Model of Interaction Between a Cardiotoxin and Dimyristoylphosphatidic Acid Bilayers Determined by Solid-State ^{31}P NMR Spectroscopy

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ABSTRACT The interaction of cardiotoxin IIa, a small basic protein extracted from *Naja mossambica mossambica* venom, with dimyristoylphosphatidic acid (DMPA) membranes has been investigated by solid-state ^{31}P nuclear magnetic resonance spectroscopy. Both the spectral lineshapes and transverse relaxation time values have been measured as a function of temperature for different lipid-to-protein molar ratios. The results indicate that the interaction of cardiotoxin with DMPA gives rise to the complete disappearance of the bilayer structure at a lipid-to-protein molar ratio of 5:1. However, a coexistence of the lamellar and isotropic phases is observed at higher lipid contents. In addition, the number of phospholipids interacting with cardiotoxin increases from about 5 at room temperature to approximately 15 at temperatures above the phase transition of the pure lipid. The isotropic structure appears to be a hydrophobic complex similar to an inverted micellar phase that can be extracted by a hydrophobic solvent. At a lipid-to-protein molar ratio of 40:1, the isotropic structure disappears at high temperature to give rise to a second anisotropic phase, which is most likely associated with the incorporation of the hydrophobic complex inside the bilayer.

INTRODUCTION

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 (Louw, 1974). Even though cardiotoxins display a lower toxicity than neurotoxins, they generally represent the major content of snake venom. The structures of several cardiotoxins have been investigated by circular dichroism (Louw and Visser, 1978; Ménez et al., 1978), Raman spectroscopy (Pézolet et al., 1982), and Fourier transform infrared (FTIR) spectroscopy (Surewicz et al., 1988; Désormeaux et al., 1992), and the complete structures of at least two cardiotoxins of *Naja mossambica mossambica* have been determined by x-ray crystallography (Rees et al., 1987) and nuclear magnetic resonance (NMR) spectroscopy (Steinmetz et al., 1988; O'Connell et al., 1993).

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 (Bougis et al., 1981, 1983; Ménez et al., 1990). 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 (Vincent et al., 1976). Fluorescence studies have established that cardiotoxins interact only with negatively charged phospholipids at a lipid-to-protein molar ratio of 7:1 (Dufourcq and Faucon, 1978; Vincent et al., 1978) for singly charged phospholipids and at a molar ratio of 3.5 for doubly charged phospholipids (Dufourcq and Faucon, 1978; Faucon et al., 1983; Batenburg et al., 1985).

Several studies have been performed to elucidate the mechanism of action of cardiotoxins on cell membranes. Many observations suggest considerable changes of the lipid organization upon binding of cardiotoxins. Such phenomena include the liberation of intramembrane proteins investigated by freeze-fracture electron microscopy (Gulik-Krzywicki et al., 1981), the liberation of trapped fluorescent species (Faucon et al., 1979), and the abolition of the thermotropic phase transition of dimyristoylphosphatidic acid (DMPA) bilayers observed by differential scanning calorimetry (DSC), fluorescence polarization, Raman spectroscopy (Faucon et al., 1981, 1983), and FTIR spectroscopy (Désormeaux et al., 1992). Furthermore, the synergetic action of cardiotoxin and phospholipase A_2 is a well-known phenomenon (Louw and Visser, 1978; Gulik-Krzywicki et al., 1981; Rivas et al., 1981; Harvey et al., 1983) that can be easily explained by a perturbation of the lipid bilayer. 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 (Désormeaux et al., 1992).

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 (Lauterwein and Wüthrich, 1978; Dufourcq et al., 1982). A third model proposed a double inverted micellar structure with a disappearance of the bilayer structure (Batenburg et al., 1985).

