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Interaction of cyclosporin A with dipalmitoylphosphatidylcholine

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Cyclosporin A, a hydrophobic cyclic peptide, is a potent immunosuppressant. In an attempt to determine the localization of cyclosporin A in phospholipid membranes, the effect of cyclosporin A on dipalmitoylphosphatidylcholine (DPPC) has been investigated using deuterium nuclear magnetic resonance (^{2}H-NMR) spectroscopy and differential scanning calorimetry (DSC). Cyclosporin A was dispersed within acyl chain per-deuterated DPPC at a concentration of 6 mole percent, hydrated with buffer, and the spectra obtained over a range of temperatures were compared with that of pure DPPC. The changes caused by cyclosporin A were assessed by the first moment (M_1) and order parameters calculated from the spectra. The presence of cyclosporin A decreases the magnitude of M_1 at temperatures below the gel to liquid-crystalline phase transition temperature but increases M_1 at temperatures above the transition. In addition, the change in M_1 at the transition temperature was also less abrupt when cyclosporin A was present. For bilayers in the liquid-cystalline state, cyclosporin A causes an increase in the order parameters along the acyl chains which suggests that cyclosporin A is located along the acyl chains of the phospholipid. For DSC, cyclosporin A was dispersed in non-deuterated DPPC at different peptide to phospholipid mole ratios. The endothermic peaks associated with the gel to liquid-crystalline phase transition and pretransition were recorded and compared with similar mole ratios of cholesterol to lipid. At 30 mole percent cyclosporin A, small decreases in the main transition temperature and associated enthalpy were observed, whereas at 30 mole percent cholesterol, the main transition is barely distinguishable from the baseline. The pretransition was not observed with the addition of 11 mole percent of either cyclosporin A or cholesterol. The results of the thermal analysis indicate that although cyclosporin A and cholesterol appear to be both located along the acyl chains of the phospholipids, they have dramatically different interactions with the membrane lipids.

Introduction

The interaction of proteins and small polypeptides with lipids is believed to be ultimately responsible for many of the physiological functions due to their influence on the structural and dynamic properties of the membrane. At present, there is considerable interest in determining not only the conformational changes of peptide drugs and hormones when associated with membranes, but also the effect of these drugs on the structure of the bilayer lipids. Both aspects are important for assessing the biological implications of lipid–peptide interactions. One system which has recently attracted attention is cyclosporin A-phospholipid.

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Cyclosporin A has revolutionized the therapy of patients undergoing organ transplant due to its effective suppression of the immune response [1]. One disadvantage to the use of cyclosporin A is the associated toxicity [2]. Presently, neither the mechanism of action nor the toxicity of cyclosporin A is known, although many hypotheses have been proposed [3]. A few of the structural requirements have been identified through studies of the structure/activity relationships [3]. Furthermore, X-ray diffraction as well as high resolution NMR spectroscopy have been used to determine the solution and solid state tertiary structure of cyclosporin A thereby providing supplementary information of the active conformation [4–6]. Cyclosporin A is a hydrophobic cyclic polypeptide composed of 11 amino acids. The limited water solubility of cyclosporin A suggests that in vivo association with the membrane lipids is likely [7]. The binding of cyclosporin A to plasma lipoproteins has been well established [8], but the location of cyclosporin A has not been identified [9–11].

One effective technique for identifying the change in the structure as well as dynamic properties of the phospholipid bilayer induced by the presence of molecules in the acyl chain region of the membrane is ^{2}H-NMR [12,13]. The basis of this method is that the orientational dependent quadrupolar interaction modulates the Zeeman interaction with the ultimate result of a splitting in the frequencies [14]. Although in solution the quadrupolar interaction is completely averaged due to the rapid rotation of the molecule, within lipid bilayers only partial averaging occurs. Specifically, phospholipids in the liquid-crystalline state undergo rapid rotation along the axis perpendicular to the bilayer surface. Thus the orientation of the carbon-deuterium bond with respect to the perpendicular direction to the bilayer surface may be estimated by measurement of the frequency difference of the observed peaks. From this information the effect of membrane perturbants may be evaluated through estimation of the change in the average order of the phospholipids [13].

