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Orientational behavior of phospholipid membranes with mastoparan studied by ^{31}P solid state NMR

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Abstract Solid state ^{31}P NMR spectroscopy was used to study the perturbing effect of the wasp venom peptide mastoparan (MP) on lipid bilayers composed of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG). The ^{31}P chemical shift anisotropy of multilamellar vesicles decreased with increasing peptide concentration, indicating that MP interacts strongly and selectively with the charged DMPG head group. Macroscopically oriented MP-lipid samples between glass plates were studied by ^{31}P NMR as a function of tilt angle. These spectra showed the coexistence of orientation-dependent lamellar signals as well as an isotropic peak, suggesting that MP can induce non-lamellar phases in DMPC/DMPG membranes.

Key words: Mastoparan; Nuclear magnetic resonance; Membrane orientation; Phospholipid; Non-lamellar phase

1. Introduction

Mastoparans (MPs) are toxic peptides with 14 amino acids, which are isolated from wasp venom and exhibit various biological activities on membranes. These effects are related to the interactions of the peptides with the lipid head groups and their ability to penetrate through the bilayer. MP undergoes a conformational change when moving from an aqueous phase into a lipidic environment. Circular dichroism (CD) suggests a largely disordered structure in water, whereas in the presence of phospholipids the spectrum is characteristic of an amphiphilic helix [1–4]. The location of MP in lipid bilayers has been investigated using fluorescence probes [5] and solution state NMR [6]. The fluorescence of both Trp-3 and of an anthryl-label at Ser-14 on MPX-A was found to be quenched in the lipid bilayer by 5-doxylstearic acid, suggesting that the peptide is located at the membrane surface with its helix axis oriented parallel to the bilayer plane [5]. Two-dimensional ^{1}H NMR was used to study MP in an isotropic solution of bilayer mimetic 'bicelles', consisting of dimyristoyl- and dihexanoylphosphatidylcholine. The pronounced periodicity of the sequential amide-^{1}H chemical shifts provided further evidence that the helix axis of this short peptide is aligned parallel, rather than perpendicular, to the membrane surface [6]. However, from these results alone it is not possible to explain several biologically relevant membrane-perturbing effects of MP, such as ion channel formation [7], or its potent stimulation of secretion in diverse mammalian cells, including histamine secretion from mast cells [8–12]. These activities suggest that MP must be able to insert into and penetrate through lipid membranes. Recently, Matsuzaki et al. [13] proposed a pore-forming model to explain the dynamic lipid-MP interactions, based on a dose-response curve of MP X-induced ion permeation. However, to date there is little information about the effect of MP on the morphology of a genuine bilayer system, and little is known about the influence of the lipid environment such as headgroup charge.

In this paper we have analyzed ^{31}P solid state NMR spectra of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) in the presence and absence of MP. The perturbations of the bilayer by the amphiphilic peptide are described, and the induction of a non-lamellar lipid phase by MP will be discussed.

2. Materials and methods

2.1. Materials

The lipids used in this work, DMPC and DMPG, were purchased from Sigma (St. Louis, MO, USA). MP (Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2) was synthesized by the stepwise elongation of Fmoc-amino acids on an Fmoc-NH-SAL resin (4-(2′,4′-dimethoxyphenyl-fluorenyl-aminomethyl)-phenoxy resin). The protecting groups and the resin were cleaved off with TFA in the presence on *m*-cresol and thioanisole at room temperature for 60 min. The crude peptides were purified by HPLC on a YMC-Pack ODS column. The purified peptides were identified by fast atom bombardment-mass spectrometry (FAB-MS). All other chemicals from Wako (Tokyo, Japan) were of special grade.

2.2. Preparation of multilamellar vesicles

Multilamellar vesicles (MLVs) were prepared by dissolving DMPC (or DMPC/DMPG, 7:3) in methanol/chloroform (1:1) and drying under a stream of dry nitrogen. The dry lipid was suspended in 10 mM Tris-HCl, 150 mM aqueous NaCl buffer adjusted at pH 7.0 and homogenized by six cycles of successive heating, cooling and vortexing for 5 min each and freezing at −20°C for 90 min. MP was dissolved in the buffer solution and mixed into the MLVs (peptide/lipid = 0, 1:100, 1:50, 1:20, and 1:10 molar ratio), followed by vortexing below 10°C. Then, the MLVs were incubated at 35°C for 24 h. The water content in the MLV and MLV-MP samples was adjusted at 200% by weight.