Solid-state ^{31}P NMR spectroscopy is a valuable technique for studying the different phases formed by model

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phospholipid membranes. The ^{31}P NMR lineshapes are very characteristic of the different phases, such as the gel and liquid-crystalline lamellar phases, the inverted hexagonal phase, and isotropic phases such as small vesicles and micelles (Seelig, 1978; Smith and Ekiel, 1984; Lindblom and Rilfors, 1989; Seddon, 1990a). On the other hand, the dynamics of the lipid headgroup can be studied by ^{31}P NMR longitudinal relaxation time (T_1) (Milburn and Jeffrey, 1987, 1989, 1990) and ^{31}P NMR transversal relaxation time (T_2) (Dufourc et al., 1992).

In the present study 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. The results indicate that the interaction of cardiotoxin with DMPA results in a complete disappearance of the bilayer structure at a lipid-to-protein molar ratio of 5:1 and in a partial disappearance at higher molar ratios (15:1 and 40:1). The isotropic structure appears to be a hydrophobic complex similar to an inverted micellar phase that gives rise to an isotropic peak in the ^{31}P NMR spectra. At a lipid-to-protein molar ratio of 40:1, a second anisotropic lineshape is present at high temperatures that is most likely associated with the incorporation of the hydrophobic complex inside the bilayer.

MATERIALS AND METHODS

Materials

The disodium salt of dimyristoylphosphatidic acid (DMPA) was obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Cardiotoxin IIa (hereafter called cardiotoxin) was purified from *Naja mossambica mossambica* venom according to the method described by Bougis et al. (1986). The concentration of cardiotoxin was determined from the absorbance at 280 nm.

Sample preparation

The sample preparation was based on the method of Désormeaux et al. (1992). Solutions of cardiotoxin at a concentration of 2% were prepared in 150 mM NaCl and 10 mM EDTA. The pH of the solutions was measured with a microelectrode (Microelectrodes, Inc., Londonderry, NH) and adjusted to 6.5 with diluted NaOH or HCl solutions. Aqueous dispersions of DMPA were prepared by mixing appropriate amounts of solid in 150 mM NaCl and 10 mM EDTA, adjusted to pH 6.5 with diluted NaOH or HCl solutions. Samples containing about 10% by weight in lipids were then heated to approximately 65°C for 10 min, stirred on a vortex mixer, and cooled down at 0°C for 10 min. This cycle was repeated at least five times the day before the analysis and five times the day of the analysis. The pH of the dispersions was adjusted to 6.5, if necessary. Lipid-protein complexes were prepared by adding the appropriate amount of 2% cardiotoxin solution to phospholipid dispersions to obtain the desired lipid-to-protein molar ratio. The addition was made after the first five temperature cycles. The samples were then centrifuged to yield white pellets that were used in the NMR experiments. All of the samples were analyzed within 4 days of their preparation.

Hydrophobic extraction

The hydrophobic extraction was performed according to the method of Batenburg et al. (1985). A total of 20 nmol of cardiotoxin in 100 μl of aqueous solution (150 mM NaCl and 10 mM EDTA) was added to 430 μl of chloroform/methanol, 5:8 (v/v), containing 900 nmol of DMPA. The phase separation was induced by the addition of 370 μl chloroform and 100 μl aqueous solution (150 mM NaCl and 10 mM EDTA), followed by a 10 min of centrifugation at 14,000 rpm. The aqueous phase was then analyzed by spectrophotometry at 280 nm.

NMR experiments

All of the ^{31}P spectra were acquired at 121.5 MHz on a Bruker ASX-300 (Bruker Spectrospin, Milton, ON) operating at a ^{1}H frequency of 300.00 MHz. Experiments were carried out with a broadband/^{1}H dual-frequency 4-mm probehead under conditions of proton decoupling. The free induction decays (2 K data points) were recorded with a spin echo sequence (4000 scans) with a 5-s repetition time. The temperature was controlled to within ±0.5°C and the chemical shifts (expressed in ppm) were referenced relative to the signal of phosphoric acid at 0 ppm. The transverse relaxation times were measured with a spin echo sequence using 20 interpulse delays (τ) varying between 10 μs and 4 ms. Five hundred twelve scans were used for the relaxation time experiments. A line broadening of 300 Hz was applied to all spectra. The T_2 values were calculated from the decay of intensity as a function of 2τ, using either a mono- or biexponential decay. The integrated areas of the whole spectra have been used in the calculations, and therefore the exponential decays reflect the relaxation behavior of the different phases present in the spectra.