DSC is a powerful complementary technique which provides thermodynamic information on phase transitions of lipids and lipid-protein mixtures. The temperature and enthalpy of the phase transition is measured and may be used to evaluate proposed molecular models of the interaction of peptides with phospholipid membranes.

Background: ^{2}H-NMR spectroscopy

Only the salient features of deuterium NMR spectroscopy are discussed here with more reviews found elsewhere [13,14]. The characteristic spectrum of a static sample obtained with ^{2}H-NMR arises from the electric quadrupolar interaction between the ^{2}H nucleus with the electric field gradient generated by the carbon-deuterium bonding environment [14]. The electric quadrupolar interaction gives rise to two allowed single-quantum transitions where the frequency separation of the resulting lines is determined by the angle between the electrostatic field gradient and the main magnetic field.

Within a membrane, motion faster than the interaction strength in units of frequency occurs giving rise to a reduced residual quadrupolar splitting. This is in contrast to the case of molecules in solution where rapid isotropic motion leads to a complete averaging of the interaction with the result of a spectrum consisting of a single line. The residual quadrupolar splitting is in turn related to the second Legendre polynomial of the angle between the electrostatic field gradient and the main magnetic field. One additional complication that exists with randomly oriented bilayers is that every angle between the plane of the bilayer and magnetic field is equally probable. The presence of all these orientations yields a powder-type spectrum, where the edge is given by the long axis of phospholipid molecules orientated at 90° with respect to the magnetic field and the shoulder represents the 0° orientation.

Fortunately the static and residual quadrupolar splitting scales in an identical manner thereby allowing a bond segmental order parameter, S_{CD}, to be defined as [13]:

$$S_{CD} = (\tfrac{1}{2})(3\cos^2\beta - 1) \quad (1)$$

where β is the angle between the carbon-deuterium bond and the direction perpendicular to the bilayer surface or bilayer normal. Since the residual quadrupolar splitting is also dependent on the same angular relationship, the average conformation of a particular segment may be estimated from the frequency separation from the following [13]:

$$\Delta\nu_Q = (\tfrac{3}{4})(e^2qQ/h)\,|S_{CD}| \quad (2)$$

where (e^2qQ/h) is the quadrupolar coupling constant and is equal to 170 KHz for aliphatic carbon-deuterium bonds [15].

Information concerning the average molecular order may be obtained from lipids in both the gel and liquid-crystalline phases using the method of moments [12]. The first moment, M_1, may be determined from the following equation

$$M_1 = \int \omega f(\omega)\,d\omega / \int f(\omega)\,d\omega \quad (3)$$

where $f(\omega)$ is the spectral intensity at frequency ω. The first moment is in turn related to the average order through the following relationship:

$$M_1 = (\pi/\sqrt{3})(e^2qQ/h)S_{CD}$$

where S_{CD} is the mean order parameter.

Materials and Methods

Sample preparation. Lipid-protein samples for ^{2}H-NMR were prepared as follows. Acyl chain per-deuterated DPPC was obtained from Avanti Polar Lipids, (Birmingham, AL). It was shown to be > 99% pure by TLC and therefore used as received. Cyclosporin A was received as a gift from Sandoz and is the highest purity available. Cyclosporin A was incorporated into the lipid membranes by first separately dissolving cyclosporin A and DPPC in chloroform and combining the resulting solutions in a test tube. After the chloroform was largely removed by blowing the solution with argon, several milliliters of cyclohexane were added The mixture was vortexed to produce a fine dispersion which was then frozen in a dry ice/acetone bath and lyophilized under high vacuum overnight. The sample weight was determined, and an equal weight of buffer, containing 67

mM sodium phosphate (pH 7.0), 0.1 mM EDTA, 0.1% NaN_3 prepared with deuterium depleted water, was added. The sample was gently vortexed to achieve a uniform dispersion and thereafter was freeze-thawed repeatedly. The material was then transferred to a 10 mm test tube which was sealed with teflon plug and then with wax. The sample was again freeze-thawed many times.

