Solid-State Nuclear Magnetic Resonance Relaxation Studies of the Interaction Mechanism of Antimicrobial Peptides with Phospholipid Bilayer Membranes†

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Received April 20, 2005; Revised Manuscript Received May 26, 2005

ABSTRACT: An 18-residue peptide, KWGAKIKIGAKIKIGAKI-NH_2 was designed to form amphiphilic β-sheet structures when bound to lipid bilayers. The peptide possesses high antimicrobial activity when compared to naturally occurring linear antimicrobial peptides, most of which adopt an amphipathic α-helical conformation upon binding to the lipids. The perturbation of the bilayer by the peptide was studied by static ^{31}P and ^{2}H solid-state NMR spectroscopy using POPC and POPG/POPC (3/1) bilayer membranes with *sn*-1 chain perdeuterated POPC and POPG as the isotopic labels. ^{31}P NMR powder spectra exhibited two components for POPG/POPC bilayers upon addition of the peptide but only a slight change in the line shape for POPC bilayers, indicating that the peptide selectively disrupted the membrane structure consisting of POPG lipids. ^{2}H NMR powder spectra indicated a reduction in the lipid chain order for POPC bilayers and no significant change in the ordering for POPG/POPC bilayers upon association of the peptide with the bilayers, suggesting that the peptide acts as a surface peptide in POPG/POPC bilayers. Relaxation rates are more sensitive to the motions of the membranes over a large range of time scales. Longer ^{31}P longitudinal relaxation times for both POPG and POPC in the presence of the peptide indicated a direct interaction between the peptide and the POPG/POPC bilayer membranes. ^{31}P longitudinal relaxation studies also suggested that the peptide prefers to interact with the POPG phospholipids. However, inversion−recovery ^{2}H NMR spectroscopic experiments demonstrated a change in the relaxation rate of the lipid acyl chains for both the POPC membranes and the POPG/POPC membranes upon interaction with the peptide. Transverse relaxation studies indicated an increase in the spectral density of the collective membrane motion caused by the interaction between the peptide and the POPG/POPC membrane. The experimental results demonstrate significant dynamic changes in the membrane in the presence of the antimicrobial peptide and support a carpet mechanism for the disruption of the membranes by the antimicrobial peptide.

A large number of antimicrobial peptides have been studied recently because of their potential to serve as medical alternatives to antibiotics (*1*, *2*). These defense peptides usually possess a net positive charge and are able to form an amphipathic structure when bound to phospholipid bilayers. Many of these antimicrobial peptides, such as PGLa (*3*, *4*) and magainins (*5*, *6*), adopt an amphipathic α-helical conformation when interacting with the membrane. Conversely, some antimicrobial peptides form an amphipathic β-sheet structure because of their intrinsic intramolecular disulfide bonds (*6*). In this paper, a novel linear amphiphilic β-sheet cationic antimicrobial peptide KIGAKI was used. This peptide was designed to have a high hydrophobic moment value (0.63) for a β-sheet structure and low hydrophobic moment value (0) for an α-helical structure (*7*). The absence of cysteine residues precludes the formation of either inter- or intramolecular disulfide bonds to stabilize the secondary structure. In a previous paper, CD and FTIR spectroscopy results showed that this peptide does indeed adopt a β-sheet conformation when bound to anionic POPG[1] lipids and forms a random structure in association with pure POPC bilayers (*7*). One important factor in determining the potential application of these antimicrobial peptides is the ability of the peptides to disrupt prokaryotic membranes at concentrations that are not harmful to host membranes. The antimicrobial and hemolytic activity of KIGAKI showed higher antimicrobial activity than PGLa and magainins and an increased selectivity between bacterial and mammalian cells (*7*, *8*).

† This work was supported by an American Heart Association Scientist Development Grant 0130396N and a National Institutes of Health Grant GM60259-01 to G.A.L. and AI047165-02 to J.B. The 500 MHz wide-bore NMR spectrometer was obtained from a National Science Foundation Grant 10116333.

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[1] Abbreviations: KIGAKI, KWGAKIKIGAKIKIGAKI-NH_2; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-glycero-3-phospholcholine; POPG, 1-palmitoyl-2-oleoyl-3-*sn*-glycero-3-[phospho-rac-1-glycerol]; POPE 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPC-d_{31}, 1-palmitoyl-d_{31}-2-oleoyl-3-*sn*-glycero-3-phospholcholine; POPG-d_{31}, 1-palmitoyl-d_{31}-2-oleoyl-3-*sn*-glycero-3-[phospho-rac-1-glycerol]; HEPES, *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid]; TFE, 2,2,2, trifluoroethanol; EDTA, ethylenediamine tetraacetic acid; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; HPLC, high-performance liquid chromatography; CP, cross-polarization; MAS, magic angle spinning; MLVs, multilamellar vesicles; LUVs, large unilamellar vesicles.

10.1021/bi050730p CCC: $30.25
Published on Web 07/07/2005

While these functional properties appear promising, no studies on KIGAKI have been reported to address the origin of the selectivity at the molecular level. To obtain a better understanding of the mechanism of the antimicrobial activity and selectivity of this peptide, solid-state NMR longitudinal (T_1) and transverse relaxation (T_2) experiments are reported here that examine the effects of KIGAKI on membrane structure and lipid mobility.

Recently, solid-state NMR spectroscopy has been widely applied to studying short membrane active peptides, such as human LL-37, HIV intracellular domain gp41, magainin, protegrin-1, and colicin Ia (*3*−*6*, *9*−*15*), using uniformly labeled or site-specifically labeled peptides while details of the dynamic and conformational information were obtained. Little useful information about the dynamics of the phospholipid bilayer membranes could be determined from these results. Static ^{31}P and ^{2}H solid-state NMR spectroscopy are the most common techniques used in studying the perturbation of the phospholipid bilayers by peptides. In fact, the bilayer membranes possess a variety of motions with a hierarchy of correlation times, including the fast *trans-gauche* isomerization of acyl chains (10^{-10}−10^{-9} s), the long axis rotation of the lipids (10^{-9}−10^{-8} s), the lipid lateral diffusion (10^{-8}−10^{-7} s), and the whole membrane slow-order director fluctuations whose correlation times depend on membrane size and thickness (*16*). Depending upon the nature of the interaction, the peptide can modify these motions in different time scales (*17*). It is very important to choose the appropriate technique to detect these motions. One type of interaction may appear fast (averaged) and featureless when viewed by one technique, but slow (discrete) with the other techniques. Solid-state NMR spectroscopy is a powerful method that can determine molecular motions over a wide range of time scales (*16*). The averaging deuterium quadrupolar interaction can detect molecular motions of ∼10−100 kHz, and the averaging ^{31}P chemical shift anisotropy is effective for probing rates of ∼4−6 kHz (*16*). Moreover, NMR relaxation time measurements are more appropriate to probe the dynamics of the phospholipid bilayers (*18*). The longitudinal relaxation time (T_1) is sensitive to the lipid molecular motions in the nanosecond-to-microsecond time scale, which includes rapid conformational changes of lipid acyl chains and polar headgroups and long axis rotations and diffusion of the lipids (fast molecular motion) (*19*−*21*). The transverse relaxation time (T_2) is sensitive to motions in the millisecond range, which is effective for measuring collective lipid motions in the bilayer (slow molecular motion) (*22*−*24*).