Spectral simulations

The powder spectra obtained for the DMPA-cardiotoxin complexes have been simulated by using different proportions of one or two powder spectra with different chemical shift anisotropies and an isotropic peak. The isotropic peak was always centered at 0 ppm and its width (denoted in Table 1 as width at half-height (WHH)) was varied between 4 ppm and 11 ppm to fit the experimental spectra. Line broadening was used in the simulations of the anisotropic spectra to fit the experimental spectra. The quality of the fit was determined by visual comparison of the experimental and simulated spectra. We estimate that the error on the calculated proportions of each phase is on the order of 3%.

RESULTS

Spectral lineshapes

The ^{31}P NMR spectra of pure DMPA bilayers as a function of temperature are shown in Fig. 1. In the temperature range between 25 and 65°C, all of the spectra are axially symmetric and characteristic of lamellar phases (Seelig, 1978). 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) (Van Dick et al., 1978; Désormeaux et al., 1992). The smaller spectral width obtained in the liquid-crystalline phase can be explained by an additional wobbling motion of the polar headgroup (Smith and Ekiel, 1984).

The addition of cardiotoxin to DMPA at a lipid-to-protein molar ratio of 5:1 (Fig. 1) 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 of the phospholipids interact with cardio-

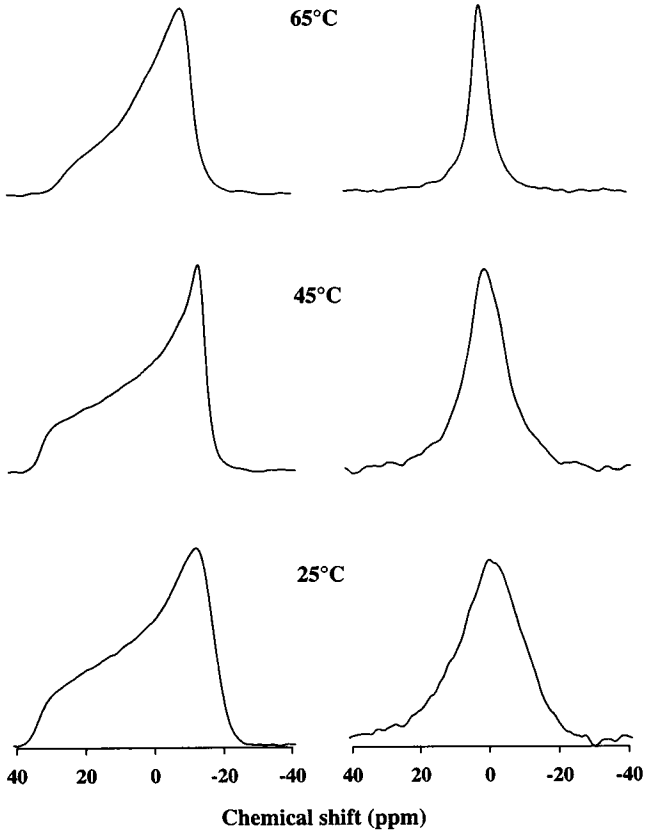

FIGURE 1 Temperature dependence of the ^{31}P NMR spectra of pure DMPA (*left*) and the complex DMPA:cardiotoxin at a lipid-to-protein molar ratio of 5:1 (*right*).