NMR spectroscopy. Spectra were obtained on a GN-500 (General Electric) spectrometer equipped with an external digitizer (Nicolet 2090 Digital oscilloscope), RF amplifier (Henry Radio Tempo 2006) and a home-built horizontal solenoid high power probe. The deuterium frequency was 76.76 MHz and a phase cycled, $(\pi/2)_x - t_1 - (\pi/2)_y - t_2$-echo sequence was employed. No 'B-channel' intensity was removed, and no first-order phase correction was applied. The samples were allowed to equilibrate at a given temperature for 30 min before acquiring data, and typically 512 signal averaged accumulations were transformed to give a spectrum. The spectra were deconvoluted using a modified program obtained from M. Bloom, University of British Columbia, Vancouver, BC, Canada [16,17]. The carbon-deuterium bond order parameter was calculated from the measured frequency separation of the peaks in the deconvoluted spectra according to Eqn. 2 [13]. The tentative assignments of the carbons were based on the results of Davis and Dodd et al. [12,18]. The assignment made by these investigators of the deuterons of the second carbon of the *sn*-2 chain differs and thus has not been included in the analysis below.

Differential scanning calorimetry. Lipid-protein samples for DSC were prepared in a similar manner as described above. The samples were hydrated to 66% (w/w) with distilled water and allowed to equilibrate at 45°C for 48 h. Calorimetric measurements were conducted on a Dupont 910 differential scanning calorimeter. The heating rate was 5 C°/min and repetitive scans were superimposable with the initial scan.

Results

The spectra obtained with ^{2}H-DPPC and mixtures of ^{2}H-DPPC and cyclosporin A at a series of temperatures are shown in Fig. 1. The spectra of pure ^{2}H-DPPC are in good agreement with previously published data [12,16]. The broad featureless spectra of pure ^{2}H-DPPC obtained at temperatures below 37°C are indicative of phospholipids residing in the gel state. At higher temperatures, the spectra are markedly narrowed reflecting the transition from the gel state to the liquid-crystalline state. In contrast to the pure lipids, the incorporation of cyclosporin A into the lipid bilayer causes the most dramatic change in the appearance of the spectra relative to that obtained with pure ^{2}H-DPPC at the same temperature of 35°C. The observed spectrum is characteristic of phospholipids undergoing rapid rotational

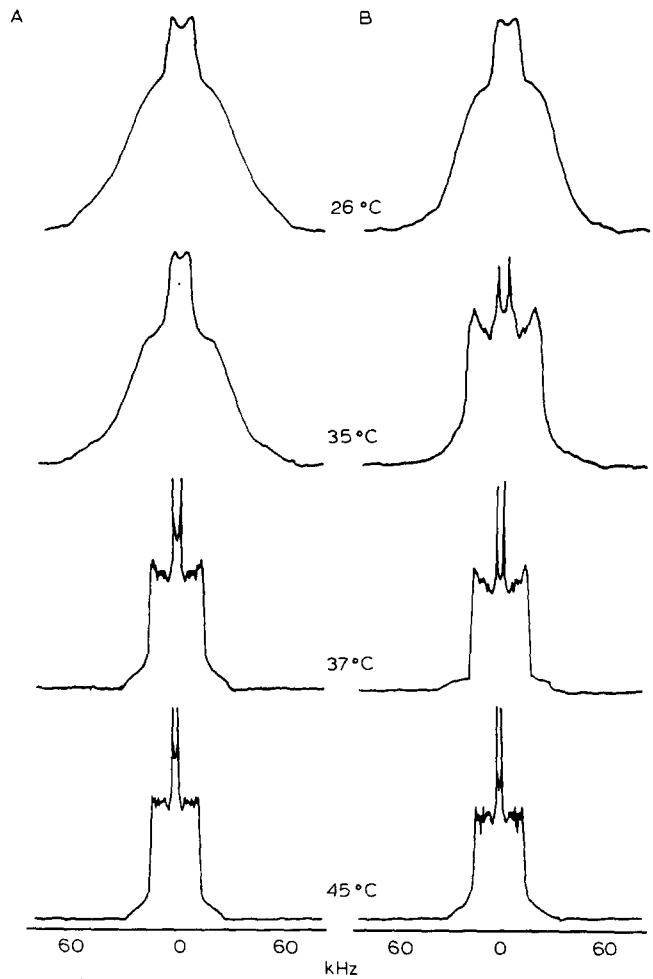

Fig. 1. ^{2}H-NMR spectra of pure ^{2}H-DPPC (A) and a 6 mole percent mixture of cyclosporin A in ^{2}H-DPPC (B) at the indicated temperatures. Spectra were scaled to similar heights.

diffusion. The phase transition temperature of acyl chain per-deuterated DPPC has been reported to be 37°C [12], as opposed to non-deuterated DPPC which undergoes a transition at 41.7°C.

