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Membrane structure and dynamics as viewed by solid-state NMR spectroscopy

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Abstract

The purpose of the present study is the investigation of the structure and dynamics of biological membranes using solid-state nuclear magnetic resonance (NMR) spectroscopy. Two approaches are used in our laboratory. The first involves the measurement of high-resolution ^{13}C and ^{1}H spectra obtained by the magic angle spinning (MAS) technique while the second approach involves the measurement of ^{31}P and ^{2}H powder spectra in static samples. This paper will present some recent results obtained by high-resolution solid-state ^{1}H NMR on the conformation of gramicidin A incorporated in a phosphatidylcholine bilayers. More specifically, we were able to observe changes in the gramicidin spectra as a function of the cosolubilization solvent initially used to prepare the samples. The interaction between lipid bilayers and an anticancer drug derived from chloroethylurea was also investigated using proton NMR spectroscopy. Finally, we have studied the interaction between cardiotoxin, a toxic protein extracted from snake venom, and negatively charged lipid bilayers using ^{31}P solid-state NMR spectroscopy. © 1997 Elsevier Science B.V.

Keywords: Solid-state; NMR; Bilayer; Protein; Gramicidin; Cardiotoxin

1. Introduction

During the last decade, nuclear magnetic resonance (NMR) spectroscopy has become a method of choice for the study of the structure and dynamics of natural and synthetic macromolecules. In particular, the introduction of multidimensional NMR techniques have brought a versatile approach to the resolution and assignment of resonances in complex NMR spectra, like those obtained in proteins [1–4]. These methods have been used successfully to study small molecules (molecular weight $< 30{,}000$) in solution. However for large proteins, membrane assemblies and polymers, problematic spectral line broadening is encountered due to reduced tumbling rates and correspondingly longer rotational correlation times. In such cases, a good alternative is the solid-state NMR technique [5–8].

The solid-state NMR methods for determining the structure of large biological molecules have developed along two directions. The first involves the study of powder spectra. In particular, deuterium and phosphorus NMR methods have been widely used in the last two decades for the study of the structure and dynamics of biological membranes [9–13]. The

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predominant source of line broadening for deuterium is the electric quadrupolar interaction while for phosphorus, the anisotropic chemical shift and the heteronuclear dipolar coupling with protons are both strong. The second class of techniques involves the restoration of high-resolution spectra using the magic angle spinning technique [5–8]. In this technique, the sample is spun in a rotor at a spinning speed typically between 1 and 15 kHz. This results in a NMR line-narrowing effect for large molecules or molecular assemblies similar to the effect of isotopic tumbling in solution. The orientational dependence of the magnetic interactions (chemical shift anisotropy and dipolar coupling), which varies with $(3\cos^2\theta - 1)/2$, averages to zero at the magic angle, $\theta = 54.7°$.

In the present paper, we will present some recent results on the interaction between lipid bilayers and intrinsic and extrinsic proteins, as well as an anticancer drug. More specifically, the conformation of gramicidin A in lipid bilayers has been investigated using high-resolution proton NMR spectroscopy [14]. We have also applied this technique to the study of a new class of antineoplastic agents incorporated in lipid bilayers. Finally, the last part of the paper will summarize the results of a study of the interaction between a toxic protein, cardiotoxin, and lipid bilayers using static solid-state ^{31}P NMR [15].

2. High-resolution solid-state ^{1}H NMR study of lipid–peptide and lipid–drug systems

2.1. Introduction

High-resolution ^{1}H nuclear magnetic resonance (NMR) spectroscopy is a powerful method for protein structure determination in solution [1]. However, this method has not been extensively applied to membrane protein systems since the strong homogeneous dipole–dipole broadening between neighboring hydrogen nuclei, which is averaged to zero by rapid isotropic reorientation of molecules in solution, is not completely averaged away in anisotropic non-spinning membrane systems and leads to spectral linewidths of 5 to 25 kHz (10 to 50 ppm depending on the magnetic field strength) [16,17]. This broadening is comparable to, or even larger than, the chemical shift dispersion obtained at the highest magnetic field available. Therefore, the spectral details found in high resolution ^{1}H NMR cannot be seen in static (non-spinning) experiments on membrane systems.