Two biologically relevant membrane systems were studied here. Multilamellar vesicles (MLVs) containing either POPC or a mixture of POPG and POPC at 3/1 molar ratio. POPC is a neutral zwitterionic phospholipid that is abundant in mammalian cell membranes. POPG is an anionic lipid that is commonly found in bacterial plasma membranes. POPE is also an important lipid in bacterial plasma membranes that regulate the lytic activity of KIGAKI (*7*). However, in this paper, only POPC and POPG lipids were studied and compared to elucidate the role that anionic lipids play upon association of KIGAKI with the membrane. The function of POPE lipids as well as the interaction between the membranes and similar antimicrobial peptides such as $(KIAGKIA)_3$ will be investigated in the future (*7*).

In this study, ^{2}H-labeled POPC and POPG acyl chains were used to localize the influence of the peptide on each lipid class in the bilayer. The static powder spectra of ^{31}P from the lipid headgroups and ^{2}H from the acyl chains showed significant difference between POPC bilayers and POPG/POPC bilayers, indicating that KIGAKI interacts with these membrane systems in a selective manner. We propose that this short cationic peptide aggregates into β-sheet structures and disrupts the membrane through an electrostatic interaction at the surface of the bilayers when it binds to the membrane containing anionic lipids, such as POPG. The relaxation studies carried out in this work reveal unique dynamic information over a large time scale range that further supports this mechanism. A significant change occurs in the ^{31}P T_1 and T_2 relaxation times at the surface of the bilayers as well as the ^{2}H relaxation times in the hydrophobic interior of the bilayers upon addition of the KIGAKI peptide. A complete picture of the membrane dynamics in the presence of the antimicrobial peptide is discussed.

MATERIAL AND METHODS

Material. POPC, POPG, POPC-d_{31}, and POPG-d_{31} were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. All phospholipids were dissolved in chloroform and stored at −20 °C prior to use. Deuterium-depleted water was obtained from Isotec (Miamisburg, OH). HEPES, TFE, and EDTA were obtained from Sigma/Aldrich (St. Louis, MO). The antimicrobial peptide KIGAKI was synthesized using Fmoc chemistry by ResGen (Huntsville, AL). The crude peptides were purified by reverse-phase HPLC. The purity of the peptide (>95%) was checked by reverse-phase HPLC and electrospray mass spectrometry.

NMR Sample Preparation. MLV samples containing either POPC MLV or POPG/POPC (3/1) MLV were prepared according to the following procedure (25). The phospholipid mixture (0.1 mmol) was first dried under a steady stream of N_2 gas for ∼30 min to remove the organic solvent. The deuterated lipids represented 15 mol % of the total phospholipids. The sample was then left under a high-vacuum desiccator overnight. MLVs were formed by resuspension of the dry lipids in 190 μL HEPES buffer (5 mM EDTA, 20 mM NaCl, and 30 mM HEPES, pH 7.0) with frequent vortexing to homogenize the sample. Samples were then allowed to sit in a warm water bath (50 °C) for 20 min. The peptides were dissolved in 100 μL TFE and sonicated for 10 min. Bilayer samples with peptides were prepared by mixing the peptides in TFE solvent with phospholipids in chloroform followed with the same drying procedure described above. Next, the samples were hydrated using 190 μL HEPES buffer (5 mM EDTA, 20 mM NaCl, and 30 mM HEPES, pH 7.0). After vortexing, the samples were put in a water bath (50 °C) for 20 min.

NMR Spectroscopy. All experiments were recorded on a Bruker Avance 500 MHz WB solid-state NMR spectrometer using a 4 mm triple resonance CPMAS probe. ^{31}P static NMR spectra were acquired at 202.4 MHz using a spin−echo pulse sequence with proton decoupling. ^{2}H static NMR experiments were performed at 76.77 MHz using a quadrupolar echo pulse sequence. The 90° pulses used for ^{31}P and ^{2}H were 4.0 and 3.0 μs, respectively.

^{31}P T_{1z} longitudinal relaxation experiments were conducted using an inversion−recovery pulse sequence 180°-*T*-90°-acq.

The delay time (T) was varied from 10.0 ms to 6.0 s. ^{31}P T_2 transverse relaxation experiments were studied using a spinecho sequence 90°-τ-180°-τ-acq. The innerpulse delay time (τ) was varied over a range from 243.7 μs to 99.994 ms. The ^{31}P solid-state NMR relaxation study was conducted when the sample was spun at 4 kHz at the magic angle. For the ^{31}P transverse relaxation experiments, rotor synchronization was used (*26*, *27*). ^{2}H T_{1z} longitudinal relaxation experiments were recorded using the inversion−recovery quadrupolar echo sequence 180°-T-90°-τ-90°-τ-acq. The delay time (T) was varied from 2.0 ms to 1.5 s. ^{2}H T_2 transverse relaxation experiments were carried out using a standard quadrupolar echo pulse sequence: 90°-τ-90°-τ-acq. The innerpulse delay time (τ) was varied between 10.0 μs to 6.0 ms (*28*). The ^{31}P and ^{2}H spin−lattice relaxation rates of membrane bilayers are anisotropic and depend on the angle between the bilayer normal and the magnetic field (*29*). In this study, unoriented MLVs were used in combination with MAS. Thus, the rapid diffusion of the lipid molecules over the curved surface of the MLVs and MAS will average out this orientation dependence (*29*).

NMR Data Analysis. Powder-type ^{2}H NMR spectra of multilamellar dispersions of POPC-d_{31} or POPG-d_{31} were deconvoluted (dePaked) using the algorithm of McCabe and Wassall (*30*). The spectra were deconvoluted such that the bilayer normal was parallel with respect to the direction of the static magnetic field. The ^{2}H order parameters S_{CD} were calculated according to the equation: $S^i_{CD} = \Delta\nu_i/(3/2(e^2qQ/h))$, where $\Delta\nu_i$ is the quadrupolar splitting for a deuteron attached to the ith carbon of the POPC or POPG acyl chain, and e^2qQ/h is the quadrupole coupling constant (168 kHz for deuteron in C−D bonds) (*31*, *32*). The order parameters of the methyl groups at the end of the acyl chain were 3 times of the calculated S_{CD} (*31*). Powder-type ^{31}P NMR spectra of POPG/POPC MLVs associated with 4 mol % peptide with respect to the total lipids concentration were fitted using two components with the DMFIT program (*33*).

For the ^{31}P and ^{2}H longitudinal relaxation study, the change in the area under a particular peak was fitted to a single-exponential function: $I(t) = I(0) - A\exp(-t/T_1)$, where $I(0)$ is close to 1 and A is about 2 (*28*). The standard deviation of each ^{31}P T_1 value is within ±0.005 ms. The ^{2}H longitudinal relaxation rate $R_{1z} = 1/T_1$. The standard deviation of each R_{1z} value is within ±0.02 s^{-1}. A double exponential decay $I(t) = A_s\exp(-2t/T_{2S}) + A_f\exp(-2t/T_{2F})$ was used to fit the ^{31}P transverse relaxation data. The standard deviation of the T_{2S} value (the slow component with longer ^{31}P transverse relaxation time) is within ±0.5 ms, whereas the standard deviation of the T_{2F} value (the fast component with shorter ^{31}P transverse relaxation time) is within ±0.1 ms. For the ^{2}H transverse relaxation study, the decay of the peak area was fitted to the equation: $I(t) = A\exp(-2t/T_2)$, where A is about 1 (*28*). The standard deviation of each T_2 value is within ±0.1 μs. The fitting was performed using Igor Pro Carbon version 4.05A (WaveMetrics Inc.) on a G5 Mac computer.

