position of the C–H methylene symmetric stretching ($\nu_{C-H}s$) band of pure POPC-d_{31} (■), and POPC-d_{31}/L_{24} (▲) and POPC-d_{31}/$(LA)_{12}$ (●) complexes (lipid/peptide molar ratio of 20:1). The *y*-axis of both graphs has been scaled to obtain an equivalent relative amplitude of a gel-to-liquid-crystalline phase transition.

bon chains of DPPC gel-state bilayers caused by these peptides. At temperatures above the transition temperature, our results indicate that the $\nu_{C-D}s$ frequencies exhibited by the $(LA)_{12}$-containing samples are higher than those exhibited by the pure lipid which, in turn, are higher than those exhibited by the L_{24}-containing mixtures. (Fig. 2A). The observations related to $(LA)_{12}$ are consistent with previous results indicating an increased of $\nu_{C-H}s$ frequency upon incorporation of $(LA)_{12}$ in perhydrogenated DPPC fluid bilayers [24]. However, it was shown that the addition of the analog P_{24} has practically no effect on the position of the $\nu_{C-H}s$ or $\nu_{C-D}s$ frequency of DPPC or DPPC-d_{62}, respectively [15]. These spectral changes were interpreted as suggesting that whereas $(LA)_{12}$ incorporation conformationally disorders liquid-crystalline DPPC-d_{62} bilayers, L_{24} incorporation has no effect on DPPC-d_{62} conformational order at temperatures above the transition temperature.

With the POPC-d_{31}-based system, data were only available for the liquid-crystalline phase. With these samples, however, one can compare the relative effects of the incorporated peptide on the conformational order of the oleoyl and perdeuterated palmitoyl chains separately by examining the frequencies of the CH_2 and CD_2 symmetric stretching bands, respectively. The data shown in Fig. 3A indicate that throughout the temperature range examined, the frequencies exhibited by the L_{24}- and $(LA)_{12}$-containing POPC-d_{31} preparations are comparable and are both higher than the those exhibited by the pure lipid. If these spectral changes are strictly interpreted in terms of conformational order, they would suggest that the two peptides cause a comparable disordering of the palmitoyl chains of liquid-crystalline POPC bilayers. In contrast, an examination of the CH_2 symmetric frequencies of the same sample (Fig. 3B) suggests that, whereas the peptide L_{24} disorders the oleoyl chains of POPC, the peptide $(LA)_{12}$ orders those chains. These FTIR spectroscopic results are apparently internally inconsistent, and, as will be shown below, they are also inconsistent with the results of our ^{2}H-NMR spectroscopic studies. The possible basis of these observations will be examined later.