In 1987–1988, it was discovered that magic angle spinning at a rate of only 3 to 4 kHz was sufficient to obtain well resolved ^{1}H spectra from multilamellar dispersions of phospholipids in water [18,19]. The most important motions which permit MAS to narrow the homogeneously broadened ^{1}H NMR spectra of lipid multilamellar dispersions is the rapid axial diffusion of the phospholipid about its long axis, which projects the intra-molecular dipolar interactions onto the diffusion axis. The dipolar broadening then depends only on the orientation of the local bilayer normal relative to the magnetic field, scaling as $(3\cos^2\theta - 1)/2$ where θ is the angle between the bilayer normal and the magnetic field. In this manner, the homogeneous dipolar broadening is converted into an inhomogeneous broadening which can be effectively averaged by magic angle spinning [20].

Although it is possible to obtain well-resolved lipid ^{1}H spectra from multilamellar dispersions, no identifiable peptide signals can be observed at spinning speeds of about 3 to 4 kHz for small peptides, such as gramicidin A, incorporated in phospholipid bilayers. Initially, it was believed that the peptide was not reorienting rapidly enough about the bilayer normal to average out the homogeneous interactions. However, several studies [21–27] have indicated that gramicidin do indeed diffuse sufficiently rapidly about the local bilayer normal, with correlation times in the range of 10^{-9} to 10^{-7} s. On the other hand, it can be shown that there are additional intermediate time scale motions which broaden the peptide resonances. ^{2}H NMR relaxation studies of gramicidin A in oriented samples of DLPC [22,23] have demonstrated that the wobble of the diffusion axis with respect to the local bilayer normal, which for that system has a correlation time of about 6×10^{-6} s, is probably the most significant cause of line broadening of the peptide's ^{1}H resonances. Therefore, it should be possible to observe ^{1}H NMR MAS spectra of membrane peptides but in order to do so, it is necessary to spin the sample at a spinning speed faster than that required for phospholipid multilamellar dispersions [20].

2.2. Structure of gramicidin A in lipid bilayers

We have demonstrated by the use of high-speed MAS one and two-dimensional solid-state ^1H NMR that it is possible to study small peptides incorporated in phospholipid membranes [14,20]. More specifically, the conformation of gramicidin A, a fifteen amino acid hydrophobic peptide which has been widely used as a model for the hydrophobic part of intrinsic membrane proteins [28], has been investigated in multilamellar dispersions of dimyristoylphosphatidylcholine (DMPC) [14]. In addition, since it has been shown by a variety of techniques such as circular dichroism [29], NMR spectroscopy [30–32], HPLC [33] and vibrational spectroscopy [34] that the conformation of gramicidin A is highly dependent upon the organic solvent initially used to codissolve the peptide with the lipid, the proton NMR spectra have been recorded for three different cosolubilization solvents, trifluoroethanol, a mixture of methanol/chloroform (1:1 v/v) and ethanol.

Fig. 1 shows the MAS solid-state proton NMR spectra at 60°C of pure DMPC and of the DMPC/gramicidin A system (lipid-to-peptide molar ratio of 10:1) initially prepared from trifluoroethanol. Both spectra were recorded in ^2H_2O and at a spinning speed of 13.000 ± 0.002 kHz. In the spectrum of the DMPC/gramicidin A system, we can observe signals between 6.0 and 9.0 ppm. In contrast, in the pure DMPC spectrum, no signal can be observed in this region. Therefore, we can conclude that the signals observed in the DMPC/gramicidin A spectrum between 6.0 and 9.0 ppm are exclusively due to the gramicidin A protons. Other resonances that can be attributed to gramicidin A are also present underneath some of the lipid resonances. Experiments performed at slower spinning speeds indicate that the linewidth of the resonances observed between 6.0 and 9.0 ppm are strongly dependent upon the spinning speed, the signal becoming significantly broader at slower speeds.