RESULTS

The effect of the antimicrobial peptide KIGAKI on the dynamic properties of phospholipid bilayers was examined using ^{31}P and ^{2}H NMR longitudinal and transverse relaxation studies. Three MLV samples consisting of POPC/POPC-d_{31}, POPG/POPC/POPC-d_{31}, and POPG/POPC/POPG-d_{31} were studied with and without peptide. First, the static ^{31}P and ^{2}H NMR powder spectra were compared for the POPC and POPG/POPC (3/1) MLVs. Then, the ^{31}P and ^{2}H NMR longitudinal relaxation times were measured. ^{31}P and ^{2}H NMR transverse relaxation were then performed only for POPG/POPC (3/1) MLVs.

^{31}P and ^{2}H Solid-State NMR Spectroscopy of POPC and POPG/POPC MLVs Interacting with KIGAKI. Static ^{31}P solid-state NMR spectra were collected for POPC lipid dispersions with different peptide concentrations (from 0 to 8 mol % with respect to the total lipid concentration) as a function of temperature (spectra not shown). The ^{31}P NMR spectra indicate the formation of MLVs in the L_α phase. The ^{31}P NMR spectrum for POPC MLVs at 25 °C revealed an axial symmetric line shape with a CSA of approximately 50 ppm (Figure 1A). The spectral line shape retained axial symmetry with no change in the CSA in the presence of 4 and 8 mol % KIGAKI. The ^{31}P NMR powder spectra with 4 mol % KIGAKI were easily simulated with one spectral component. The phosphocholine headgroup has a large dipole moment and behaves like a sensitive "molecular electrometer" (*34*). An increase in the surface positive electric charge should change both the orientation of the $^-$P−N$^+$ dipole and the corresponding ^{31}P CSA. The lack of a significant CSA change suggests little or no electrostatic interaction between the cationic peptide and the POPC headgroups. The intensity ratio between the $\sigma_{||}$ edge and the $\sigma_\perp$ edge, however, changes upon addition of the peptides. The slight change in the line shape indicates that the peptide perturbs the fast motion of the lipid headgroup (*35*, *36*).

The static ^{31}P NMR spectra of POPG/POPC MLVs are shown in Figure 1B,C. The single CSA of ∼40 ppm observed for POPG/POPC MLVs is much smaller than the CSA of pure POPC MLVs (∼50 ppm). ^{31}P CSA values of ∼37 ppm for pure POPG bilayers (*37*) and ∼48 ppm for pure POPC bilayers (*34*) have been reported. The single CSA value for POPG/POPC MLVs supports a direct interaction between the headgroups of POPC and POPG and no lateral phase separation of the lipids in this binary lipid mixture over the NMR time scale. However, the powder pattern spectra of mixed POPG/POPC MLVs at 4 mol % peptide concentration were able to be deconvoluted into two different components using the DMFIT simulation program. The isotropic component contributes about 28% to the whole peak area, and the anisotropic component with a CSA of approximate 40 ppm contributes about 72% (Figure 1D). The two components correspond to two different phospholipid species. These results clearly indicate that the antimicrobial peptide selectively perturbs and alters the POPG membranes.

Solid-state ^{2}H NMR spectra were obtained for POPC and POPG/POPC phospholipid bilayers with peptide concentrations of 0 and 4 mol % with respect to the phospholipids. The ^{2}H NMR spectra for POPC MLVs with peptide concentrations of 0 and 4 mol % at 30 °C are shown in Figure 2A. The ^{2}H NMR spectra for POPG/POPC MLVs with either POPC-d_{31} or POPG-d_{31} as the deuterium labels at 25 °C are shown in Figure 2, panels B and C, respectively. The resolution decreased significantly at a peptide concentration of 4 mol %. The phospholipid molecule in the bilayer is characterized by an axial-symmetric motion with fast rotation

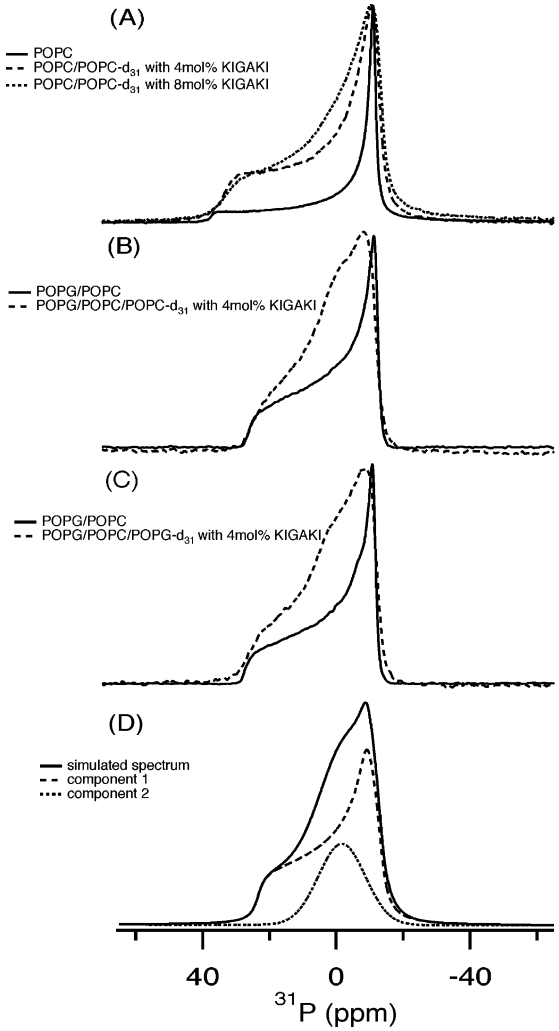

FIGURE 1: (A) ^{31}P NMR powder spectra of POPC MLVs at 25 °C. The *solid line* represents the spectrum of the control POPC MLVs. The dash line represents the spectrum of the POPC MLVs with 4 mol % antimicrobial peptide. The dotted line represents the spectrum of the POPC MLVs with 8 mol % antimicrobial peptide. (B and C) ^{31}P NMR powder spectra of POPG/POPC/POPC-d_{31} (B) MLVs and POPG/POPC/POPG-d_{31} (C) MLVs at 25 °C. The solid line represents the spectra of the control sample, while the dash line represents the spectra of MLVs with 4 mol % peptide. (D) The best fitting of ^{31}P NMR spectrum of POPG/POPC/POPC-d_{31} MLVs at 25 °C using the DMFIT program (*33*). Two spectral components are used to simulate the spectrum. One anisotropic component (dash line) and one isotropic component (dotted line), contributing approximately 72% and 28%, respectively, to the total peak area. The sum of the two components is shown in solid line.