The spectra obtained with pure ^{2}H-DPPC and the mixture of ^{2}H-DPPC and cyclosporin A at temperatures greater than 37°C are similar in their overall appearance; however, the presence of cyclosporin A causes an increase in the frequency separation of the symmetric peaks about the center. This is more apparent in the deconvoluted spectra obtained at 40°C shown in Fig. 2. The order parameters calculated according to Eqn. 2 as a function of position along the acyl chain for ^{2}H-DPPC and ^{2}H-DPPC and cyclosporin A are given in Fig. 3. The order parameters are not the same for each carbon position in the acyl chains due to the different conformations of the carbonyl moieties in the *sn*-1 and *sn*-2 chains. An increase in the calculated order parameters was found except in the terminal methyl group. This suggests that the presence of cyclosporin A has the effect of slightly increasing the order along the entire acyl chain.

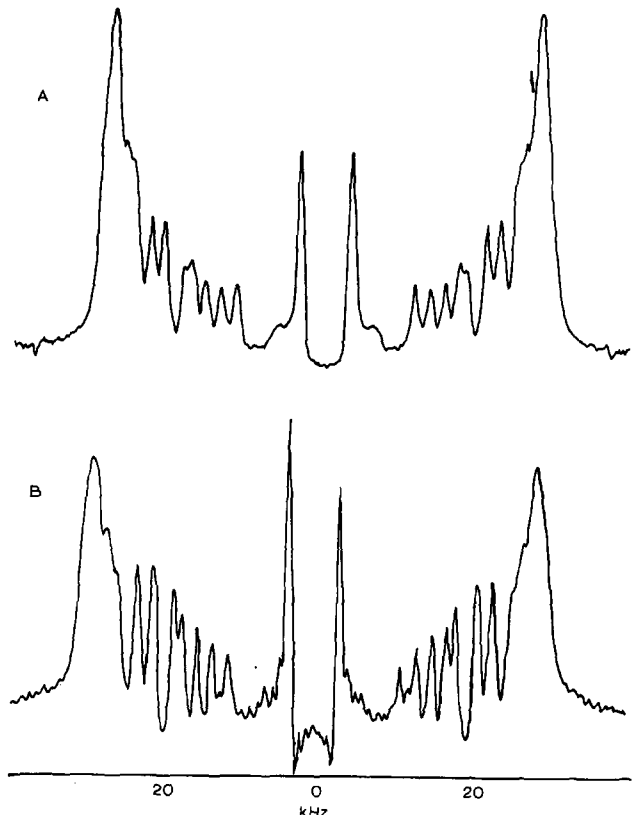

Fig. 2. Depaked ^{2}H-NMR spectra obtained at 40°C of ^{2}H-DPPC (A) and a ^{2}H-DPPC-Cyclosporin A mixture (6 mole percent) (B).

