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Structural changes in a binary mixed phospholipid bilayer of DOPG and DOPS upon saposin C interaction at acidic pH utilizing ^{31}P and ^{2}H solid-state NMR spectroscopy

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Abstract

Saposin C (Sap C) is known to stimulate the catalytic activity of the lysosomal enzyme glucosylceramidase (GCase) that facilitates the hydrolysis of glucosylceramide to ceramide and glucose. Both Sap C and acidic phospholipids are required for full activity of GCase. In order to better understand this interaction, mixed bilayer samples prepared from dioleoylphosphatidylglycerol (DOPG) and dioleoylphosphatidylserine (DOPS) (5:3 ratio) and Sap C were investigated using ^{2}H and ^{31}P solid-state NMR spectroscopy at temperatures ranging from 25 to 50 °C at pH 4.7. The Sap C concentrations used to carry out these experiments were 0 mol%, 1 mol% and 3 mol% with respect to the phospholipids. The molecular order parameters (S_{CD}) were calculated from the dePaked ^{2}H solid-state NMR spectra of Distearoyl-d70-phosphatidylglycerol (DSPG-d70) incorporated with DOPG and DOPS binary mixed bilayers. The S_{CD} profiles indicate that the addition of Sap C to the negatively charged phospholipids is concentration dependent. S_{CD} profiles of 1 mol% of the Sap C protein show only a very slight decrease in the acyl chain order. However, the S_{CD} profiles of the 3 mol% of Sap C protein indicate that the interaction is predominantly increasing the disorder in the first half of the acyl chain near the head group (C1–C8) indicating that the amino and the carboxyl termini of Sap C are not inserting deep into the DOPG and DOPS mixed bilayers. The ^{31}P solid-state NMR spectra show that the chemical shift anisotropy (CSA) for both phospholipids decrease and the spectral broadening increases upon addition of Sap C to the mixed bilayers. The data indicate that Sap C interacts similarly with the head groups of both acidic phospholipids and that Sap C has no preference to DOPS over DOPG. Moreover, our solid-state NMR spectroscopic data agree with the structural model previously proposed in the literature [X. Qi, G.A. Grabowski, Differential membrane interactions of saposins A and C. Implication for the functional specificity, J. Biol. Chem. 276 (2001) 27010–27017] [1].

Keywords: Saposin C; Dioleoylphosphatidylglycerol; Dioleoylphosphatidylserine; Solid-state NMR spectroscopy; Molecular order parameter; Mixed bilayer

1. Introduction

Saposins are a family of a small 80 amino acid heat stable glycoproteins that are essential for the in vivo hydrolytic activity of several lysosomal enzymes in the catalytic pathway of glycosphingolipids [2,3]. Four members of the saposins family, A, B, C and D are proteolytically derived from a single precursor protein, named prosaposin [4,5]. Saposin C (Sap C) is known to stimulate the catalytic activity of the lysosomal enzyme Glucosylceramidase (GCase) and thereby to facilitate the hydrolysis of glucosylceramide to ceramide and glucose [6,7]. In addition, the presence of both Sap C and acidic phospholipids such as phosphatidylserine (PS) is required for

Abbreviations: Sap C, Saposin C; S_{CD}, Molecular Order Parameters; CSA, Chemical Shift Anisotropy; GCase, Glucosylceramidase; CL, Cardiolipin; DOPG, Dioleoylphosphatidylglycerol; DOPS, Dioleoylphosphatidylserine; PC, Phosphoatidyecholines; PG, Phosphatidylglycerol; PS, Phosphatidylserine; DSPG-d70, Distearoyl-d70-phosphatidylglycerol; DMPC, Dimyristylphosphatidylcholine; HCL, Hydrochloric Acid; TFE, 2,2,2 Triflouroethanol; PLL, Poly(L-lysine); NMR, Nuclear Magnetic Resonance; CP-MAS, Cross-Polarization Magic-Angle Spinning; HPLC, High Performance Liquid Chromatography; MALDI-TOF, Matrix Assisted Laser Desorption Ionization Time-of-flight Mass Spectrometry; MLVs, Multilamellar vesicles

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full activity of GCase [8–10]. Deficiency in either Sap C or GCase leads to different variant forms of Gaucher's disease [9–12]. Gaucher's disease is the most common genetic disease affecting Jewish people of Eastern European ancestry and is the most common lipid-storage disorder [13]. In addition, the disease course is quite variable, ranging from no outward symptoms to severe disability and death [13].