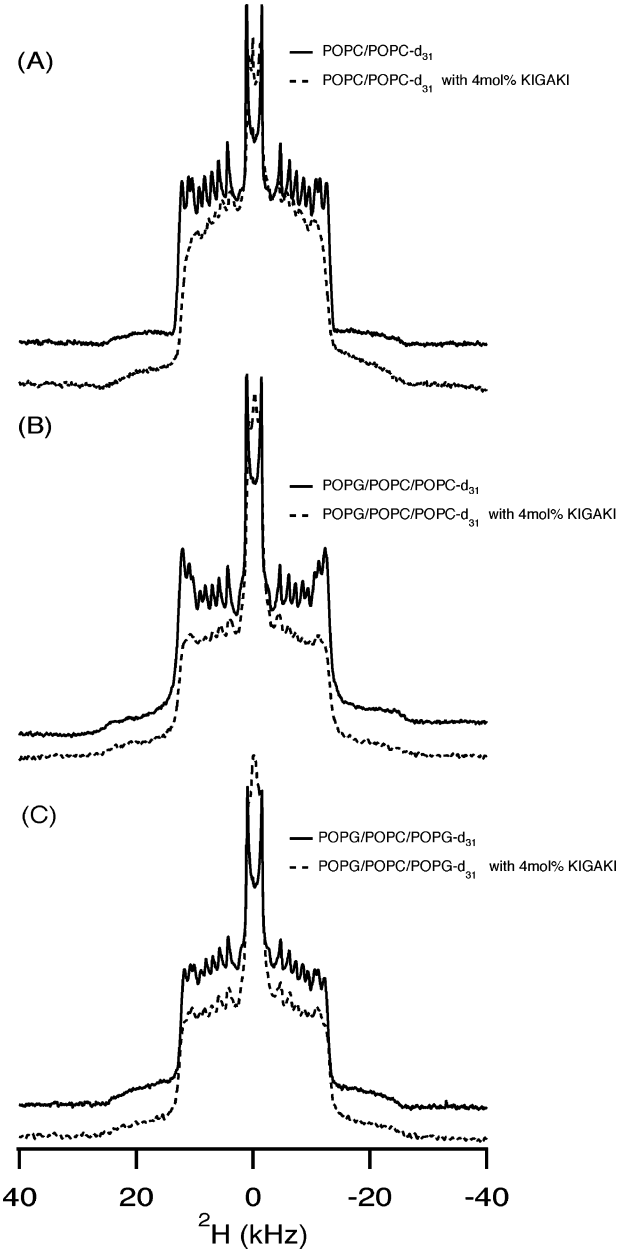

FIGURE 2: ^{2}H NMR powder spectra of POPC MLVs with and without the antimicrobial peptide, using POPC-d_{31} as the isotopic label at 30 °C (A). ^{2}H NMR powder spectra of POPG/POPC MLVs with and without the antimicrobial peptide, using POPC-d_{31} as the isotopic label (B) or using POPG-d_{31} as the isotopic label (C) at 25 °C. The solid line represents the spectra of the control sample, while the dash line represents the spectra of the MLVs with 4 mol % peptide. The ^{2}H quadrupolar splittings decrease down the acyl chain toward the center of the bilayer, with the smallest ^{2}H quadrupolar splitting corresponding to the methyl groups (CD_3) at the end of the lipid acyl chain (*47*, *48*).

around the long molecular axis and a slower reorientation rate perpendicular to the axis (*38*). The fast rotation gives a sharp peak at the $\sigma_{\perp}$ edge. The decrease in resolution indicates that interactions with the peptide have changed the motion of the lipids. An isotropic peak also appears at the higher peptide concentration, indicative of an isotropic reorientation, which is in slow exchange with the remaining lipids in the bilayers on the solid-state deuterium NMR time scale ($\sim 10^{-6}$ s) (*16*). The results clearly show that the antimicrobial peptide perturbs the membranes for all of the samples.

The order parameters for each individual C$-^{2}$H were calculated after dePakeing the ^{2}H NMR spectra. The order parameter profiles for ^{2}H at different carbon positions along the *sn*-1 POPC or *sn*-1 POPG phospholipids acyl chain are shown in Figures 3 for each MLV sample. The degree of ordering slightly decreased along the whole acyl chains for POPC MLVs in the presence of KIGAKI. Conversely, for the mixed POPG/POPC MLVs, the degree of ordering of each C$-^{2}$H did not change in the presence of the peptide using either POPC or POPG as the deuterium label (Figure 3B,C). The difference in the order parameter changes between Figure 3A and Figure 3B,C indicates that the mechanism by which the lipids and peptides interact is slightly different for the POPC MLVs and the POPG/POPC MLVs.

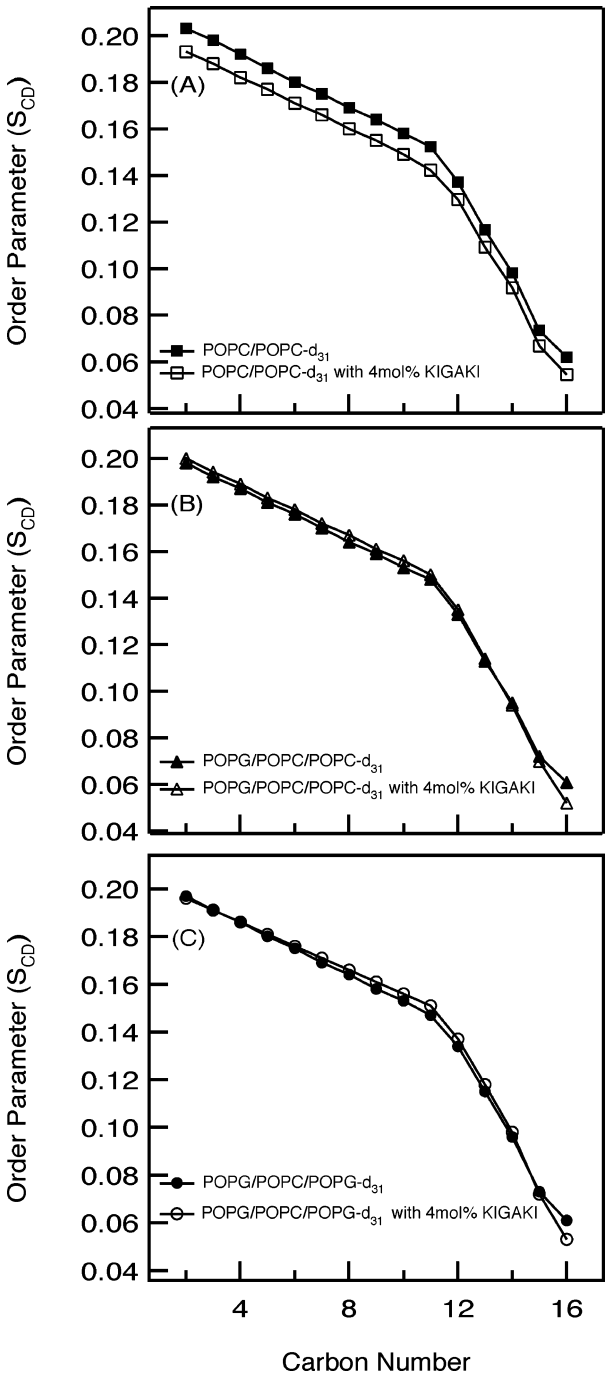

FIGURE 3: Molecular order parameter profiles with respect to the carbon positions along the acyl chain of deuterium labeled phospholipids with and without 4 mol % peptide, calculated from the dePaked ^{2}H NMR powder spectra. (A) POPC/POPC-d_{31} MLVs at 30 °C, (B) POPG/POPC/POPC-d_{31} MLVs at 25 °C, (C) POPG/POPC/POPG-d_{31} MLVs 25 °C. The solid symbols represent the data from control MLVs. The open symbols represent the MLVs with 4 mol % peptide.