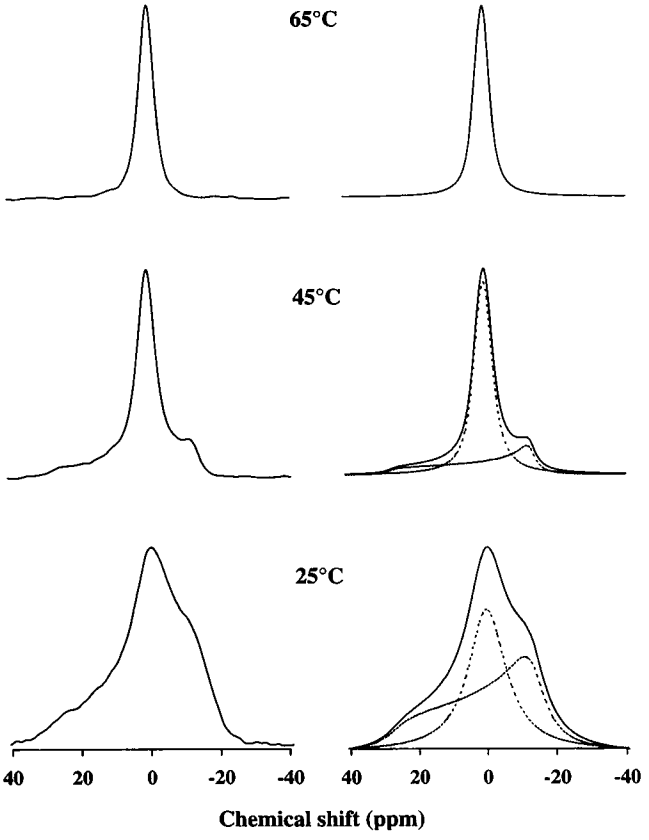

FIGURE 2 Experimental (*left*) and simulated (*right*) ^{31}P NMR spectra as a function of temperature for the complex DMPA:cardiotoxin at a lipid-to-protein molar ratio of 15:1. The spectra used in the simulations are represented by dotted lines.

toxin to form an isotropic phase in which the motions are sufficiently fast to completely average the chemical shift anisotropy.

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 on a lamellar phase spectrum (Fig. 2). This suggests that at that temperature, only a fraction of cardiotoxin molecules interact with the lipids, giving rise to an isotropic peak similar to that observed at a lipid-to-protein 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. 2 as a function of temperature. The parameters used in the simulations are given in Table 1 *A*. These results clearly indicate that the width at half-height of the isotropic peak decreases and its intensity increases with increasing temperature. The width at half-height of the isotropic peak is 11 ppm at 25°C and 6 ppm at 65°C, and its relative intensity increases from 46% to 100% between 25 and 55°C. Above 55°C, only the isotropic peak is present, suggesting that all of 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 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.

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 on a nonperturbed lamellar spectrum (Fig. 3). 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 on 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 (CSAs). The comparison between the experimental and simulated spectra is presented in Fig. 3 as a function of temperature, and the parameters used in the