2.3. Preparation of oriented samples

MP and DMPC at a 1:10 molar ratio (or MP/DMPC/DMPG at a ratio of 1:7:3) was co-solubilized in methanol/chloroform (1:1). The solution was spread onto 0.1 mm thick glass plates (5×9 mm) and dried under vacuum for 24 h. The dry plates and a small amount of water were then stacked in a sample tube (6.5 mm external diameter×20 mm length). The water was added to achieve 60% by weight hydration. Then, the sample was sealed with Teflon cap and epoxide resin. The sample tubes were incubated at 45°C for 1–2 weeks [14].

2.4. Solid state NMR experiments

Proton-decoupled ^{31}P solid state NMR spectra were recorded for the MLV and the oriented samples on a JEOL EX-400 NMR spec-

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trometer, using a double-resonance $^{31}P/^{1}H$ probe. The ^{31}P resonance frequency was 161.6 MHz, and a recycle delay of 3 s and a 90° pulse width of 6.5 μs was used. All computational work was performed on a Silicon Graphics Indy R4400 workstation.

3. Results and discussion

3.1. Interaction between MP and phospholipid head groups in MLVs

The ^{31}P chemical shift anisotropy (CSA) of MLVs is analyzed in Fig. 1 as a function of the amount of MP. The ^{31}P solid state NMR spectrum of pure DMPC shows a typical axially symmetric powder pattern, with a CSA of approximately $\Delta\sigma$ ($=\sigma_{\parallel}-\sigma_{\perp}$) = 47 ppm. With increasing concentration of MP the intensity at $\sigma_{\parallel}$ is decreased. This effect may be induced by a partial alignment of the molecules in the strong magnetic field, which affects both the lipid as well as peptide. These results are reminiscent to those obtained by Moll and Cross [14] for oriented DMPC bilayers containing gramicidin D. For the mixed MLVs of DMPC/DMPG the ^{31}P CSA decreases significantly with increasing amounts of MP in Fig. 1 (right), and a peak splitting is observed at $\sigma_{\perp}$. We note that the DMPC/DMPG = 7:3 corresponds to the optimum range for MP binding [13], and indeed the strongest effect is observed for this negatively charged lipid mixture at the highest peptide concentration, i.e. in the sample composed of MP/DMPC/DMPG = 1:7:3. The decrease in the ^{31}P CSA indicates that MP exerts a significant conformational and dynamic perturbation on the lipid bilayer. The appearance of two peaks at $\sigma_{\perp}$ (with a shift difference of 4 ppm) suggests that the positively charged peptide interacts preferentially with the negative lipid head groups and thereby induces a local phase separation of the acidic and zwitterionic phospholipids. This explanation is consistent with previous ^{31}P NMR data from multilamellar POPC/POPG vesicles under magic angle spinning (MAS) and from DMPC/DMPG/DHPC bicelles, where a discrete isotropic DMPG signal was observed downfield of an isotropic DMPC signal [15].

Alterations in the ^{31}P CSA may be due to many reasons, such as changes in the order of the lipid molecules, in the amplitude of head group motion, in the head group orientation, or in the electrostatic environment around the phosphorus nucleus. Given that the lipids maintain a lamellar liquid crystalline (Lα) phase, the observed reduction in the ^{31}P chemical shift anisotropy is explained by such perturbations of the axially symmetric lipid motion. In several other lipid systems the occurrence of an isotropic ^{31}P peak in a powder pattern has been reported, as will also be described below [16–19]. In those cases the ^{31}P CSA can be used as a reporter on any phase transitions triggered by the presence of the peptide. Under the present experimental conditions of MLVs in excess hydration, however, we note that no isotropic peak at zero ppm was observed for DMPC or DMPC/DMPG with MP.