^{31}P and ^{2}H Longitudinal Relaxation Study. ^{31}P longitudinal relaxation solid-state NMR studies were carried out for both POPC and POPG/POPC phospholipid bilayers with and without KIGAKI. Magic angle spinning was employed for this section of the relaxation study to give high-resolution solid-state NMR spectra. The isotropic peak position of POPC does not change upon addition of the peptide into pure POPC bilayers. For POPG/POPC mixed bilayers, POPG and POPC exhibit ^{31}P isotropic peaks at 0.68 and −0.41 ppm,

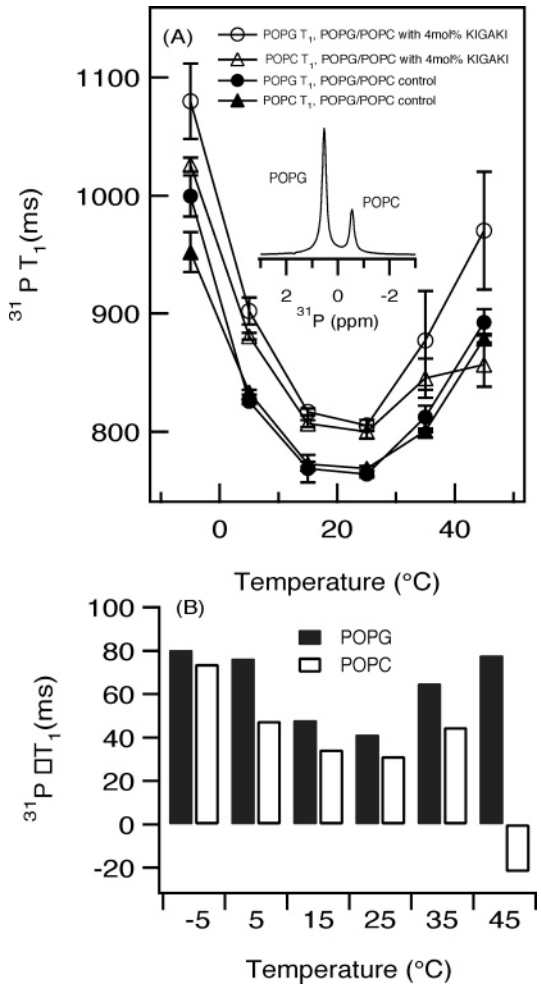

FIGURE 4: (A) ^{31}P longitudinal relaxation times (T_1) as a function of temperature for POPG/POPC MLVs. The solid symbols represent the control MLVs. The open symbols represent the samples with 4 mol % peptide. The circles represent POPG phospholipids. The triangle represents POPC phospholipids. The error bar was obtained by averaging ^{31}P T_1 values obtained from three samples with the same sample components. The inset is the NMR spectrum of control POPG/POPC bilayers spun at 4 K at the magic angle. (B) The ^{31}P T_1 values difference (ΔT_1) was calculated by subtracting the control T_1 value from the T_1 value of MLVs in the presence of KIGAKI for both POPG (black bars) and POPC (white bars) phospholipids at variable temperatures.

respectively (see inset in Figure 4). In the presence of KIGAKI, both ^{31}P peaks position shift upfield by about 0.05 ppm with a slight line broadening (spectrum not shown). The ^{31}P chemical shift of the lipids headgroup is sensitive to the surface charge density (*39*). The binding of the cationic peptides to the anionic bilayers will shift the ^{31}P peak of the phospholipids headgroup upfield slightly (*39*). These results demonstrate that the peptide interacts with the POPG/POPC membrane surface, and the surface charge of the membrane is reduced.

The ^{31}P longitudinal relaxation time (T_1) changes upon addition of the peptide to the membrane at 25 °C as shown in Table 1. The ^{31}P relaxation time T_1 of POPC MLVs decreases slightly from 763 to 755 ms within the experimental error when the peptide is added into the bilayers. The error was calculated by the average ^{31}P T_1 obtained from three individual samples with the same composition. Conversely, the ^{31}P T_1 values increase significantly for both POPG lipids and POPC lipids once the peptide is integrated

Table 1: The Comparison of ^{31}P Longitudinal Relaxation Times T_1 between Control MLV Samples and MLVs with 4 mol % KIGAKI for Both POPC MLVs and POPG/POPC MLVs at 25 °C

		^{31}P T_1 (ms) MLVs control	^{31}P T_1 (ms) MLVs with 4% peptide	^{31}P ΔT_1 (ms)[a]
POPC MLVs	POPC	763 ± 3	755 ± 5	−8
POPG/POPC MLVs	POPG	764 ± 3	805 ± 5	+41
	POPC	768 ± 2	800 ± 6	+32

[a] ^{31}P ΔT_1 (ms) = ^{31}P T_1 (MLVs with 4 mol % peptide) − ^{31}P T_1 (MLVs control).

into the POPG/POPC MLVs at 25 °C (Figure 4). The increase in the spin−lattice relaxation time upon addition of the peptide indicates a less efficient longitudinal relaxation mechanism, probably caused by the interaction between the peptide and lipids and therefore a reduction in the fast axis rotational motion of the lipids.

Figure 4A exhibits the ^{31}P T_1 value changes as a function of temperature before and after addition of the peptide into the POPG/POPC MLVs. The ^{31}P T_1 value of POPG/POPC MLVs decreases with increasing temperature from 5 to around 15 °C, and then increases at temperatures above 15 °C. The ^{31}P longitudinal relaxation time T_1 is related to the correlation time (τ_c) of fast molecular motion according to $1/T_1 \propto \tau_c/(1 + \omega_0{}^2\tau_c{}^2)$, where ω_0 is the Larmor frequency. If the molecular correlation time fits the equation $\omega_0\tau_c \cong 1$, the relaxation mechanism is the most efficient, and the T_1 relaxation time is at the minimum (*26*, *27*). The molecular correlation time changes as the corresponding molecular motion increases at higher temperature. The relaxation process is most efficient around 15 °C for both POPC and POPG, where the molecular correlation time τ_c is approximately equal to $1/\omega_0$. A significant difference between POPC and POPG lipids is found at 35 and 45 °C for POPG/POPC membranes with the addition of 4 mol % peptide. POPG lipids experience a large increase in their corresponding ^{31}P T_1 values, while the ^{31}P T_1 values of POPC lipids only increase slightly at high temperatures.

Figure 4B shows the difference in the ^{31}P relaxation time T_1 values (^{31}P ΔT_1) between the control sample and POPG/POPC MLVs with 4 mol % KIGAKI at variable temperatures for both POPG and POPC phospholipids. Generally, the ^{31}P T_1 value increases upon association of KIGAKI with the lipid bilayers. Over a wide temperature range (−5 to 45 °C), the increase in the value of the POPG ^{31}P relaxation time T_1 is greater than the corresponding increase in the POPC ^{31}P relaxation time T_1. However, at 45 °C, the POPG ^{31}P relaxation time T_1 increases in the presence of KIGAKI, whereas the POPC ^{31}P T_1 decreases in the presence of the antimicrobial peptide.