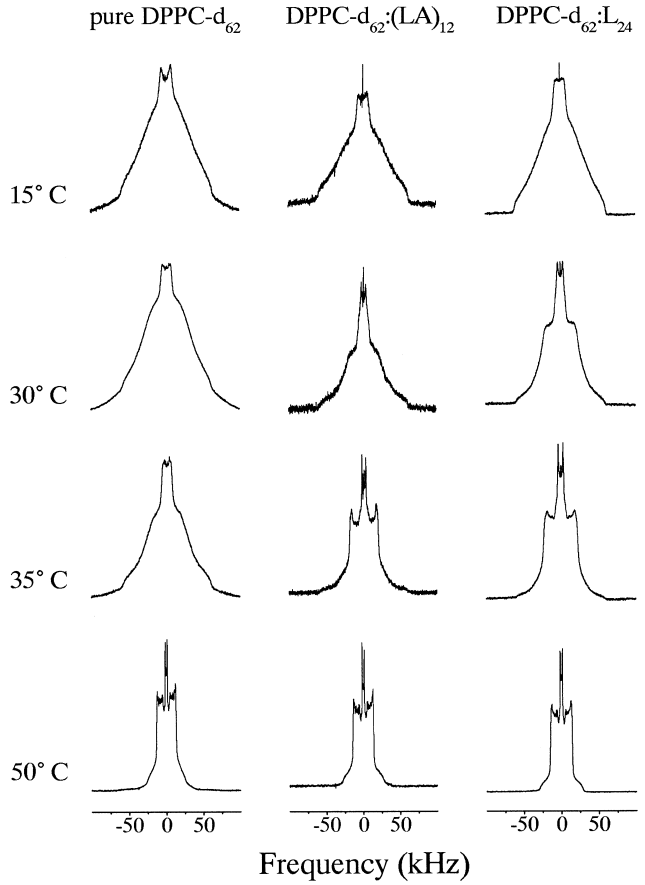

Fig. 4. ^{2}H-NMR spectra of pure DPPC-d_{62}, and DPPC-d_{62}/L_{24} and DPPC-d_{62}/$(LA)_{12}$ mixtures (lipid/peptide molar ratio of 20:1). The temperatures are indicated on the left.

3.3. ^{2}H-NMR spectroscopy

Illustrated in Fig. 4 are the ^{2}H-NMR spectra obtained with DPPC-d_{62} in the presence and absence of the transmembrane peptides. At temperatures near 15°C, the spectra of pure DPPC-d_{62} and its mixtures with the peptides all show powder patterns typical of gel-phase bilayers [31]. Upon heating, a component typical of fluid-phase bilayers, composed of overlapping powder patterns associated with axially symmetric systems, appears in the spectra of the lipid–peptide mixtures. The shift of the gel-to-fluid phase transition towards the low temperatures caused by the presence of the peptides, as detected by FTIR spectroscopy and DSC, can also be observed in the NMR spectra at 35°C, since the spectrum of pure DPPC-d_{62} still shows exclusively a typical gel-phase profile whereas the spectra of the peptide-containing samples display a significant proportion of a fluid component, indicating that, in these conditions, lipids form a two-phase (gel and fluid) system. Similar spectra were obtained for the complex DPPC-d_{62}:L_{24} (31:1) by Huschilt et al. [19]. At 50°C, the spectra of pure DPPC-d_{62} and DPPC-d_{62} in the presence of L_{24} or $(LA)_{12}$ are typical of the lamellar liquid-crystalline phase and the presence of the peptides leads to an increase of the spectral width. Thus, for example, the