3.2. Orientational behavior of DMPC and DMPC/DMPG membranes without MP

The alignment of DMPC as bilayers was characterized by ^{31}P NMR using oriented samples, which are placed with the glass plates at different angles θ relative to the magnetic field

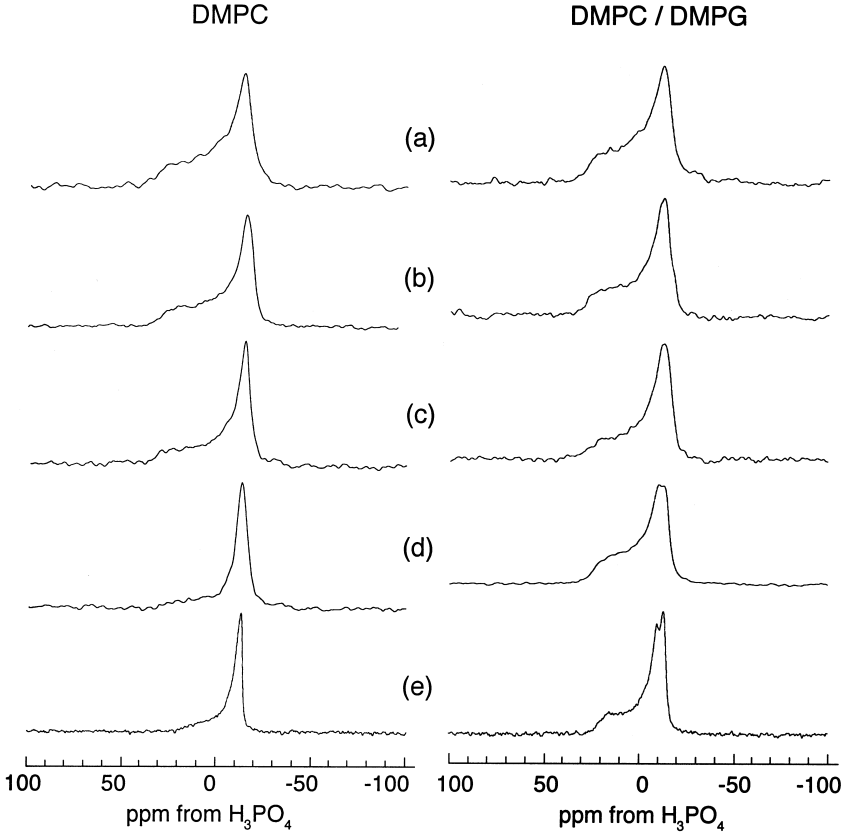

Fig. 1. ^{31}P solid state NMR spectra of DMPC and DMPC/DMPG multilamellar vesicles as a function of the fraction MP after incubation for 24 h at 35°C. Peptide/lipid molar ratio: (a) 0, (b) 1:100, (c) 1:50, (d) 1:20, and (e) 1:10.

B_0. Fig. 2 (left) shows the ^{31}P NMR spectra of an aligned DMPC sample at 35°C, where the single sharp line shifts from $\sigma_{\parallel}$ to $\sigma_{\perp}$ upon changing the angle from $\theta = 0°$ to 90°. A plot of the resonance position versus θ shows the expected angular dependence as a function of $(3\cos^2\theta - 1)$ (Fig. 2 upper). This confirms that the motion of the phosphate group is indeed axially symmetric around the bilayer normal [20]. Only a small fraction at $\sigma_{\perp}$ exhibits no angular dependence, but this is attributed to imperfections in the macroscopically oriented sample.

The alignment of DMPC/DMPG bilayers was also examined (Fig. 2 right), and the peak showed a similar angular dependence as in DMPC. However, different half-height widths of main peak at $\theta = 0°$ were obtained, that is 2.7 ppm for the DMPC bilayer and 4.5 ppm for the DMPC/DMPG bilayer. This observation is consistent with the observed ^{31}P chemical shifts at $\sigma_{\perp}$ of DMPC and DMPG in MLVs (Fig. 1 right), suggesting that the two types of lipid in the DMPC/DMPG mixture differ not only in terms of their isotropic chemical shifts but also in terms of the axially averaged tensor values.