Additionally, a ^2H longitudinal relaxation study for POPC bilayers with POPC-d_{31} utilizing an inversion−recovery pulse sequence was carried out at 30 °C. The longitudinal relaxation rate (R_{1z}) profiles for ^2H at different carbon positions along the POPC phospholipids acyl chain are shown in Figure 5A. The relaxation rates ^2H R_{1z} decrease monotonically with C−^2H group position going from the interfacial segments of the membrane to the terminal methyl group of the lipid acyl chain. In the presence of KIGAKI, the ^2H relaxation rates R_{1z} decrease. This is consistent with the results obtained from the ^2H order parameter studies, which

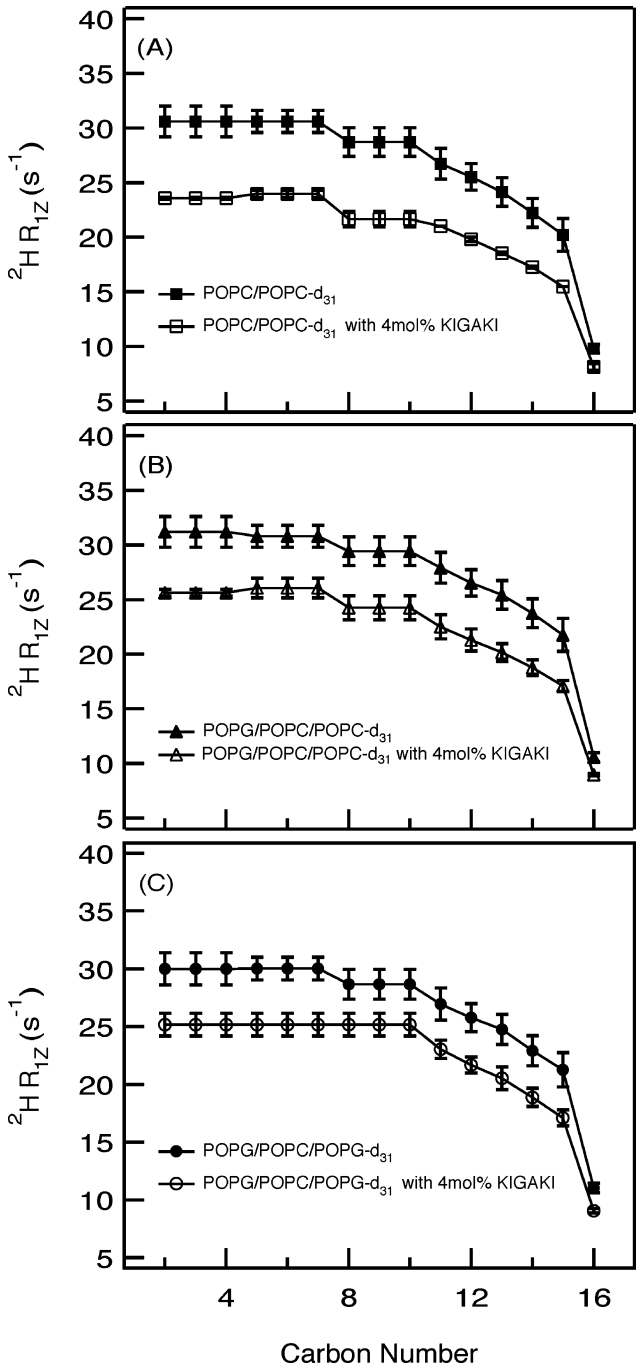

FIGURE 5: ^2H NMR longitudinal relaxation rate R_{1z} profiles with respect to the carbon positions along the acyl chain of deuterium-labeled phospholipids with and without 4 mol % peptide. (A) POPC/POPC-d_{31} MLVs at 30 °C, (B) POPG/POPC/POPC-d_{31} MLVs at 25 °C, (C) POPG/POPC/POPG-d_{31} MLVs at 25 °C. The solid symbols represent the control MLVs data. The open symbols represent the data with 4 mol % peptide. The error of each individual ^2H R_{1z} value is obtained by averaging ^2H R_{1z} values from two samples with the same sample components.

show a slight decrease in the ordering of the C−^2H groups along the whole acyl chain of POPC in the presence of the peptide (Figure 3A). Figure 5, panels B and C, exhibits the ^2H longitudinal relaxation rates R_{1z} of POPC-d_{31} and POPG-d_{31}, respectively in the POPG/POPC (3/1) phospholipid bilayers with and without 4 mol % KIGAKI at 25 °C. The ^2H R_{1z} rates of both lipids decrease upon association of the antimicrobial peptide with the bilayer.

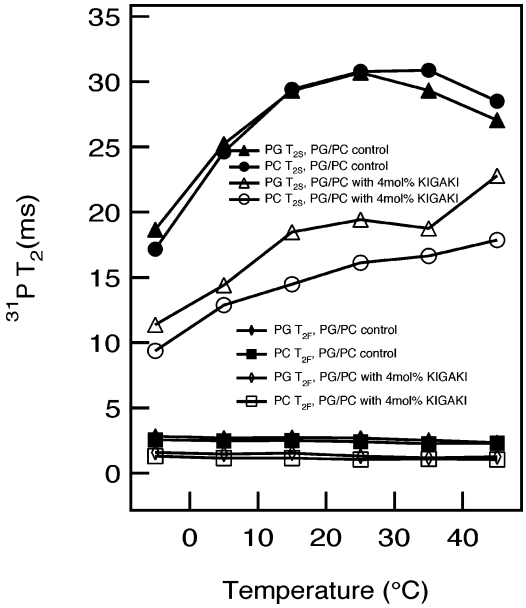

FIGURE 6: ^{31}P transverse relaxation time T_2 as a function of temperature for POPG/POPC MLVs. The solid symbols represent the control POPG/POPC MLVs. The open symbols represent the POPG/POPC MLVs with 4 mol % peptide. The triangles represent the POPG slow relaxation component. The circles represent the POPC slow relaxation component. The diamonds represent the POPG fast relaxation component. The squares represent the POPC fast relaxation component.

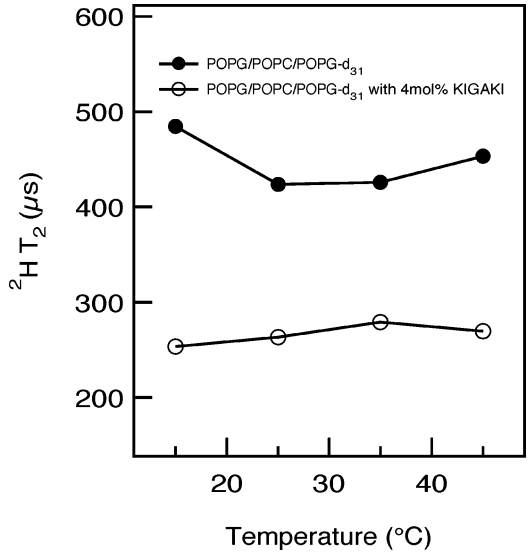

FIGURE 7: ^{2}H transverse relaxation times (T_2) as a function of temperature for POPG/POPC MLVs with POPG-d_{31} as the isotopic label. The solid symbols represent the control MLVs data. The open symbols represent the sample with 4 mol % antimicrobial peptide.