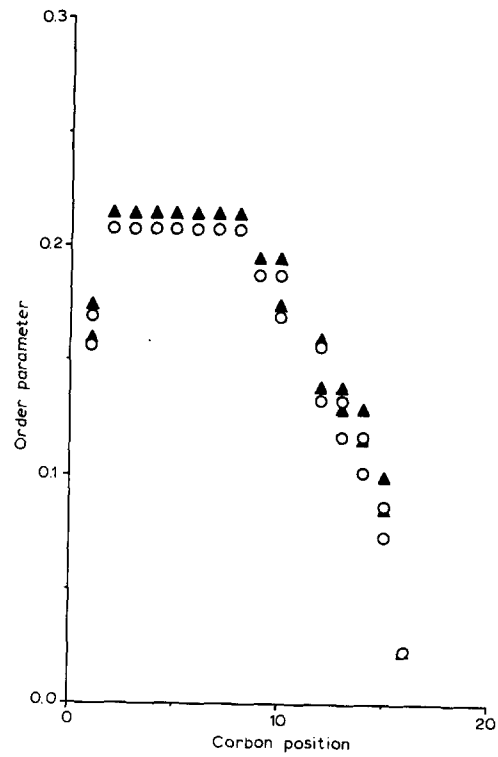

Fig. 3. Carbon-deuterium order parameter calculated from Eqn. 2 given as a function of carbon position for ^{2}H-DPPC (○) and ^{2}H-DPPC-Cyclosporin A mixture (6 mole percent) (▲) for the spectra obtained at 40°C. Due to the inequivalence of the order parameters of the two acyl chains, there are two values of the order parameter for the deuterons of the carbons near the terminal portion of the chain. The larger value arises from the *sn*-2 acyl chain and the smaller from the *sn*-1 acyl chain.

In Fig. 4, the first moments calculated from the spectra obtained with and without cyclosporin A are given as a function of temperature. Several features are apparent. One is that at temperatures below the phase transition temperature, M_1 is less in the presence of cyclosporin A. On the other hand at temperatures above the phase transition, M_1 is greater in the presence of cyclosporin A. As a consequence, the change in M_1 is less abrupt at the phase transition in the presence of cyclosporin A.

The observed DSC traces of various mole ratios of cyclosporin A in DPPC are given in Fig. 5a. The upper curve was obtained with pure DPPC dispersed in water with the lower curves obtained at increasing mole ratios of cyclosporin A. At a concentration of 2 mole percent of cyclosporin A, the pretransition peak is no longer seen. Cyclosporin A had little effect on the main transition. The maximum decrease in the temperature was about one degree, which is consistent with the changes observed in the first moment, and the endotherm was progressively broadened with greater peptide to lipid ratios. The area under the curve, reflecting the enthalpy of the transition, was also slightly decreased.

In Fig. 5b, the DSC traces obtained with a series of cholesterol-DPPC mixtures are shown. The presence of cholesterol has a relatively dramatic effect on the observed endotherms of DPPC. At 2% cholesterol, the pretransition is almost indistinguishable from the baseline and is shifted to lower temperatures. The main transition is also decreased and the transition broadened with increasing ratios of cholesterol to DPPC. In

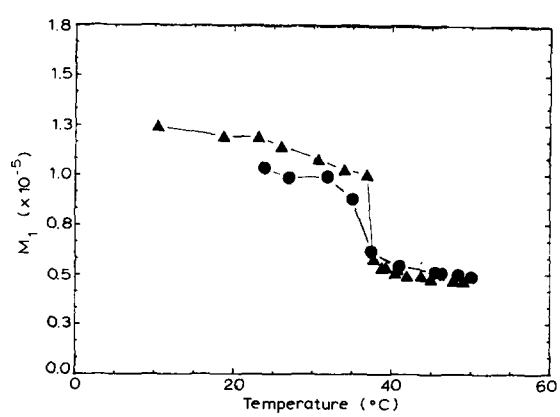

Fig. 4. The first moment, M_1, of pure ^{2}H-DPPC (▲) and 6 mole percent cyclosporin A in ^{2}H-DPPC (●) as a function of temperature.