3.3. Perturbation of oriented phospholipid membranes by MP

The angular dependence of ^{31}P NMR spectra was examined

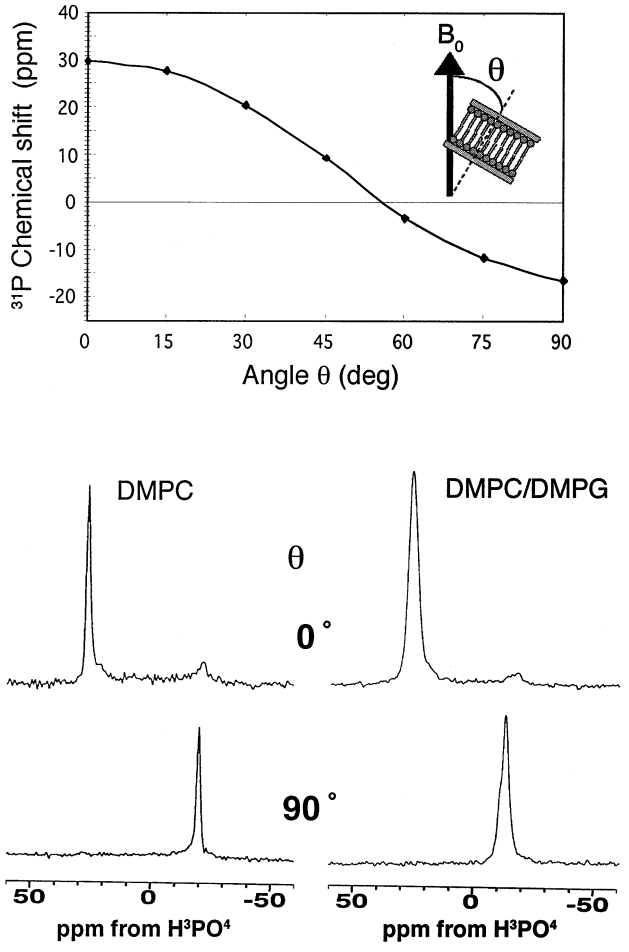

Fig. 2. ^{31}P solid state NMR spectra of a macroscopically oriented DMPC (left) and DMPC/DMPG (right) samples at 35°C as a function of the angle θ between the bilayer normal and magnetic field (lower). Plot of the ^{31}P chemical shift of the oriented peaks as a function of θ (upper).

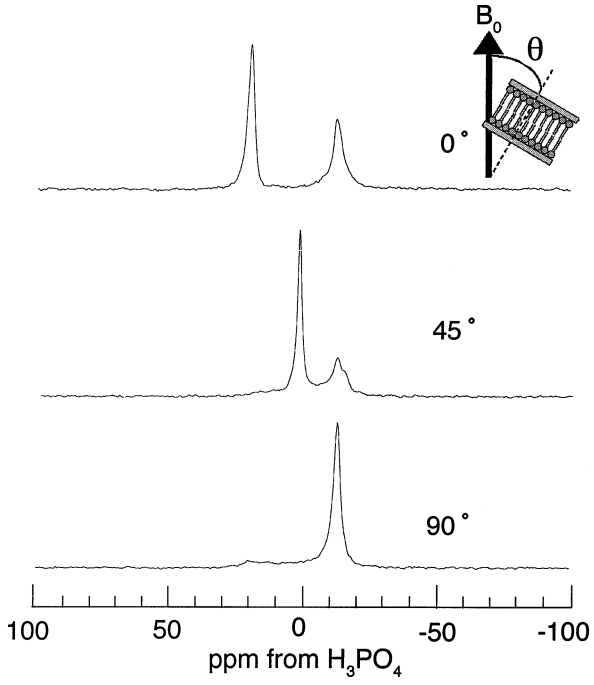

Fig. 3. ^{31}P solid state NMR spectra of a macroscopically oriented DMPC sample with MP at 35°C as a function of the angle θ between the bilayer normal and the magnetic field. DMPC/MP molar ratio was 10:1.