^{31}P and ^{2}H Transverse Relaxation NMR Study. ^{31}P solid-state NMR transverse relaxation times (T_2) were investigated on POPG/POPC lipid mixtures as a function of temperature from −5 to 45 °C (Figure 6). Since a single-exponential fit of the data was not adequate, a biexponential fit for both POPG and POPC phospholipids was used. For the POPG/POPC control sample, the ^{31}P transverse relaxation time T_2 of the fast relaxation component is about 2∼3 ms for both POPG and POPC over the temperature range studied. The T_2 value of the slow relaxation component varies over the range of 20∼30 ms. Upon addition of 4 mol % peptide into the membrane, the relaxation times of both the slow component and the fast component decrease. The transverse relaxation rate is sensitive to changes in slow collective membrane motions (*23*). The reduction in the slow component T_2 relaxation time in the presence of the peptide indicates a more efficient relaxation mechanism caused by the interaction of the antimicrobial peptide with the lipids.

^{2}H NMR transverse relaxation experiments were used to examine the overall slow collective membrane motion for POPG/POPC membranes with POPC-d_{31} or POPG-d_{31}. The transverse relaxation rates at different carbon positions along the acyl chain of POPC-d_{31} or POPG-d_{31} were calculated using the dePaked ^{2}H NMR spectra, and did not show any significant changes between the different C−^{2}H groups. These results confirm that only the collective phospholipid motion dominates the relaxation mechanism. Fast molecular motions, such as *trans-gauche* isomerizations, which are different for each C−^{2}H group, is not the dominant transverse relaxation mechanism. Therefore, the final transverse relaxation rates were calculated by fitting the total area of all the ^{2}H NMR peaks. Figure 7 shows the ^{2}H transverse relaxation time T_2 at four different temperatures for POPG/POPC bilayers with and without KIGAKI using POPG-d_{31} as the deuterium labels. The relaxation time T_2 decreases by a factor of 2 when the peptide is added into the mixed membrane. Similar results were obtained for POPG/POPC bilayers with POPC-d_{31} (data not shown). These results also indicate that the transverse relaxation is more efficient in the presence of the antimicrobial peptide, which agrees with the result obtained from the ^{31}P transverse relaxation experiments. The ^{2}H transverse relaxation time is not very sensitive to temperature changes. Over the experimental temperature range (15−45 °C), the ^{2}H T_2 relaxation time does not change significantly.

DISCUSSION

KIGAKI is known to form a β-sheet structure when bound to anionic POPG lipids (*7*). The minimum inhibitory concentration of the peptide against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* is much lower than the naturally occurring peptides magainin2 and PGLa peptides (*7*, *8*). It is believed that the high percentage of anionic lipids in the bacterial membrane plays a crucial role in the function of antimicrobial peptides. POPC MLVs and POPG/POPC MLVs were used in this study to mimic the mammalian membrane and bacterial membrane, respectively, to test this hypothesis.

The Phospholipid Selectivity of the Antimicrobial Peptide KIGAKI. Static ^{31}P powder pattern NMR spectra clearly indicate that KIGAKI disrupts POPG/POPC bilayers into two major species at 4 mol % concentration, whereas the peptide only slightly perturbs POPC bilayers at twice (8 mol %) this peptide concentration (Figure 1). The ^{31}P NMR powder pattern spectrum of POPC MLVs at 4 mol % peptide concentration only revealed one axial symmetric component. This result supports that KIGAKI has a greater ability to perturb the membrane structure that mimics bacterial membranes (*7*).

The ^{31}P longitudinal relaxation study exhibits a significant increase in the values of both POPG and POPC T_1 upon association of KIGAKI with POPG/POPC MLVs. However, the ^{31}P T_1 values do not change significantly upon association

of KIGAKI with POPC MLVs (Table 1). The ^{31}P longitudinal relaxation time T_1 is sensitive to fast rotational phospholipid headgroup motions (*16*). The increase in the longitudinal relaxation time T_1 indicates a less efficient relaxation mechanism caused by a reduction in the molecular motion of the lipids. The decrease in motion of the lipid headgroups clearly indicates that a strong interaction exists between the peptide and the POPG/POPC phospholipid bilayers. Conversely, there is no significant interaction between KIGAKI and POPC phospholipid bilayers. The slight decrease in the ^{31}P T_1 value upon addition of the peptide to the POPC bilayers indicates a small increase in lipid motion. A subtle increase in the lipid motion would result in a slight decrease in ordering for both the lipid headgroups and the acyl chains. This agrees well with the static ^{31}P and ^{2}H solid-state NMR data (Figures 2a and 3A).

The KIGAKI−Phospholipid Interaction Mechanism. The interaction mechanism between KIGAKI and the membrane was analyzed by ^{2}H solid-state NMR spectroscopy. ^{2}H order parameters along the lipid acyl chains were calculated for both POPC MLVs and POPG/POPC MLVs. Both POPG-d_{31} and POPC-d_{31} deuterium labels were used in the POPG/POPC bilayers to distinguish between the different motional properties of POPG and POPC in the presence of KIGAKI. The ordering along the POPC and POPG acyl chains (Figure 3B,C) does not change upon association of the peptide with POPG/POPC MLVs. This result suggests that there is no significant influence on the packing of the lipid acyl chain upon peptide binding to the POPG/POPC membranes, indicating that the peptide binds to the POPG/POPC membranes near the membrane surface.

The ^{2}H order parameter reveals information about the amplitudes of the acyl chain fluctuations at different carbon positions, while the longitudinal relaxation rates depend on both the amplitude of the motion and the corresponding rate of the motion (*40*). ^{2}H longitudinal relaxation rate R_{1z} profiles exhibit a decrease in the relaxation rate along the POPC acyl chains as well as the POPG acyl chains upon association of the peptide with POPG/POPC MLVs (Figure 5B,C). Considering the ordering of the lipids acyl chains does not show much change in the presence of the peptide, this result clearly indicates that only the rates of the lipids motion are disturbed upon peptide binding to the lipid bilayers.

The ^{31}P MAS spectra of POPG/POPC MLVs show an upfield shift in both POPG and POPC isotropic peaks upon addition of the antimicrobial peptide. The ^{31}P chemical shift of the lipid headgroup is sensitive to the surface charge density (*39*). A decrease in the surface charge density will cause an upfield shift in the ^{31}P isotropic peak (*35*, *39*). Our result indicates that the binding of the cationic antimicrobial peptide to the anionic membrane surface decreases the membrane surface charge density. Conversely, the ^{31}P isotropic peak position of POPC MLVs does not show any shift when the peptide is added. The ^{31}P CSA value of the POPC bilayers is also very sensitive to the changes in membrane surface charge because of its phosphocholine headgroup with a large dipole moment (*34*, *39*). The ^{31}P CSA value of pure POPC bilayer remains unchanged upon addition of the peptide (Figure 1A). These results suggest that the electrostatic interaction plays an important role in the peptide binding to the POPG/POPC phospholipid bilayers but not with pure POPC phospholipid bilayers.