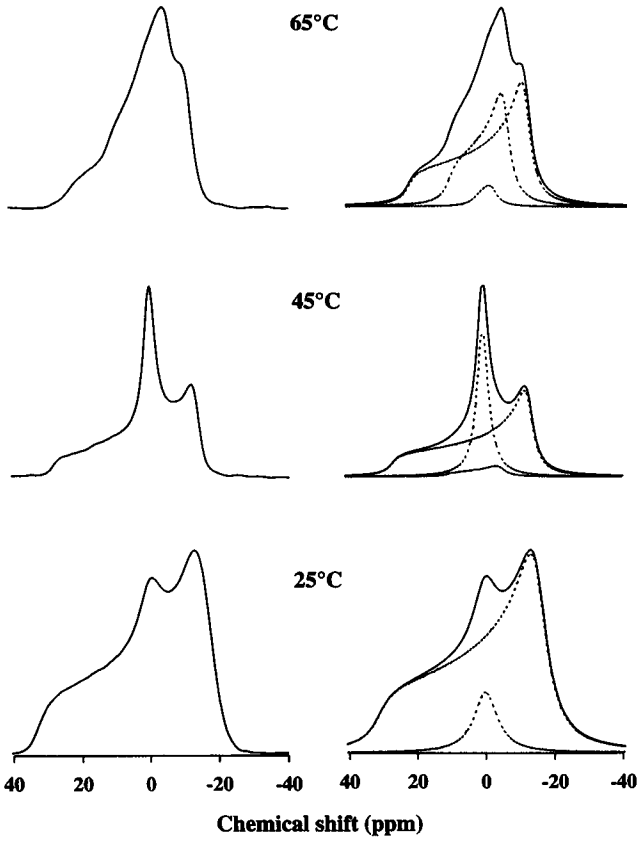

FIGURE 3 Experimental (*left*) and simulated (*right*) ^{31}P NMR spectra as a function of temperature for the complex DMPA:cardiotoxin at a lipid-to-protein molar ratio of 40:1. The spectra used in the simulations are represented by dotted lines.

simulations are given in Table 1 *B*. 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.

Transverse relaxation times

The transverse relaxation time (T_2), like other NMR observables, is sensitive to motions occurring on a specific time scale. The T_2 sensitivity reflects processes with correlation times equal to the inverse chemical shift anisotropy, thus offering a means of studying molecular dynamics in the range of 10^{-7} to 10^{-2} s (Dufourc et al., 1992). Therefore, the study of the transverse relaxation times allows the investigation of a very useful range of correlation times.

The effect of temperature on the transverse relaxation times obtained for DMPA in the absence and presence of cardiotoxin is shown in Fig. 4. All transverse relaxation times measured are characteristic of an organized phase (Dufourc et al., 1992). The important changes in relaxation times between lipid-to-protein molar ratios of 1:0 and 5:1 suggest significant differences in the dynamics of the isotropic and anisotropic phases. These differences become more important at temperatures close to the phase transition temperature. For the pure lipid system, all curves have been fitted with a monoexponential decay. The relaxation time starts to increase at 40°C and reaches a maximum at 45°C. At 50°C, the T_2 is back to its value measured before the phase transition. Such an increase of T_2 at the gel to liquid-crystalline phase transition has also been observed for other phospholipids (Dufourc et al., 1992).

For the complex at a lipid-to-protein molar ratio of 40:1, the T_2 obtained at low temperatures were fitted with a monoexponetial function and are very similar to those obtained for pure DMPA. However, after the phase transition temperature of the pure lipid, the T_2 decay was best fitted with two exponentials. The shortest T_2, presented in Fig. 4, is longer than that observed for pure DMPA. In this system, a second component is also present with a T_2 greater than 10 ms. The exact value of this T_2 could not be calculated

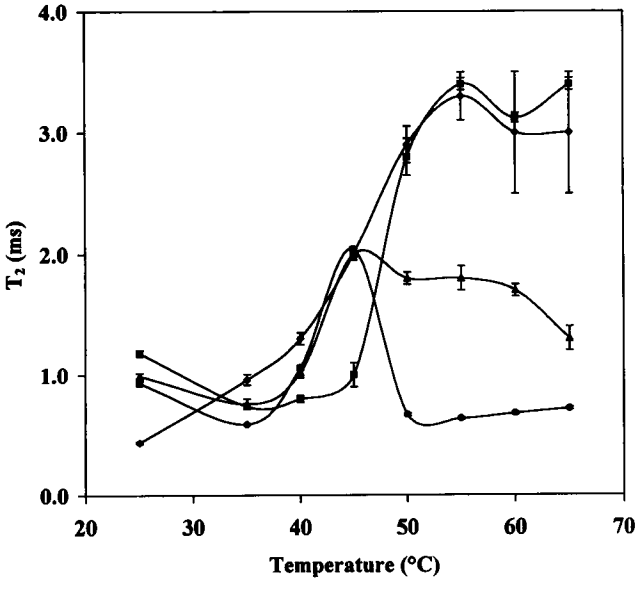

FIGURE 4 ^{31}P NMR transverse relaxation times (T_2) as a function of temperature for different DMPA-cardiotoxin molar ratios. ●, 1:0; ▲, 40:1; ■, 15:1; ◆, 5:1;. The T_2 curves were fitted either with a mono- or biexponential decay. The shortest T_2 obtained from the fits are shown on this figure.