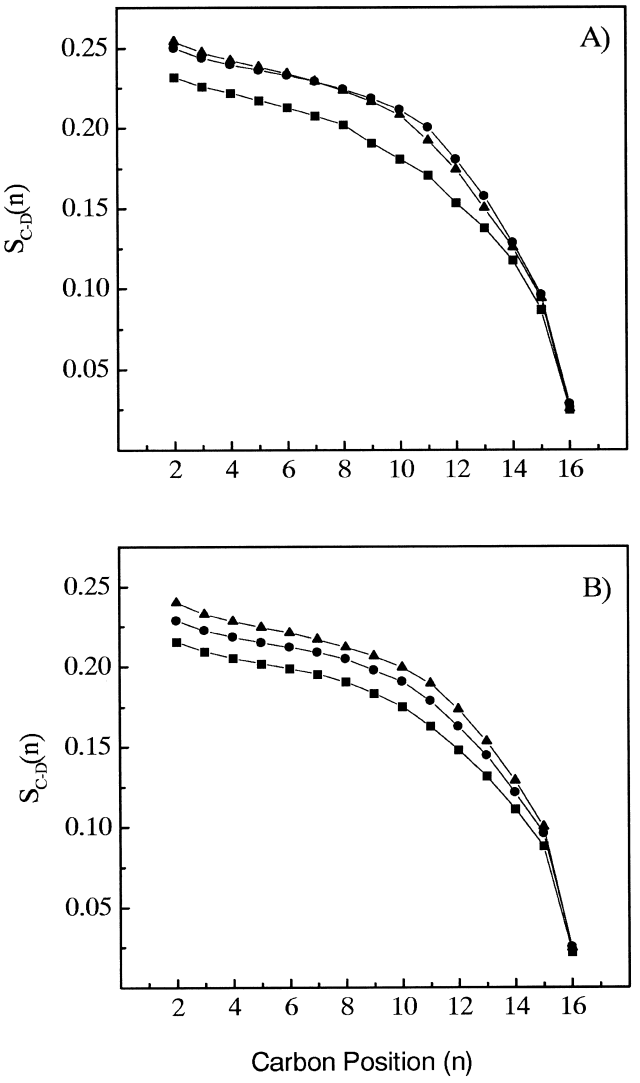

Fig. 5. (A) Smoothed order profiles determined for pure POPC-d_{31} (■), and POPC-d_{31}/L_{24} (▲) and POPC-d_{31}/$(LA)_{12}$ (●) mixtures (lipid/peptide molar ratio of 20:1), at 15°C. (B) Smoothed order profiles determined for pure DPPC-d_{62} (■), and DPPC-d_{62}/L_{24} (▲) and DPPC-d_{62}/$(LA)_{12}$ (●) mixtures (lipid/peptide molar ratio of 20:1), at 50°C.

quadrupolar splittings associated with lipid CD_2 groups located near to the bilayer polar/apolar interface increases from values near 24.6 kHz with the pure phospholipid samples to values near 26.3 and 27.2 kHz with the $(LA)_{12}$- and L_{24}-containing samples, respectively. A similar increase of orientational order caused by the peptide P_{24} has been already reported from an increase of the first moment of the spectra [19]. The smoothed segmental order profiles constructed from spectra obtained at 50°C (see Fig. 5B) show that throughout the entire length of the fatty acyl chain, segmental order parameters derived from the L_{24}- and $(LA)_{12}$-containing membranes are both higher than those derived from the pure lipid preparations, except possibly for the terminal CD_3 groups, whose mobility is strongly influenced by the free rotation along the CD_2–CD_3 bond. The order parameters obtained with the L_{24}-containing samples appears to be consistently higher than those obtained with the $(LA)_{12}$-containing preparations (Fig. 5B), suggesting that L_{24} may exert a greater ordering effect on liquid-crystalline DPPC hydrocarbon chains than does $(LA)_{12}$. As illustrated in Fig. 7B, this effect is observed at all temperatures examined where the lipid is in the liquid-crystalline state.

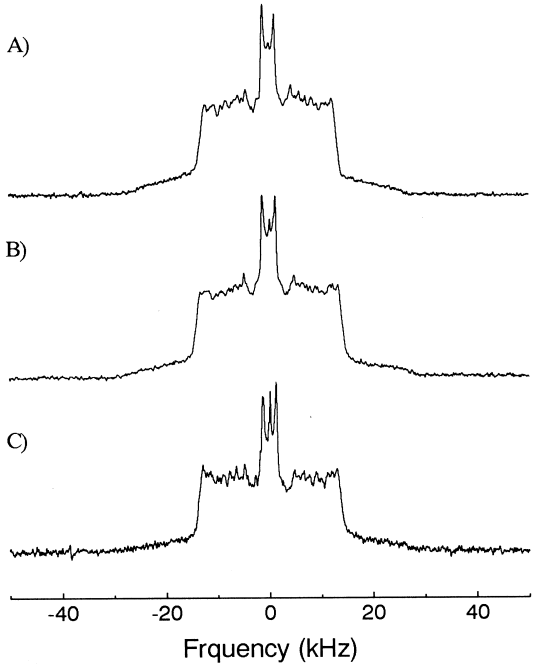

Fig. 6. ^{2}H-NMR spectra of (A) pure POPC-d_{31}, and (B) POPC-d_{31}/L_{24} and (C) POPC-d_{31}/$(LA)_{12}$ mixtures (lipid/peptide molar ratio of 20:1), recorded at 30°C.