On the basis of high-resolution liquid NMR studies of gramicidin A [27,35], the spectral region between 6.0 and 9.0 ppm can be assigned to the aromatic protons of the tryptophan residues (resonances at 7.0 and 7.4 ppm) and to the formyl group proton of gramicidin A (resonance at 8.4 ppm). In addition, the width of the resonances at 7.0 and 7.4 ppm also reflects the distribution of isotropic chemical shifts for the indole protons of the four tryptophan residues in each gramicidin molecule. The spectrum presented in Fig. 1B is very similar to that obtained by Le Guernevé and Seigneuret [36] for gramicidin A incorporated in dilauroylphosphatidylcholine bilayers at a lipid-to-peptide molar ratio of 20:1. This study has also demonstrated that it is possible to record such spectra in H_2O, even at relatively high water content, by the use of water suppression techniques. This therefore allows the observation of peptide-exchangeable ^1H. In addition, regioselective excitation of ^1H peptide resonances can be used to obtain an efficient suppression of lipid resonances.

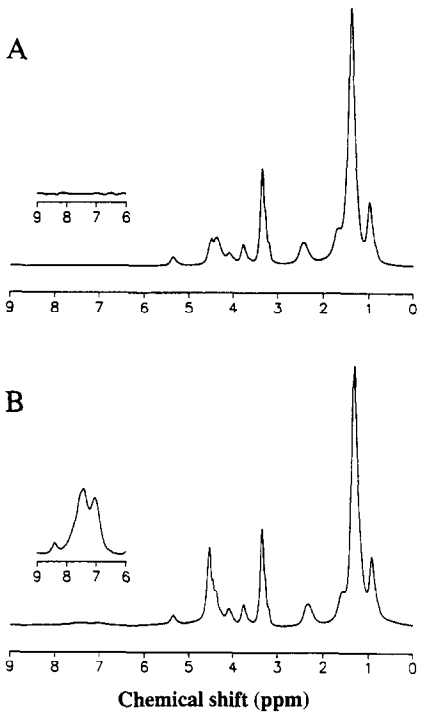

Fig. 1. MAS solid-state ^1H spectra at 60°C of (A) pure DMPC and (B) DMPC/gramicidin A system (lipid-to-peptide molar ratio of 10:1) initially prepared from trifluoroethanol. The spectra were recorded in ^2H_2O and the spinning speed was set to 13.000 ± 0.002 kHz, (from Ref. [14], reproduced with permission of Biophysical J.).

Fig. 2 shows the MAS solid-state proton NMR spectra at 60°C between 6.3 ppm and 8.7 ppm for the DMPC/gramicidin A systems initially prepared from

three different organic solvents: ethanol, a mixture of methanol/chloroform (1:1 v/v) and trifluoroethanol. The spectra clearly show significant changes in the lineshape and chemical shifts of the gramicidin A signals in the three systems. The changes observed in the spectra presented in Fig. 2 can be explained by the fact that the conformation adopted by gramicidin A varies with the organic solvent initially used in the preparation of the samples. When trifluoroethanol is used as the cosolubilization solvent, it has been shown that gramicidin A is incorporated in lipid bilayers as an amino terminal to amino terminal hydrogen bonded dimer in the single-stranded $\beta^{6.3}$ helical conformation [29,31,32,37]. In this conformation, the four tryptophan residues (at position 9, 11, 13 and 15) are located near the bilayer surface, close to the lipid carbonyl groups [34]. By contrast, when ethanol is used, in which gramicidin A tends to form intertwined dimers, it incorporates in the non-channel state and the channel structure is slow to appear [29,30,34]. In such systems, the dominant conformation of gramicidin A is the double helix in which the tryptophan residues are located uniformly along the peptide axis, therefore having chemical environments different from those obtained in a $\beta^{6.3}$ helix. When the cosolubilization solvent is a methanol/chloroform (1:1 v/v) solution, a mixture of conformations is obtained, including the channel and the double helix conformations [29,30,34].