TABLE 1 Parameters used in the spectral simulations

T (°C)	Lamellar phase 1 Proportion‡ (%)	Lamellar phase 1 CSA§ (ppm)	Lamellar phase 2 Proportion (%)	Lamellar phase 2 CSA (ppm)	Isotropic phase Proportion (%)	Isotropic phase WHH¶ (ppm)	Interaction ratio*
A. DMPA-to-cardiotoxin molar ratio of 15:1							
25	54	39	—	—	46	11	7
30	52	39	—	—	48	11	7
35	44	39	—	—	56	8	8
40	31	39	—	—	69	8	10
45	27	39	—	—	73	6	11
50	16	38	—	—	84	6	13
55	—	—	—	—	100	6	15
65	—	—	—	—	100	6	15
B. DMPA-to-cardiotoxin molar ratio of 40:1							
25	89	44	—	—	11	8	5
30	83	44	—	—	17	8	7
35	79	46	—	—	21	6	9
40	61	42	—	—	39	5	16
45	64	38	5	14	31	4	15
50	68	33	30	14	3	7	13
55	66	32	32	14	2	7	14
60	62	32	35	14	3	7	15
65	59	31	37	14	4	7	16

* Calculated number of lipids in interaction with cardiotoxin.
‡ The estimated error on the proportion of each phase is 3%.
§ Chemical shift anisotropy.
¶ Width of half-height.

precisely because of the very small intensities obtained at longer τ values in the T_2 experiments.

For the complex with a lipid-to-protein molar ratio of 15:1, every T_2 curve was fitted with a monoexponential decay. At this lipid-to-protein molar ratio, the temperature dependence of T_2 is characterized by two plateau regions, below and above the phase transition temperature of the pure lipid. At low temperatures, the T_2 are similar to those observed for pure DMPA, whereas at high temperatures, higher T_2 values are obtained.

For the complex with a lipid-to-protein molar ratio of 5:1, the T_2 curves were fitted with a monoexponential decay at temperatures below that of the phase transition of the pure lipid. The T_2 increases gradually between 25°C and 50°C, where a plateau is reached, as was observed for the 15:1 molar ratio. At higher temperatures, the T_2 curves were best fitted with a biexponential decay, and the shortest T_2 shown on Fig. 4 are very similar to those obtained at the 15:1 molar ratio. A second component with a T_2 greater than 10 ms was detected at high temperatures for the lipid-to-protein molar ratio of 5:1.

Hydrophobic extraction

Several structures can give rise to an isotropic peak in ^{31}P NMR spectra. To test the possible formation of a hydrophobic phase, we have performed a hydrophobic extraction based on the method of Batenburg et al. (1985). By this method, an aqueous solution of cardiotoxin and DMPA is mixed with chloroform. Usually, when cardiotoxin is mixed with DMPA in water at a lipid-to-protein molar ratio of 5:1, a precipitate is formed. However, in the presence of chloroform, the solution became clear, indicating that the complex is soluble in this solvent. Analysis by spectrophotometry showed that 80% of the cardiotoxin is transferred from the aqueous solution to the hydrophobic phase. Because Batenburg et al. (1985) have shown that no detectable amount of cardiotoxin is transferred when an aqueous solution of pure cardiotoxin is mixed with an organic phase, the hydrophobic extraction of cardiotoxin in the presence of lipids indicates that the protein is present within a hydrophobic complex.