for DMPC with MP in Fig. 3. There are two peaks in the spectrum of the sample that is oriented with its normal parallel to the magnetic field ($\theta = 0°$). The peak at lower field shows an orientation dependence as a function of $(3\cos^2\theta - 1)$, just as it was shown in Fig. 2 (upper). The anisotropy decreased in the presence of MP ($\Delta\sigma = 32$ ppm) in the same way as observed for the MLVs. The second peak at $\sigma_{\perp}$ exhibits no angular dependence and is attributed to non-oriented material in the sample. By analyzing the intensities of the spectrum at $\theta = 0°$, it appears that the lamellar fraction of DMPC has decreased to 60% compared with that of DMPC without MP. A similar loss of orientational quality of phospholipids on glass plates was reported in the presence of other membrane-bound peptides, too [14,21].

The ^{31}P NMR spectrum of oriented DMPC/DMPG showed a dramatic change upon the addition of MP (Fig. 4). Now there are three peaks in the spectrum when the sample is aligned with its normal parallel to the magnetic field ($\theta = 0°$). Since the two peaks at lower field exhibit an angular dependence as a function of $(3\cos^2\theta - 1)$, just as it was seen in Fig. 2 (upper), they must correspond to the headgroups of DMPC and DMPG in a lamellar lipid arrangement. Most likely they reflect the two distinct lipid phases, which had been previously identified in the MLV samples, one of which is enriched in DMPG and the other one consisting of the remaining DMPC. The third peak at zero ppm, on the other hand, exhibits no angular dependence and therefore represents an isotropic fraction. Also, we note that there appears to be hardly any contribution from non-oriented lipids at $\sigma_{\perp}$. The stability of all orientational states of the phospholipids was confirmed by measuring duplicate samples and by repeating the experiments on the same sample with approximately 2 weeks delay to avoid non-equilibrium effects. Our observations therefore suggest that MP is able to induce a phase

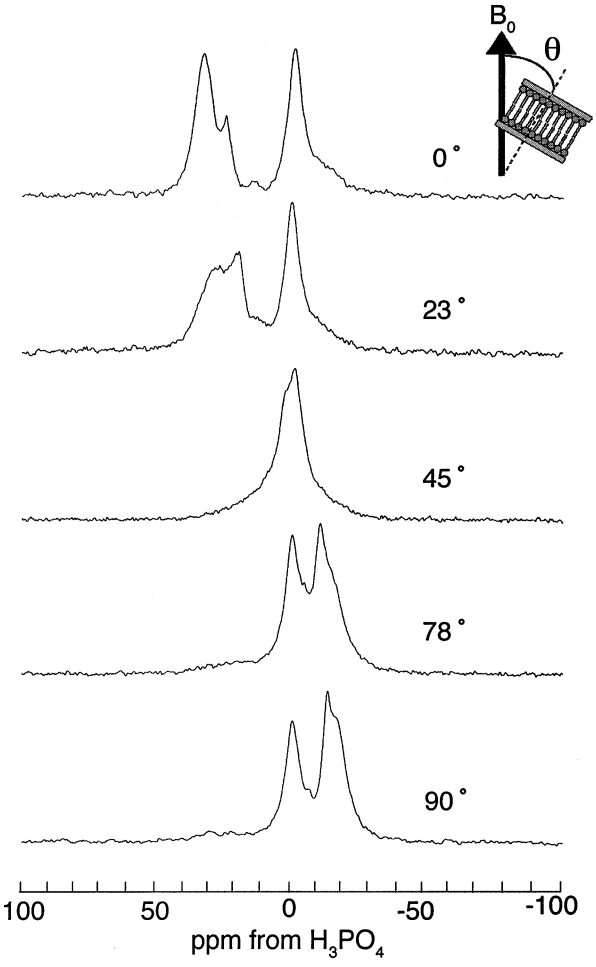

Fig. 4. ^{31}P solid state NMR spectra of a macroscopically oriented DMPC/DMPG (7/3) sample with MP at 35°C as a function of angle θ of bilayer normal with respect to magnetic field. Lipids/MP molar ratio was 10:1.

transition from a lamellar to a non-lamellar state for a significant fraction of the lipids.