Since the electrostatic interaction is important to the binding between KIGAKI and the mixed POPG/POPC phospholipid bilayers, one would expect that the cationic peptide would prefer to interact with anionic phospholipid POPG. ^{31}P and ^{2}H longitudinal relaxation experiments on POPG/POPC MLVs would show a big difference in the change of T_1 value between POPG and POPC lipids upon addition of the peptide. However, the ^{31}P longitudinal relaxation experiments indicate that both the POPC and POPG lipid T_1 values increase upon association of the peptide with the mixed POPG/POPC phospholipid bilayers at 25 °C. Similarly, the ^{2}H longitudinal relaxation study displays a similar relaxation rate R_{1z} reduction for both POPG and POPC samples in the presence of the KIGAKI peptide. To further elucidate the molecular mechanism of the interaction between the antimicrobial peptide and the POPG/POPC lipid bilayers, ^{31}P longitudinal relaxation studies on POPG/POPC MLVs as a function of temperature were carried out. It is very interesting to observe that the change in the ^{31}P T_1 value for POPG phospholipids is larger than POPC lipids over a wide temperature range (Figure 4B). This result supports the peptide's preference to interact with the POPG lipids and indicates that the interaction between the peptide and the POPG headgroup is greater than that between the peptide and the POPC headgroup.

At 45 °C, the POPC and the POPG phospholipids ^{31}P longitudinal relaxation T_1 values exhibit totally different changes upon association of the antimicrobial peptide with the POPG/POPC bilayers (Figure 4B). The POPC ^{31}P longitudinal relaxation T_1 value decreases, while the POPG ^{31}P longitudinal relaxation T_1 value increases significantly. The increase in T_1 value indicates an interaction between the peptide and lipids and a more restricted phospholipid headgroup motion, while a decrease in T_1 value suggests a relatively less-restricted lipid headgroup motion than the lipids in the control POPG/POPC MLVs. This result can be explained by the changes in peptide−lipid and lipid−lipid interactions upon association of KIGAKI with POPG/POPC bilayers. For the binary mixed bilayer POPG/POPC system, only one ^{31}P CSA value was obtained (Figure 1B,C) in the ^{31}P powder pattern spectra. Since the ^{31}P CSAs of pure POPG and pure POPC bilayers are significantly different, the result indicates a strong interaction between the POPG and POPC headgroups (*37*). Once the peptide is associated with bilayer, the ^{31}P relaxation time T_1 of both of the phospholipids increases at low temperature, suggesting there are probable interactions between the peptide and both of the lipids. The intensity of all these interactions changes as the temperature increases. At high temperatures (45 °C), if the interaction between the POPC headgroup and the peptide becomes very weak and finally broken and the interaction between the POPG headgroup and the peptide is retained, the POPC ^{31}P relaxation time T_1 value would be close to the POPC ^{31}P T_1 value of the control. However, the result demonstrates a decrease in the POPC ^{31}P T_1 value when compared to the control, suggesting that it is important to consider the interaction between the POPC and POPG phospholipid molecules together. It is more possible that the peptide interacts with POPG directly and restricts the POPG headgroup motion. The POPC headgroup motion is only affected indirectly by the interaction between the POPC and POPG headgroups. At 45 °C, the interaction between the POPC

and POPG headgroups decreases in the presence of the peptide, causing the POPC headgroup motion to be faster than the POPC headgroup motion in the control POPG/POPC MLVs. These results suggest that the interaction between the phospholipids is strongly affected upon the peptide binding to the POPG/POPC membranes.

Magainin is a surface peptide and disrupts the membrane via a carpet mechanism (*41*). The antimicrobial peptide first binds at the membrane surface at low concentrations such as a carpet spreading on the floor (*41*). When the peptide concentration reaches a threshold value, the membrane will be disintegrated into small vesicles. It has been reported that magainin associates with the lipid headgroups without significantly disturbing the lipid acyl chain packing even at very high peptide concentrations (*42*). In this paper, the ^{2}H order parameter shows that the acyl chain packing of both POPG and POPC is unperturbed in the presence of the peptide and supports that the peptide binds to the POPG/POPC membranes near the membrane surface. ^{31}P NMR chemical shift and CSA analysis indicate that the electrostatic interaction is important in the binding of the antimicrobial peptide KIGAKI to the POPG/POPC membranes. At 4 mol % peptide concentration, the peptide is able to disrupt the POPG/POPC membranes into two species with one isotropic component, which possesses a faster vesicle tumbling motion than the normal MLVs (*43*). The ^{31}P longitudinal relaxation data suggest that the interaction between the POPG and POPC molecules decreases upon association of the peptide, providing the probable mechanism of the disruption of the POPG/POPC membranes. All of these results suggest that a carpet mechanism may also apply to the KIGAKI peptide perturbing the POPG/POPC membranes.

As to the pure POPC MLVs, ^{31}P NMR powder type spectra and longitudinal relaxation data indicate that there is no strong interaction between KIGAKI and the POPC membranes, although the lipids headgroup motion is slightly perturbed. ^{2}H order parameters show a decrease in the ordering of each C$-^2$H group along the POPC-d_{31} acyl chain upon association of the antimicrobial peptide with the POPC MLVs (Figure 3A). Additionally, the longitudinal relaxation rate ^{2}H R_{1z} of POPC acyl chains also decreases in the presence of the peptide (Figure 5A). The ^{2}H NMR data suggest that the acyl chain packing of the POPC bilayers is disturbed. Considering the amphipathic characteristics of the peptide, the peptide may randomly aggregate in the phospholipid headgroup region and create space between different phospholipid molecules. The loose packing phospholipid molecules, therefore, are able to move more freely and exhibit a decrease in the ordering of the entire acyl chains. Similar effects on ^{31}P and ^{2}H NMR spectra were also observed on DMPC membranes by antimicrobial peptides derived from Australian frogs (*44*). A previous study on KIGAKI using fluorescence spectroscopy indicates that there were no tryptophan emission intensity changes at 330 nm upon peptide binding to pure POPC large unilamellar vesicles (LUVs), suggesting that the peptide remains in a very hydrophilic environment upon peptide binding (*8*). LUVs have a significantly high hydration level (lipids concentration ~1 mg/mL in buffer) when compared to MLVs (lipids concentration ~76 mg/190 μL in buffer). The peptide may prefer to stay closer to the membrane aqueous surface when a bulk aqueous solution exists in LUVs. However, in MLVs, it is more likely that KIGAKI inserts into the lipid headgroup region since the perturbation in the motion of the lipids is clearly observed in both the lipid headgroup and acyl chain regions.

In conclusion, the cationic antimicrobial peptide acts differently in POPC and POPG/POPC bilayers. For POPC bilayers, the antimicrobial peptide has a weak interaction with the membranes although it does perturb the lipids motion slightly in this study. For POPG/POPC mixed bilayer membranes, the peptide has a strong ability to disrupt the membrane. The electrostatic interaction between the peptide and the membrane surface is very critical in the initiation of the binding. The binding of the antimicrobial peptide KIGAKI to the membrane surface disturbs the interaction between the phospholipids, changes the dynamics of the lipid motion, and thereby makes the membrane more fragile. The results suggest a carpet mechanism for bilayer disruption (*41*, *45*, *46*).

This research obtains information purely from the perspective of the phospholipid membranes and demonstrates that solid-state NMR relaxation experiments, specially designed to probe molecular motions, can be extremely powerful in probing membrane−peptide interactions.

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BI050730P