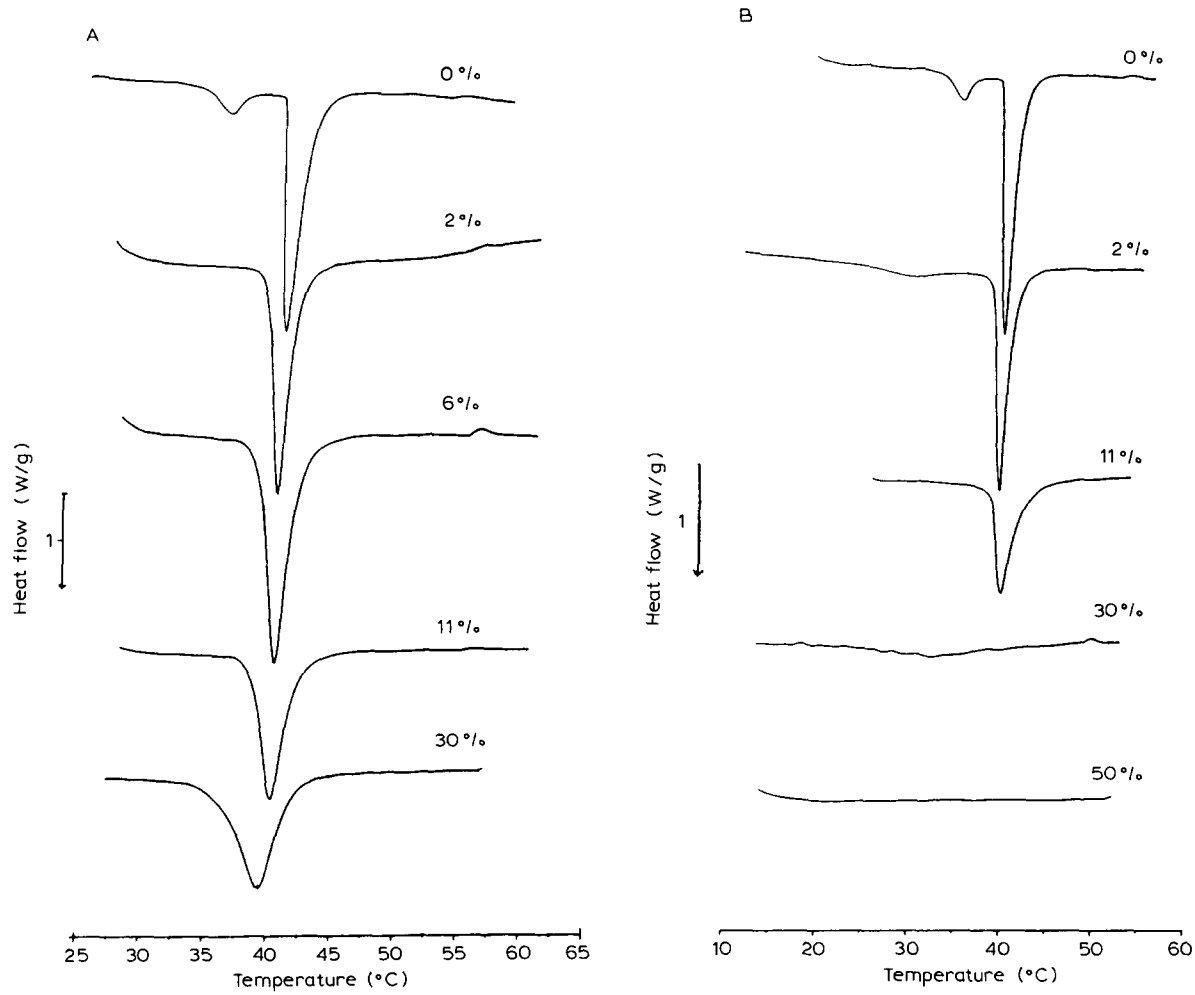

Fig. 5. DSC thermograms of 66 (w/w) % aqueous mixtures of cyclosporin A in DPPC (A) and cholesterol in DPPC (B) at the indicated mole ratios of solute to phospholipid.

addition, there is a reduction in the area under the curve such that at 50% cholesterol the transition is not observed.

Discussion

^{2}H-NMR has been frequently used to determine the interaction of solutes with the acyl chains of phospholipid membranes [13,19]. In the liquid-crystalline state, the phospholipids undergo rapid rotation about the long molecular axis thereby partially averaging the orientational dependent quadrupolar interaction [14]. This greatly symplifies the spectra without obliterating the structural information and allows quantification of the average orientation of the carbon-deuterium bond [20,21]. Earlier work with specifically deuterated phospholipids have shown that the conformational order of the carbons in the acyl chain decreases with distance from the carbonyl [20,21]. This has been explained on the basis of a tethering effect of the phospholipid head group to the membrane/aqueous interface [13].