We have compared the chemical shifts obtained for the systems prepared from trifluoroethanol, which favors the channel conformation, with those obtained from ethanol, which favors the double helix conformation. For the system prepared from trifluoroethanol, the formyl proton is located at 8.39 ppm while two major resonances can be observed for the tryptophan protons, at 7.02 and 7.42 ppm. For the system prepared from ethanol, three resonances can be observed at 7.05, 7.36 and 7.72 ppm for the tryptophan protons while the formyl proton is still present at 8.38 ppm. These results have in turn been compared with those obtained from solution NMR spectra of gramicidin A in both the channel conformation (incorporated into sodium dodecyl-d_{25} sulphate micelles) [35] and in the double helix conformation (gramicidin A species 4 in dioxane) [27]. When gramicidin A is in the channel structure, the formyl group proton is located at 8.35 ppm and the indole proton resonances are distributed between 6.90 ppm and 7.55 ppm [35]. This dispersion is much smaller than that obtained for gramicidin A in the double helix conformation in dioxane [27] and for the multilamellar dispersions prepared in ethanol, in which resonances up to 7.9 ppm can be detected. Therefore, the chemical shifts measured in the solid-state NMR spectra of gramicidin A incorporated in different conformations in DMPC bilayers are similar to those measured by solution NMR. This demonstrates that solid-state proton NMR spectra is very sensitive to structural changes of small peptides incorporated in lipid bilayers.

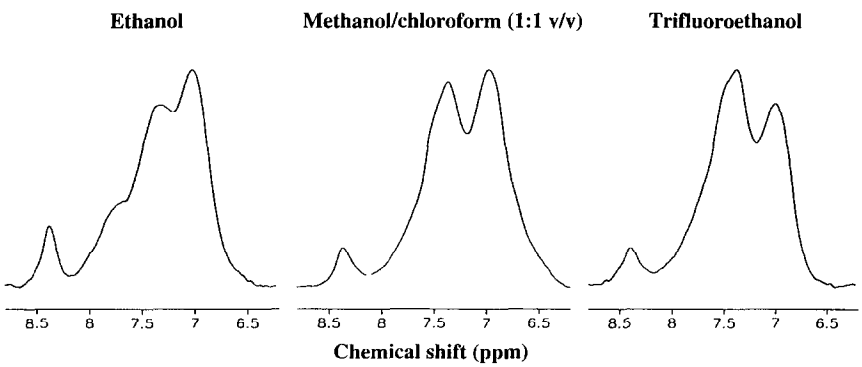

Fig. 2. MAS solid-state ^1H spectra at 60°C between 6.3 ppm and 8.7 ppm for DMPC/gramicidin A systems (lipid-to-peptide molar ratio of 10:1) initially prepared from the three organic solvents: ethanol, a mixture of methanol/chloroform (1:1 v/v) and trifluoroethanol. The spectra were recorded in ^2H_2O and the spinning speed was set to 13.000 ± 0.002 kHz, (from Ref. [14], reproduced with permission of Biophysical J.).

2.3. Interactions between anticancer drugs and lipid bilayers

We have investigated the interactions between 1-aryl-3-(2-chlorethyl)ureas (CEU), a new class of antineoplastic agents, and lipid bilayers. These molecules are active both in vitro and in vivo [38–40] but their mechanisms of action are not yet defined. However, it has been shown that the effects of different CEUs on model membranes are strongly dependent on the aryl substituent and are related to the cytotoxicity of the drug [41]. In addition, the amphiphilic structure of the CEUs suggests that biological membranes are one of the most likely site for the binding of CEUs. The interaction between one of the CEU derivatives, 4-*tert*-butyl-[3-(2-chloroethyl)ureido] benzene (*t*BCEU), and model phosphatidylcholine membranes has been investigated by infrared spectroscopy and differential scanning calorimetry [42]. The results indicate that the hydrophobic portion of the drug is intercalated between the lipid acyl chains while the hydrophilic region is located close to the interfacial region of the bilayer.