The appearance of a non-lamellar phase was detected by ^{31}P NMR also for melittin in bovine heart cardiolipin membranes [22], for the gp32 glycoprotein of SIV [17], for alamethicin in dielaidoylphosphatidylethanolamine [23], and for the cyclic peptide gramicidin S in dimyristoylphosphatidylethanolamine [19]. In addition, the effects of temperature, hydration, and peptide concentration have been shown to affect the phase behavior of lipids [18,24]. Two major types of non-lamellar phases known for phospholipids, namely the inverted hexagonal (H_{II}) and cubic (I_2) phases [25]. Cubic phases give rise to an isotropic component in the ^{31}P NMR spectrum. There are also several other possible structures that may give rise to narrow ^{31}P NMR peaks, e.g. small vesicles, micelles, or inverted micelles. In our case (Fig. 4), the ^{31}P NMR spectra were obtained with aligned bilayers between glass plates, similar to a previous ^{31}P NMR study of oriented lipids [26]. In that study, distinct Lα, H_{II}, and cubic (I_2) resonances were assigned in the ^{31}P NMR spectra of DOPE/DOPC (7:3) and of DOPC/monoolein (MO) (3:7). Very slow transitions (seconds timescale) between Lα and H_{II} and between Lα and I_2 were observed by two-dimensional exchange NMR. In addition, in mixed lipid systems it was shown that the isotropic component involves all types of lipid. A comparison of these previous and our present results suggests that indeed three kinds of lipid populations may be classified. That is, we observed a lamellar morphology for the DMPC-enriched phase (low field peak) and for the DMPG-enriched phase (middle peak), as well as one cubic phase (isotropic resonance) consisting of both DMPC and DMPG. The peak intensities of the two lamellar populations agree with the ca. 7:3 composition of the mixed lipids. The basic MP interacts preferentially with DMPG, as the orientational averaged tilt angle of the DMPG headgroup is more strongly affected than that of DMPC. It should be noted that non-lamellar phases of DMPC/DMPG were observed in the presence of MP only under the experimental conditions of the oriented samples, but not in the case of MLVs in excess hydration.

Recently, the MP-DMPC/DMPG interaction has been interpreted in terms of a pore-forming model, based on the dose-response curve of MP X-induced ion permeation [13]. The peptide induces a transbilayer movement of both negatively charged and zwitterionic lipid probes. The flip-flop of the negatively charged probe on a timescale of minutes was slightly faster than that of the zwitterionic probe. This suggests a 'pore-mediated flip-flop' in which the lipids are involved in the pore structure together with the amphiphilic peptide, by lining the pore with their head groups. This situation brings the outer and inner monolayers into a continuum, allowing the lipid molecules to diffuse freely under slow exchange within NMR timescale. If the pore-mediated continuum across the lipid bilayer is induced by the amphiphilic peptide, the ^{31}P NMR spectra would be expected to show an isotropic component. In addition, this isotropic component will consist of contributions from both the negatively charged and the zwitterionic lipid. Thus our results are in good agreement with this pore-mediated flip-flop model. Likewise, we have observed that the ^{2}H quadrupolar splittings of DMPC-d_4 (α- and β-methylene deuterons at headgroup) are strongly narrowed in the presence of MP (Y. Hori, in preparation), which also supports the presence of peptide-induced pores in the oriented sample of DMPC/DMPG with MP. A ^{15}N solid state NMR analysis [27] of ^{15}N labeled MP in phospholipid membranes is currently in progress to characterize the peptide orientation in the phospholipid bilayer.

4. Conclusion

The effect of MP on the behavior of phospholipid bilayers of DMPC and DMPG was investigated using ^{31}P solid state NMR of MLVs and of oriented samples. A local phase separation of DMPC/DMPG MLVs is induced by MP, suggesting a stronger interaction of the cationic MP with acidic head groups than with the zwitterionic lipids. The mixed bilayer of DMPC and DMPG was converted by MP into two distinct lamellar populations and a non-lamellar state. The isotropic ^{31}P resonance is interpreted to reflect the formation of pores in the lipid bilayer, induced by the amphiphilic peptide helix.

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