At temperatures below the gel to liquid-crystalline phase transition, the lack of rotational averaging in the pure ^{2}H-DPPC bilayer prevents determination of the segmental conformation. However, the bilayer remains in the fluid state at 35°C in the presence of cyclosporin A as indicated by the spectrum which is characteristic of phospholipids undergoing rapid rotation. The fact that most of the lipids remain in this state suggests that cyclosporin A is dispersed throughout the membrane. It is also apparent that a portion of the phospholipids give rise to a broad spectrum, which is most apparent in the wings of the spectrum. Thus the phospholipids coexist in at least two phases. A DSC scan of a portion of the sample used in ^{2}H-NMR gave an endotherm over the temperature range consistent with these results obtained with ^{2}H-NMR. Whether cyclosporin A is uniformly dispersed in lipids in the gel state is unknown.

The moment analysis provides additional information concerning the interaction of cyclosporin A with the phospholipid membrane. The pure ^{2}H-DPPC is seen to have a large first moment at temperatures below the phase transition with an abrupt decrease at the transition. The presence of cyclosporin A causes a reduction

in the spectral width at temperatures below the phase transition but increases the width at temperatures above the transition. Furthermore, the change at the transition is less abrupt. These effects suggest that the presence of cyclosporin A induces disorder in the acyl chains below the phase transition but induces order above the transition.

While ^{2}H-NMR provides information concerning the conformational order of the acyl chains, DSC is sensitive to the thermal properties of the transition from the gel state to the liquid-crystalline state [21]. With pure DPPC, two endotherms are apparent. The first peak corresponds to what is known as the pretransition which involves the change from the gel state, L_{β}, to the ripple state, $P_{\beta'}$. The second, larger peak is referred to as the main transition and corresponds to the change from the $P_{\beta'}$ state to the liquid crystalline state, L_{α}. Incorporation of small amounts, about 6 mole percent, of either cyclosporin A or cholesterol into the lipid bilayer results in the disappearance of the pretransition. Although both cholesterol and cyclosporin A decrease the temperature and enthalpy of the main transition, the effect of the cholesterol is much greater than that seen with cyclosporin A. Thus, cholesterol is more effective in reducing the cooperative melting of the lipid molecules. These results of the effect of cyclosporin A on the thermal properties of DPPC largely confirm those reported by O'Leary et al. [9].

Although the thermodynamic information obtained with DSC does not establish the molecular details of the system, all models of the lipid–protein interactions must be consistent with the measured thermal properties. With this in mind, Papahadjopoulos et al. [22] have classified the interactions of proteins with lipids into three groups. Type I proteins cause an increase in the enthalpy and little or no increase in the temperature of the main transition. Proteins in this class are hydrophilic and are thought to adsorb to the surface of the bilayer. Type II proteins cause a decrease in the temperature as well as enthalpy of the transition. These proteins are adsorbed to the surface but also penetrate into the head group region. Type III proteins cause a decrease in the enthalpy but have little or no effect on decreasing the transition temperature. Proteins in this group are hydrophobic and generally reside within the bilayer interior. Thus based on the results obtained with DSC, cyclosporin A would be classified as a type III protein and thereby would be expected to reside in the bilayer interior.

Conformational analyses of cyclosporin A in the solid state or in solution have shown that six of the amino acids, Abu-2, Val-5, Ala-7, MeVal-11, MeBmt-1 and MeLeu-6 have their side chains projected in a quasi perpendicular direction from the plane of the back bone ring [5,6]. If cyclosporin A retains this conformation within the membrane, molecular models reveal that such a structure would not provide a suitable surface for packing of the *trans* conformation of the carbon-carbon bonds as is largely found in the gel state of phospholipids. This also provides an explanation for the lower first moments calculated from spectra of bilayers with cyclosporin A.

The location within the bilayer may also be evaluated by examination of the structural perturbation as measured by ^{2}H-NMR. The segmental order parameter has been calculated for ^{2}H-DPPC and ^{2}H-DPPC-cyclosporin A bilayers above the phase transition from the well-resolved, depaked spectra. This permits the conformational disturbances arising from the incorporation of cyclosporin A to be quantified. From Fig. 3, it is apparent that the order parameter profile is altered in the presence of cyclosporin A. Specifically, at temperatures above the phase transition the plateau region proximal to the carbonyl of the acyl chain has a greater order as reflected by a larger value of the order parameter.