Fig. 3 shows the proton solid-state NMR spectrum of *t*BCEU incorporated in a model membrane made of dimyristoylphosphatidylcholine (DMPC), at a lipid-to-drug molar ratio of 5:1. The spectrum has been obtained at a spinning speed of 11 kHz. The results indicate that resonances attributed to the aromatic ring of *t*BCEU are clearly distinguishable in the spectrum. This suggests the rapid axial rotation of the drug molecule in the membrane. We have also recorded some preliminary NOESY experiments on this sample. The results show the presence of cross peaks between the aromatic protons of *t*BCEU and the protons located close to the carbonyl groups on the lipid acyl chains, indicating that two dimensional proton solid-state NMR appears to be a promising technique to determine the exact location of drug molecules in lipid bilayers.

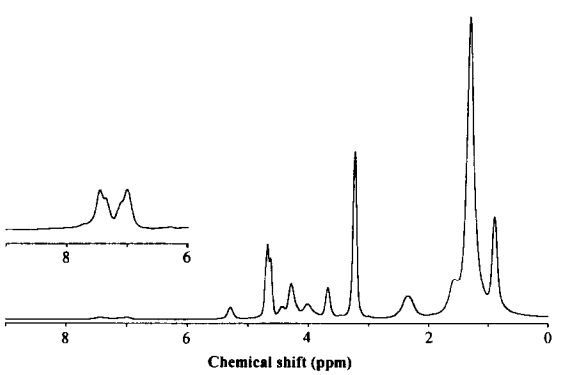

Fig. 3. MAS solid-state ^{1}H spectrum of DMPC/*t*BCEU at a lipid-to-drug molar ratio of 5:1. The spectrum was recorded in $^{2}H_2O$ at a spinning speed of 11 kHz and at a temperature of 40°C. The inset shows the vertical expansion of the spectral region between 6 ppm and 9 ppm.

3. Solid-state ^{31}P NMR study of the interaction between a cardiotoxin and dimyristoylphosphatidic acid bilayers

3.1. Introduction

Solid-state ^{31}P NMR spectroscopy is a valuable technique to study the different phases formed by model phospholipid membranes. The ^{31}P NMR lineshapes are very characteristic of the different lipid phases such as the gel and liquid–crystalline lamellar phases, the inverted hexagonal phase and isotropic phases such as small vesicles and micelles [12,13,43,44].

We have investigated the interaction between the cardiotoxin IIa of *Naja mossambica mossambica* with dimyristoylphosphatidic acid at different lipid-to-protein molar ratios by ^{31}P solid-state NMR [15]. Cardiotoxins are basic proteins extracted from snake venom. They have a molecular weight of about 7000 and are highly stabilized by the presence of four disulfide bridges [45]. The mechanism of action of cardiotoxins on cell membranes is still a matter of controversy but there is a general agreement that cardiotoxins act by perturbing the lipid phase of cell membranes [46–48]. On the basis of the large amount of cardiotoxins that were observed to be bound to biological membranes, it has been proposed that lipids were involved in this binding [49]. In addition, fluorescence studies have established that cardiotoxins interact only with negatively charged phospholipids at a lipid-to-protein molar ratio of 7:1 [50,51] for singly charged phospholipids and at a molar ratio of 3.5 for doubly charged phospholipids [50–53].

Several studies have been performed in order to elucidate the mechanism of action of cardiotoxins on cell membranes. Many observations suggest considerable changes of the lipid organization upon binding of cardiotoxins. On the other hand, a study by attenuated total reflection FTIR spectroscopy and X-ray crystallography has shown that the complex between DMPA and cardiotoxin is poorly ordered [54]. In order to explain these data, three models of interaction have been suggested. Two models proposed a bilayer structure with a partial incorporation of cardiotoxin in the hydrophobic region of the bilayer [55,56]. A third model proposed a double inverted micellar structure with a disappearance of the bilayer structure [53].