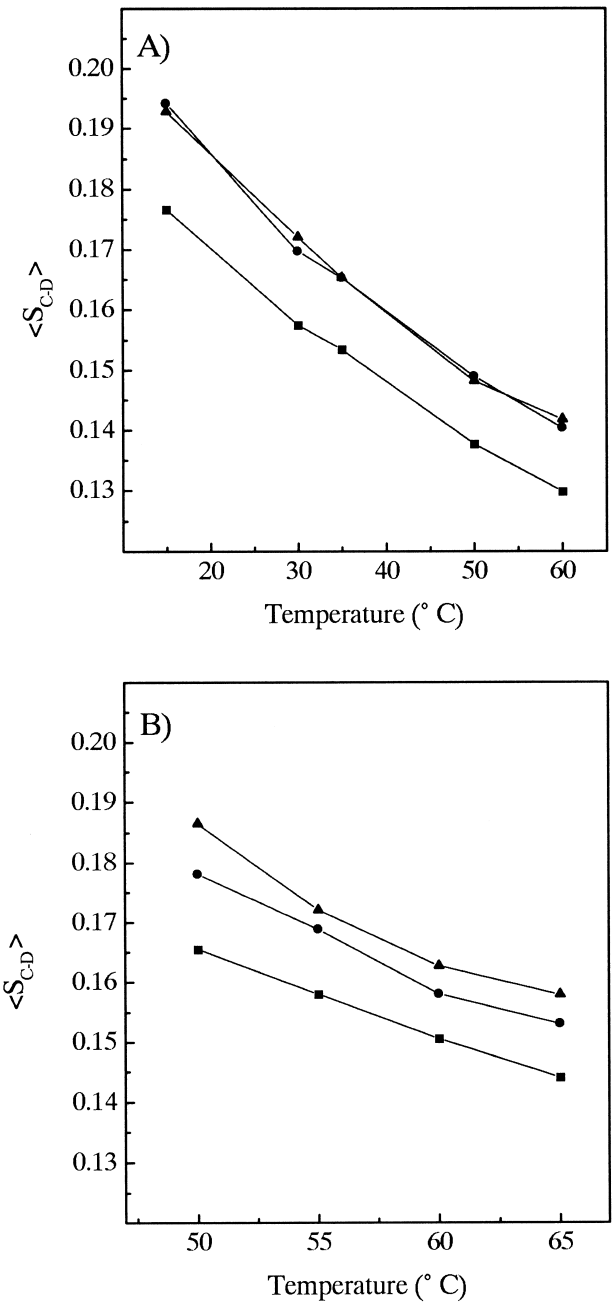

Fig. 7. Plots of chain-averaged order parameters as a function of temperature. (A) POPC-d_{31} (■), POPC-d_{31}/L_{24} (▲) and POPC-d_{31}/$(LA)_{12}$ (●) mixtures at a lipid/peptide molar ratio of 20:1. (B) DPPC-d_{62} (■), DPPC-d_{62}/L_{24} (▲) and DPPC-d_{62}/$(LA)_{12}$ (●) mixtures at a lipid/peptide molar ratio of 20:1.

The effect of $(LA)_{12}$ and L_{24} on the orientational order of the lipid acyl chains was also determined in

POPC-d_{31} bilayers. Fig. 6 displays ^{2}H-NMR spectra obtained for pure POPC-d_{31} and its mixtures with $(LA)_{12}$ and L_{24} at a molar lipid/peptide ratio of 20:1 at temperatures near 30°C. Because the gel/liquid-crystalline phase transitions of these samples occur at temperatures below 0°C, these three samples all exhibit exclusively axially symmetric powder patterns typical of lipids in the lamellar liquid-crystalline phase throughout the entire temperature range examined (5–60°C) and, as observed with the DPPC-d_{62}-based mixtures, quadrupolar splittings exhibited by the peptide-containing preparations are higher than those observed by the pure lipid. Thus, for example, the splitting of the widest doublet increases from values near 24.4 kHz with pure POPC-d_{31} to values near 25.9 and 26.6 kHz for the $(LA)_{12}$- and L_{24}-containing mixtures, respectively. The smoothed segmental order profiles derived from the spectra (see Fig. 5A) indicate that with the both the L_{24}- and $(LA)_{12}$-containing preparations, a comparable peptide-induced increase of the orientational order occurs at all positions along the acyl chain, except the terminal CD_3 group and, as illustrated in Fig. 7A, this effect is observed at all temperatures examined. These peptide-induced increases in CD_2 segmental order parameters are comparable to those observed in previous ^{2}H-NMR spectroscopic studies of P_{24}-containing POPC membranes [25], and are consistent with the L_{24}-induced orientational ordering of POPC observed in previous reported ESR spectroscopic studies [22]. The present results suggest that both L_{24} and $(LA)_{12}$ tend to order hydrocarbon chains in POPC bilayers to a comparable extent.