DISCUSSION

Disappearance of the bilayer structure

The results presented above indicate that cardiotoxin interacts with DMPA bilayers and that at all molar ratios studied there is the apparition of a new isotropic phase. 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 that give rise to a complete averaging of the chemical shift anisotropy in the ^{31}P spectra.

Isotropic spectra in solid-state ^{31}P NMR spectroscopy can be due to the formation of small vesicles, micelles,

cubic phases, or inverted micelles. The formation of small vesicles or hydrophilic micelles is very unlikely, based on the results of the hydrophobic extraction, which suggested the formation of a hydrophobic (inverted) structure. This structure has to be small to explain the isotropic band in the ^{31}P NMR spectra and therefore could not be an inverted hexagonal (H_{II}) phase. The most likely candidate is therefore the formation of a hydrophobic complex similar to an inverted micelle, as it has also been suggested for complexes of cardiotoxin with cardiolipin (Batenburg et al., 1985).

Mixed inverted micelles of DMPA and cardiotoxin

The results obtained strongly suggest the formation of inverted micelles that are most likely formed by DMPA molecules interacting directly with cardiotoxin. This interaction is electrostatic and most likely localized at the charges of the protein. The interaction should therefore result in a neutralization of the charges of cardiotoxin and of the phospholipids interacting with the protein. A hydrophobic complex is therefore formed. Because there are few lipid molecules (5 to 15) associated with each cardiotoxin molecule, the hydrophobic complex has some similarities with an inverted micelle but has an irregular shape.

Inverted micellar structures have been observed in some lipid systems as intermediate phases in cell fusion or phase transition (Siegel, 1984, 1986a,b). They are often present between bilayers (i.e., in a hydrophobic region; Cullis and Hope, 1978), and they are mostly observed in systems that show a tendency to form inverted hexagonal phases. Inverted micelles were originally observed by freeze-fracture microscopy and NMR spectroscopy (de Kruijff et al., 1979; for a review see Verkleij, 1984). Recently, the possibility of stacked inverted micelles of membrane lipids in a cubic phase was suggested (Lindblom and Rilfors, 1989) and confirmed by several groups for systems such as phosphatidylcholine-diacylglycerol-water (Seddon, 1990b; Lindblom and Orädd, 1994; Orädd et al., 1995).

Our results show that at room temperature, the formation of the isotropic phase is complete at a lipid-to-protein molar ratio of 5:1 and incomplete for ratios of 15:1 and 40:1. This suggests that there are about 5 to 10 phospholipids and one cardiotoxin by inverted micelle at that temperature. On the other hand, the spectra obtained at higher temperatures for the molar ratios of 15:1 and 40:1 indicate that there is an increase in the number of phospholipids interacting with cardiotoxin with increasing temperature, with up to 15 phospholipids per cardiotoxin above the phase transition of the pure lipid system.

Because the isotropic structure is formed at every phospholipid-to-cardiotoxin molar ratio, it appears to be more stable than the DMPA bilayers. The hydrophobic complex could be formed by membrane fusion, according to the model of Cullis and Hope (1978), so that the cardiotoxin would not have to penetrate into the lipid bilayers. It is interesting to note that the peaks observed at 65°C for the lipid-to-protein molar ratios of 5:1 and 15:1 are very similar to the isotropic peak characteristic of a cubic phase made of inverted micelles (Seddon, 1990b; Lindblom and Orädd, 1994; Orädd et al., 1995). Therefore, the disappearance of the bilayer structure most likely results in hydrophobic complexes stabilized by hydrophobic interactions.