3.2. Disappearance of the bilayer structure

The ^{31}P NMR spectra of pure DMPA bilayers as a function of temperature are shown in Fig. 4. In the temperature range between 25 and 65°C, all the spectra are axially symmetric and characteristic of lamellar phases [12]. The spectral width decreases gradually with increasing temperature with a major change between 40 and 50°C, which corresponds to the gel to liquid–crystalline phase transition temperature of DMPA (48 to 50°C). The smaller spectral width obtained in the liquid–crystalline phase can be explained by an additional wobbling motion of the polar head group [13].

The addition of cardiotoxin to DMPA at a lipid-to-protein molar ratio of 5:1 (Fig. 4) causes the complete disappearance of the lamellar phase spectrum and only a broad isotropic peak is present. The width at half-height of the isotropic peak decreases gradually from 18 ppm at 25°C to 5 ppm at 65°C. This suggests that at that lipid-to-protein molar ratio, all the phospholipids interact with cardiotoxin to form an isotropic phase in which the motions are sufficiently fast to completely average the chemical shift anisotropy. On the other hand, the addition of cardiotoxin to DMPA at a lipid-to-protein molar ratio of 15:1 at 25°C induces the apparition of an isotropic peak at 0 ppm superimposed to a lamellar phase spectrum (Fig. 5). This suggests that at that temperature, only a fraction of cardiotoxin molecules interacts with the lipids, giving rise to an isotropic peak similar to that observed at a lipid-to-protein

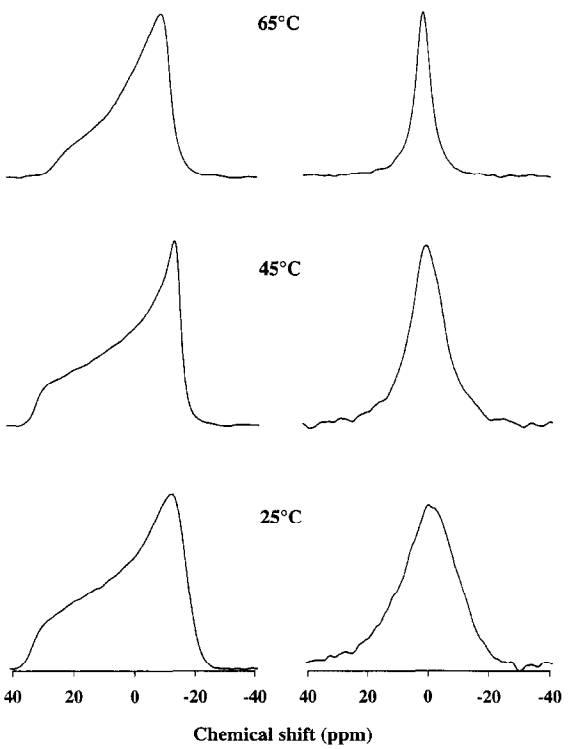

Fig. 4. Temperature dependence of the ^{31}P NMR spectra of pure DMPA (left) and the complex DMPA:cardiotoxin at a lipid-to-protein molar ratio of 5:1 (right), (from Ref. [15], reproduced with permission of Biophysical J.).

molar ratio of 5:1. With increasing temperature, the intensity of the isotropic peak increases at the expense of the lamellar phase spectrum. It is important to note that the changes observed as a function of temperature are fully reversible.

Spectral simulations have been performed by varying the amounts of isotropic and lamellar spectra and the comparison with the experimental spectra obtained for a lipid-to-protein molar ratio of 15:1 is presented in Fig. 5 as a function of temperature. The parameters used in the simulations have been given elsewhere [15]. The results indicate that the width at half-height of the isotropic peak decreases and its intensity increases with increasing temperature. Above 55°C, only the isotropic peak is present, suggesting that all the phospholipids are interacting with cardiotoxin. We have calculated from the relative intensities of the two subspectra the number of lipids in interaction with cardiotoxin as a function of