4. Discussion

An objective of the present work was to evaluate the impact of two transmembrane peptides with different surface topology on the lipid acyl chains of fluid PC bilayers. Our ^{2}H-NMR spectroscopic results clearly indicate that the incorporation of both L_{24} and $(LA)_{12}$ significantly increase the time-averaged orientational order of hydrocarbon chains in liquid-crystalline DPPC-d_{62} and POPC-d_{31} bilayers, an effect observed at all positions along the hydrocarbon chain and at all temperatures examined. The results with L_{24} are consistent with those of previous ^{2}H-NMR spectroscopic studies where increases in hydrocarbon chain orientational order were observed when the structurally related peptide P_{24} was incorporated into liquid-crystalline DPPC-d_{62} [13,19] and POPC-d_{31} [25] bilayers. The present work reveals that $(LA)_{12}$ also orders PC bilayers. This finding is supported by our ESR spectroscopic studies in progress, where the incorporation of L_{24} and $(LA)_{12}$ into liquid-crystalline POPC bilayers causes an increase in the orientational order and a decrease in the rates of motion of the lipid hydrocarbon chains ([22]; W.K. Subczynski, A. Kusumi, personal communication). However, these findings are not in accord with the FTIR spectroscopic results of the present and previous work [15,24], where in most cases, increases in the frequencies of CH_2 and CD_2 stretching bands are observed, and these spectral changes have been empirically associated with a disordering of the lipid chains in the liquid-crystalline state by these peptides.

The puzzling and often inconsistent conclusions derived from FTIR spectroscopic data raises the issue of the reliability of the widespread practice of using CH_2 and CD_2 stretching frequencies as indicators of relative hydrocarbon chain conformational order or disorder. This molecular interpretation is empirically based on the fact that the gel to liquid/crystalline phase transition temperature is accompanied by an increase (typically 2–5 cm^{-1}) in the frequency of the CH_2 (or CD_2) symmetric stretching vibrations because of the conversion of the conformationally highly ordered all-*trans* hydrocarbon chains characteristic of the gel state into the conformationally disordered chains characteristic of the liquid-crystalline state (see [29,32]). Moreover, the relative frequency of the CH_2 (or CD_2) symmetric stretching vibration in the liquid-crystalline state seems to be quantitatively related to the relative degree of hydrocarbon order in many, but not in all, phospholipid model membrane systems (see [29,32, 33]). For this reason, shifts in the CH_2 (or CD_2) symmetric stretching frequencies have often been used to assess the effects of the addition of cholesterol [34,35], peptides [15,24] or proteins (see [36]) on hydrocarbon chain conformational order in the gel and liquid-crystalline states of the host lipid bilayer. However, it has been shown that other phenomena,

such as changes in interchain coupling and librotorsional motions, can also induce shifts in the frequency of the methylene stretching bands, even in the absence of changes in hydrocarbon chain conformational order [37]. For example, interchain coupling is significant enough even in fluid lipid bilayers that its alteration by isotopic dilution can lead to a $\nu_{C-D}s$ frequency shift of more than 2 cm^{-1} [37]. The incorporation of transmembrane peptides in the lipid bilayer is likely to affect the interchain coupling in addition to its potential effect on lipid chain order. In addition, the CH_2 stretching band should also contain contributions from the amino acid side chains of the peptide; such potential spectral interference, that is dependent on the side chain composition, is difficult to correct adequately and can alter the absolute value of the frequency of the measured for the band maximum. Given this, the molecular interpretation of a shift in the frequency of these bands exclusively in terms of hydrocarbon chain conformational order is probably not justified. We also note that there are other examples in the literature in which the frequency of the CH_2 symmetric stretching mode also does not appear to be well correlated with lipid hydrocarbon chain order (e.g. [34,35,38]).