The T_2 values obtained for the lipid-to-protein molar ratios of 15:1 and 5:1 are characteristic of an organized phase despite the observation of isotropic spectra. This is understandable if the small structures formed upon the interaction of DMPA with cardiotoxin are motionally restricted because of hydrophobic interactions. For the lipid-to-protein molar ratio of 15:1, the T_2 are similar to those observed for pure DMPA at low temperatures, whereas at high temperatures the higher T_2 values reflect the greater motions of the lipids in the hydrophobic complex. For the complex with a lipid-to-protein molar ratio of 5:1, the shortest T_2 shown on Fig. 4 are very similar to those obtained at the 15:1 molar ratio. This suggests that the motions dominating the T_2 relaxation in these systems are the internal motions of the lipid molecules. The second component with a T_2 greater than 10 ms detected at high temperatures can most likely be attributed to the faster rotation of the hydrophobic complex.

Formation of a second anisotropic phase

For the lipid-to-protein molar ratio of 40:1, 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 nonperturbed 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 (Pott et al., 1995) 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. On the other hand, the T_2 decay was best fitted with two exponentials in this system at high temperatures. The shortest T_2 (presented in Fig. 4) is longer than that observed for pure DMPA, which suggests that the lamellar phase lipids are perturbed, most likely because of the incorporation of the hydrophobic complex inside the bilayer.

Literature results explained by an inverted micelle structure

It is interesting to note that the inverted micelle model can satisfactorily explain several observations reported in the literature. Hence, the hydrophobicity of the complex could explain the precipitate observed by several groups (Vincent et al., 1976; Faucon et al., 1979). In addition, the synergetic effect between cardiotoxin and phospholipase A_2 (Louw and Visser, 1978; Gulik-Krzywicki et al., 1981; Rivas et al., 1981; Harvey et al., 1983) could also be explained by the formation of an inverted structure in which the acyl chains are more accessible.

The abolition of the thermotropic phase transition of DMPA in the presence of cardiotoxin has been observed by DSC, fluorescence polarization, and infrared spectroscopy (Faucon et al., 1981, 1983; Désormeaux et al., 1992). In addition, it has been determined by attenuated total reflection FTIR spectroscopy (Désormeaux et al., 1992) that both the lipid and cardiotoxin in the complex are poorly ordered. In addition, the FTIR results indicate that at a lipid-to-protein molar ratio of 5:1, the number of *gauche* conformers in the DMPA acyl chains is the same in both the gel and the liquid-crystalline phases. This large increase of the number of *gauche* conformers and the fact that the complex is poorly ordered are consistent with a micellar structure.

Comparison with other models

Three other models have been suggested in the literature to explain the interactions between cardiotoxin and negatively charged phospholipids. In the model of Lauterwein and Wüthrich (1978), cardiotoxin spans the membrane completely to interact with the phosphate group on the two sides of the bilayer. On the other hand, Dufourcq et al. (1982) have suggested that the initial electrostatic interactions of the basic residues of cardiotoxins with negatively charged phospholipids is followed by the incorporation of at least the N-terminal hydrophobic loop into the bilayers. Finally, Batenburg et al. (1985) have suggested that in the case of the interaction of cardiotoxin with cardiolipin (a doubly charged phospholipid), the protein resides with its polar part in the interior of two inverted micelles and its hydrophobic β-pleated sheet in the acyl chain region.

The two models involving lipid bilayers cannot satisfactorily explain the isotropic peak obtained by ^{31}P NMR spectroscopy or the solubility of the complex in a hydrophobic solvent observed in the present study at low lipid-to-protein molar ratios. In this case, the hydrophobic complex is most likely similar to the model proposed by Batenburg et al. (1985) for the interaction between cardiolipin and cardiotoxin. However, it is important to note that cardiolipin is known to form inverted structures (de Kruijff and Cullis, 1980) in conditions where DMPA does not (Laroche et al., 1988, 1991). For the lipid-to-protein molar ratio of 40:1, the formation of a second lamellar phase at high temperatures suggests that the hydrophobic complex is incorporated into the lipid lamellar phase, in a model similar to that suggested by Lauterwein and Wüthrich (1978).

In conclusion, the strong electrostatic interactions between cardiotoxin IIa extracted from *Naja 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 into the lipid bilayer in the liquid-crystalline phase.

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