Since cyclosporin A increases the order along the length of the acyl chain, one possible position of the molecule is a parallel alignment along the long axis of the phospholipid molecules. The results of DSC also indicate that cyclosporin A is a type III protein thus resides within the bilayer interior. Since little or no disordering effect was observed in the terminal portion of the acyl chain, appreciable accumulation between the two halves of the bilayer is less likely. This is also consistent with its poor solubility in completely non-polar solvents, such as hexane [6].

Other investigators have used ^{31}P-NMR to evaluate the interaction of cyclosporin A with the phospholipid membrane [23]. Through calculation of the chemical shift anisotropy, which is obtained by measurement of the spectral width, inference may be made as to the orientation and rate of rotation of the phospholipid headgroup with respect to the membrane surface [24]. The result was that an appreciable broadening in the chemical shift anisotropy was found for dielaidoyl-phosphatidylethanolamine (DEPE) in the presence of cyclosporin A. This suggests that major changes in the geometry and/or dynamic properties of the headgroups are induced by the presence of cyclosporin A, although care must be taken to avoid artifacts which cause line broadening arising from instrumental limitations of the NMR spectroscopy [25].

In addition, the effect of cyclosporin A on the lamellar to hexagonal phase transition temperature of DEPE, depended on the peptide-lipid mole ratio [23]. At low peptide to lipid ratios, the L_{α} to H_{II} (hexagonal) phase transition temperature was increased whereas at higher mole ratios, the temperature was decreased. This provides important information as to the location of cyclosporin A in the membrane, since the H_{II} phase has been shown to be favored in systems with a small area

ratio of headgroup to acyl chain. The implication is that solutes which are primarily located within the acyl chains lower the L_α to H_{II} phase transition temperature, whereas solutes which are primarily located in the headgroup region increase the transition temperature. Since the effect of cyclosporin A on the phase transition depends on the peptide to lipid ratio, the location of cyclosporin A may also depend on the amount of cyclosporin A within the bilayer. One possible explanation offered is that at high peptide to lipid ratios, the self association of cyclosporin A within the acyl chains of the bilayer is favored which effectively increases the area ratio of the hydrocarbon to headgroup [26]. On the other hand, polar solvents enhance dimerization which would argue against association within the bilayer interior.

Cyclosporin A has been found to concentrate within the inner monolayer of small vesicles as determined from ^{19}F-NMR [11]. The observation would indicate that cyclosporin A packs better in phospholipid monolayers with a smaller radius of curvature. The implication is that the relative cross sectional area occupied at the membrane/aqueous interface is less than that within the bilayer core which would occur when cyclosporin A would be located within the acyl chain region of the bilayer. It is of interest that cholesterol also shares this feature in having a greater volume within the bilayer core relative to the bilayer surface and thereby lowering the L_α to H_{II} phase transition temperature [27].

Fourier transform infrared spectroscopy has also been used to investigate cyclosporin A–DPPC interactions [10]. These investigators found that there is little influence of cyclosporin A on the C–O stretching mode even at peptide-lipid mole ratios of 40 mole percent. This is in contrast to cholesterol which has a major effect on the carbonyl–carbonyl interactions. It is clear that additional studies are required to unambiguously determine the distribution of cyclosporin A within phospholipid bilayers. These should include an examination of the distribution of cyclosporin A at various lipid to peptide mole ratios as well as other techniques such as X-ray diffraction to allow estimation of the geometric arrangement of the phospholipids. The results obtained may then be used to provide a greater understanding of the role of lipid–peptide interactions in the mechanism of action and also toxicity of cyclosporin A.

In summary, cyclosporin A has relatively minor effect on the structure of the phospholipid bilayer as compared with other peptides or cholesterol. At temperatures above the gel to liquid-crystalline phase transition, cyclosporin A increases the magnitude of the order parameters in the plateau region of the acyl chains of the phospholipids. On the other hand, cyclosporin A causes a small decrease as well as broadening in the phase transition temperature.

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