The ^{2}H-NMR and IR measurements do not probe the chain order in the same way. As discussed elsewhere [1,33], the two techniques do not report the same type of order (conformational versus orientational), do not respond on the same time scale, and do not express the order distribution along the acyl chain the same way. The order parameters in ^{2}H-NMR spectroscopy are primarily sensitive to *trans/gauche* isomerization, as is the case of FTIR spectroscopy, even though the wobbling of the director axis associated with the lipid fast rotation may play some role in the averaging of the NMR quadrupolar interactions [33]. Thus the increased ^{2}H-NMR order parameters caused by the transmembrane peptides are almost certainly a consequence of reduced segmental motions along the lipid acyl chains resulting from a decrease of rotational isomerism. Therefore, the contradictory and unreliable molecular interpretation drawn from the frequency of the methylene stretching modes in IR spectroscopy are likely attributed to the sensitivity of the band position to phenomena other than *trans/gauche* isomerization such as the interchain coupling and the contribution of peptide in the methylene and methyl stretching regions.

A key factor which may influence the effect of α-helical transmembrane peptides on the orientational order of lipid hydrocarbon chains is the degree of mismatch between the length of the hydrophobic segment of the peptide and the intrinsic hydrophobic thickness of the host lipid bilayer. The effect of such hydrophobic mismatch has been considered in the so-called mattress model [39], which postulates that the phospholipid chains will either extend (become more ordered) or shorten (become more disordered) in order to match their length as closely as possible to the hydrophobic segment of the peptide, so as to minimize contact between water and hydrophobic surfaces on the lipid or peptide [25,39]. Thus, if one assumes that the peptides L_{24} and $(LA)_{12}$ are oriented perpendicular to the lipid bilayer [17,24], then the actual and effective lengths of their 24-amino-acid hydrophobic core should be about 30–31 Å [15,24], a value larger than the ~26 Å expected for liquid-crystalline DPPC and POPC bilayers at temperatures just above their gel/liquid-crystalline phase transition temperatures [40]. Given these observations, hydrophobic mismatch considerations would predict that with the systems studied here, both peptides should induce some lengthening (i.e., ordering) of the phospholipid hydrocarbon chains at temperatures above T_m as observed. Such an ordering has been observed previously for P_{24} in POPC [25] and DPPC-d_{62} bilayers [13,19]. The present study extends the observation of this behavior to the analog L_{24} peptide and to a peptide composed of alternating Ala and Leu residues, leading to a rougher surface topology. It should be mentioned that $(LA)_{12}$, the peptide with the more compositionally complex transmembrane core and rougher surface topology, seems to exert a smaller ordering effect on liquid-crystalline DPPC bilayers than does L_{24}, but its overall effect is still a considerable ordering of the lipid chains. Interestingly, the introduction of peptides containing an alternating leucine and alanine segment flanked on both sides with tryptophan residues (WALP peptides) in PC membranes has also shown systematic changes of bilayer thickness related to the hydrophobic mismatch [41].

It is not yet established how the surface topology of the helix surface influences the order of the neigh-