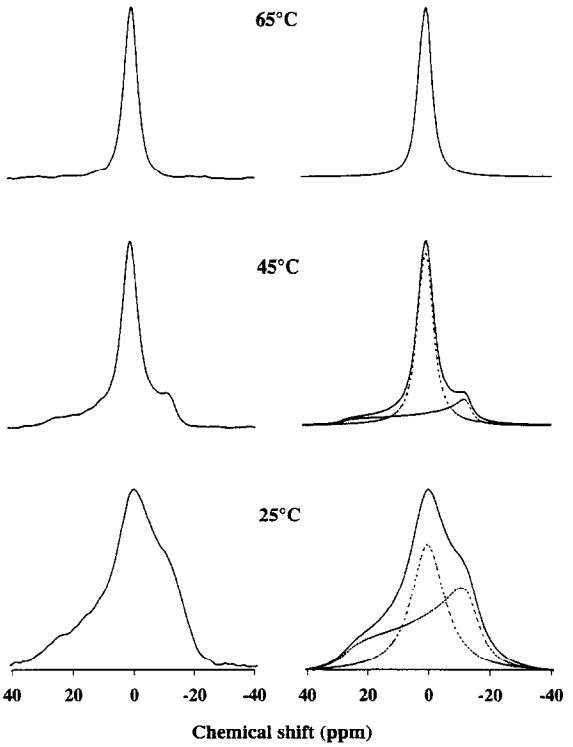

Fig. 5. Experimental (left) and simulated (right) ^{31}P NMR spectra as a function of temperature for the complex DMPA:cardiotoxin at a lipid-to-protein molar ratio of 15:1. The spectra used in the simulations are represented by dotted lines, (from Ref. [15], reproduced with permission of Biophysical J.).

temperature. This number varies from 7 at 25°C to 15 at temperatures above 55°C, indicating that there is a change in the proportion of phospholipids that interact with cardiotoxin as a function of temperature.

The isotropic peak can be associated with phospholipids interacting with cardiotoxin and the lamellar lineshape with pure DMPA bilayers. The disappearance of the lamellar lineshape suggests the formation of small isotropic structures which give rise to a complete averaging of the chemical shift anisotropy in the ^{31}P spectra. The most likely candidate is therefore the formation of a hydrophobic complex similar to an inverted micelle [15], as it has also been suggested for complexes of cardiotoxin with cardiolipin [53].

3.3. Formation of a second anisotropic phase

At a lipid-to-protein molar ratio of 40:1, there is still an interaction between cardiotoxin and DMPA at room temperature, as indicated by the presence of a small isotropic peak superimposed to a non-perturbed lamellar spectrum (Fig. 6). The isotropic peak is present at 25°C and its intensity increases with increasing temperature until the phase transition to the liquid–crystalline state. At that temperature, another spectrum is superimposed to the isotropic and lamellar spectra. This new band does not exhibit a maximum at 0 ppm, which suggests that it is due to an anisotropic phase. In this case, spectral simulations have been performed by varying the amounts of isotropic peak and one or two lamellar phase spectra with different chemical shift anisotropies (CSA).

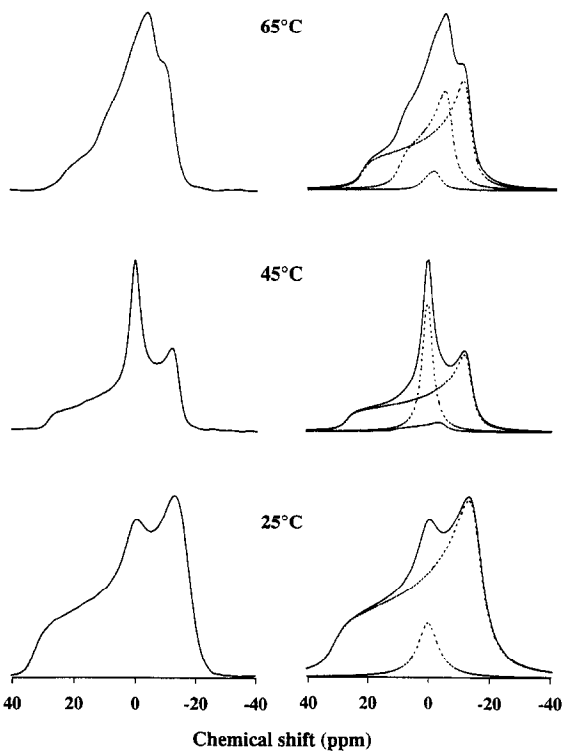

Fig. 6. Experimental (left) and simulated (right) ^{31}P NMR spectra as a function of temperature for the complex DMPA:cardiotoxin at a lipid-to-protein molar ratio of 40:1. The spectra used in the simulations are represented by dotted lines, (from Ref. [15], reproduced with permission of Biophysical J.).

The comparison between the experimental and simulated spectra is presented in Fig. 6 as a function of temperature. These results indicate that at temperatures below that of the phase transition, only two components are present, a typical lamellar phase spectrum with a width corresponding to that of pure DMPA and a small isotropic peak. The relative intensity of the isotropic peak increases from 11% at 25°C to 39% at 40°C, at the expense of the lamellar phase component. However, at temperatures above the phase transition temperature of the pure lipid, a second spectrum with a spectral width of about 14 ppm is necessary to adequately simulate the experimental spectra, in a proportion ranging from 30 to 37% at temperatures between 50 and 65°C. In these spectra, the amount of isotropic peak is very small (less than 5%). Assuming that the lamellar spectrum with a smaller CSA is due to DMPA interacting with cardiotoxin, the number of lipids in interaction with cardiotoxin varies from 5 at 25°C to about 15 at temperatures above 45°C. This behavior is very similar to that observed at a lipid-to-protein molar ratio of 15:1.

The coexistence of a lamellar lineshape and of an isotropic peak at low temperatures suggests that the hydrophobic complex is outside the bilayers. At high temperatures, the spectra indicate the coexistence of two anisotropic phases, a non-perturbed lamellar phase and a perturbed phase with a chemical shift anisotropy of 14 ppm. The formation of a second lamellar phase at high temperatures suggests that the hydrophobic complex is incorporated into the lipid lamellar phase. The smaller CSA obtained for the second lamellar phase can be explained by the neutralization of the charges of the DMPA molecules in the complex, resulting from the electrostatic interaction with cardiotoxin. In a recent article [57], it has been demonstrated that the chemical shift anisotropy of uncharged DMPA in the liquid–crystalline phase is 14 ppm. This corresponds exactly to the chemical shift anisotropy observed above the phase transition temperature for the DMPA:cardiotoxin complex at a lipid-to-protein molar ratio of 40:1.

4. Conclusions

The novel possibility of using high-speed magic angle spinning to obtain solid-state proton NMR spectra of small peptides and drugs incorporated in lipid membranes has been demonstrated in this paper. With the resolution achieved in our spectra, signals that can be attributed to the formyl group and the indole ring protons can be distinguished in the spectra of gramicidin A incorporated into DMPC bilayers. In addition, it has been demonstrated that the resonances of the tryptophan indole protons vary significantly for the different conformations adopted by gramicidin A in lipid bilayers, indicating that solid-state proton NMR is very sensitive to structural changes of small peptides incorporated in lipid membranes. On the other hand, we have also demonstrated that static ^{31}P NMR can be very useful to elucidate the different phases formed in phospholipid membranes in the presence of proteins. More specifically, we have shown that the strong electrostatic interactions between cardiotoxin IIa extracted from *N. mossambica mossambica* and dimyristoylphosphatidic acid results in the disappearance of the bilayer structure and the formation of an isotropic phase at small lipid-to-protein molar ratios, which gives rise to an isotropic peak in the solid-state ^{31}P NMR spectra. At high lipid-to-protein molar ratios, our results suggest that the hydrophobic complex is incorporated in the lipid bilayer in the liquid–crystalline phase.

Acknowledgements

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