boring lipid chains. The set of data presented here provides some insights into this aspect since the effect of two peptides with different surface topology on lipid chain order has been examined in the same conditions. We have found that despite the replacement of one half of the leucine residues by smaller and less hydrophobic alanine, there is no considerable difference in their chain stiffening effect, suggesting that the different surface topology of the two investigated transmembrane helices does not have a considerable effect on fluid-lipid chain order. The primary sequence variations between L_{24} and $(LA)_{12}$ are relatively modest, but one should note that they are sufficient to have a marked effect on the stability of the helical structure of the peptide [23]. Moreover, the surface topology effects may also explain why L_{24} causes smaller reductions in the temperature and enthalpy of the gel/liquid-crystalline phase transition of DPPC bilayers than does $(LA)_{12}$. The DSC and FTIR spectroscopic results presented here indicate that the incorporation of $(LA)_{12}$ into DPPC-d_{62} MLVs produces a greater reduction in the lipid gel/liquid-crystalline phase transition temperature and enthalpy than does the incorporation of a comparable amount of L_{24}. These findings are consistent with the results of previous calorimetric studies of the effects of the peptides $(LA)_{12}$ and P_{24} on DPPC and other linear saturated PC bilayers [14,24]. Since the peptides L_{24} and $(LA)_{12}$ have roughly comparable ordering effects in the liquid-crystalline states of both DPPC and POPC bilayers, we presume that the greater reduction in the gel/liquid-crystalline phase transition temperature and enthalpy by $(LA)_{12}$ arises primarily from exerting a greater destabilizing effect on of the gel state of PC bilayers. The results of previous FTIR spectroscopic studies of the peptides P_{24} and $(LA)_{12}$ in PC bilayers [15,24] would seem to support this conclusion. However, since our present findings indicate that the CH_2 and CD_2 symmetric stretching frequencies are not reliable indicators of hydrocarbon chain conformational order in these systems, firm conclusions about the effect of these peptides on lipid chains in gel-state bilayers cannot be drawn from this and previous FTIR spectroscopic studies; additional studies using more reliable techniques will be required to address this question adequately.

In biological membranes, it has been reported that the presence of integral transmembrane proteins either does not alter hydrocarbon chain orientational order in the liquid-crystalline state [42] or may orientationally disorder liquid-crystalline phospholipid chains [43]. The evolutionary selection of proteins for which the match between the length of their apolar transmembrane segments and the hydrophobic section of the membrane that are inserted in is good has been proposed to be at the origin of the very limited effect of membrane proteins on the lipid chain order observed on various bacterial membranes [39]. Other phenomena like peptide tilting could also take place to compensate for the hydrophobic mismatch when the length of the peptide exceeds the hydrophobic thickness of the host lipid bilayers [12]. Moreover, previous studies have shown that the transmembrane α-helical peptides P_{24} and $(LA)_{12}$ may not behave as rigid cylinders in lipid bilayers, as often assumed in theoretical studies, but may exhibit some changes in helical conformation in response to changes in the hydrophobic thickness of the host bilayer [15,24]. In addition, the more compositionally complex peptide $(LA)_{12}$ appears to be more conformationally flexible than is the more compositionally homogenous peptide L_{24}. It is therefore possible that the very compositionally heterogeneous transmembrane α-helices of natural membrane proteins may more readily alter their conformation in order to match their hydrophobic thickness with that of the host lipid bilayer, which could, in turn, attenuate their ordering effect on the hydrocarbon chains of adjacent lipid molecules. The idea of membrane lipid thickness-induced conformational distortions of the transmembrane segments of membrane proteins is compatible with the so-called squishy protein hypothesis [44] which proposes that the surface of transmembrane helical protein segments is fairly soft and malleable, leading to a smooth interfacing of the peptide and the lipid bilayer [44]. The limited effect of the peptide surface topology on lipid chain order presented here is compatible with the squishy protein hypothesis [44]. However, natural transmembrane proteins and these α-helical transmembrane peptides may display important differences in their surface topologies. Unlike peptides such as P_{24}, L_{24}, or even $(LA)_{12}$, which have a rather compositionally homogenous hydrophobic core and consequently relatively smooth cylindrically symmetrical

surface topologies, the transmembrane segments of natural transmembrane proteins are compositionally heterogeneous entities with rougher and more varied surface topologies, which may therefore interact differently with adjacent lipids. In this regard one should note that in a recent ^{2}H-NMR and ESR spectroscopic study of a series of synthetic transmembrane α-helical peptides and gramicidin A, it was concluded that both the effective hydrophobic length and the structure of the peptide surface can affect hydrocarbon chain order in liquid-crystalline phosphatidylcholine bilayers [41]. It would clearly be valuable to extend the ^{2}H-NMR spectroscopic experiments reported here to a series of PCs of widely varying hydrocarbon chain length in order to better quantify any hydrophobic mismatch effects between a single transmembrane peptide and phospholipid bilayers of different thicknesses, and to transmembrane peptides with more pronounced changes in amino acid composition to separate this effect from any surface topology effects.

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