Under acidic pH conditions, Sap C is thought to destabilize the phospholipid membrane and facilitate the association of GCase with acidic phospholipid [12,14]. The binding of Sap C to phospholipid vesicles is a pH-controlled reversible process [14]. Since Sap C is a lysosomal protein and pH gradients occur in lysosomes in vivo, the degradation of lipids in the lysosome is proposed to be switched on and off by Sap C [14]. The three-dimensional structure of Sap C has been determined via solution NMR spectroscopy and Sap C was found to have five α-helices [14]. The negatively charged electrostatic surface of Sap C, resulting from its eleven Glu residues (See Fig. 1), needs to be partially neutralized to promote membrane binding [14]. In addition, the solution NMR structure of human Sap C in a detergent environment has been determined [15]. Using fluorescence emission spectroscopy and quenching analysis, a hypothetical model of membrane interactions with Sap C was proposed by Xiaoyang Qi and coworkers [1]. In this model, the amphipathic amino terminus of helix 1 and the carboxyl terminus of helix 5 of Sap C are inserted into the membrane and the middle region of Sap C is exposed to the aqueous phase. The positively charged Lys residues of Sap C, consolidated at 50% of the amino terminal half, are important in recognizing the negative surface of PS-containing membranes for initial binding of Sap C [1].

In addition to its role as GCase activator in the lysosome, Sap C participates in the fusion and destabilization of acidic phospholipids vesicles [12]. Membrane fusion is an important event in secretion, endocytosis, exocytosis, fertilization and intracellular transport [16]. Ying Wang and coworkers studied the mechanism of Sap C-induced membrane fusion using a particle size analyzer and fluorescence spectroscopy [17]. They proposed a "clip-on" model for Sap C-induced membrane fusion. This model proposes that helices 1 and 5 of two Sap C molecules are embedded into two different lysosomal phospholipid bilayer membranes and that membrane fusion is facilitated by the dimerization of Sap C (via helices 2–4) on opposing liposomal membranes [17]. Sap C is fusogenic, since it promotes the biological membrane fusion under acidic pH condition [6,18]. The fusogenic activity of Sap C is influenced by the composition of the membrane [6].

Solid-state NMR spectroscopy has been widely used to study the structure and dynamic of membrane–protein systems [19–24]. Solid-state NMR spectroscopy is an excellent technique for investigating lipid–protein interactions in membranes. Liposomes or phospholipid dispersions are commonly used to mimic and study biological membranes upon peptide insertion and interaction [20,25]. One aim of this study is to study the effect of anchoring of Sap C on the dynamics of the acyl chain of the phospholipid bilayers. ^{2}H solid-state NMR spectroscopy has been reported in the literature to study the effect of peptides altering the dynamic properties of phospholipid acyl chains [26,27]. Measuring the ^{2}H quadrupolar splittings and the disorder over the entire chain length of predeuterated phospholipids upon peptide binding provide a powerful tool to achieve this aim [28].

Recently, the interaction of the fusogenic peptide B18 in its amyloid-state with lipid membranes has been studied via solid-state NMR spectroscopy [29]. On the basis of ^{2}H and ^{31}P solid-state NMR spectroscopy results, it was possible to obtain a differentiated picture of the influence of the B18 peptide on different regions of the phospholipid bilayer. Moreover, ^{2}H and ^{31}P solid-state NMR spectroscopy has been used to study the interaction of Lantibiotic Nisin with mixed lipid bilayers [22]. Additionally, cytochrome *c* has been reported to effect phosphoatidyecholines (PC) more than Cardiolipin (CL) in the PC/CL bilayers using ^{31}P solid-state NMR spectroscopy [30]. In this paper, for the first time, ^{2}H and ^{31}P solid-state NMR spectroscopy has been used to investigate the structural changes in the lipid bilayer upon interaction of the fusogenic peptide Sap C with a PS-containing mixed binary lipid bilayer. Acidic phospholipids such as phosphatidylserine (PS) are required for the full activity of GCase [8,9]. Comparing the magnitude of interaction of Sap C with mixed acidic phospholipids is crucial to understanding the mechanism of Sap C interacting with membranes. Therefore, the second aim of this study is to determine whether Sap C interacts stronger with PS phospholipid than another acidic phospholipid such as phosphatidylglycerol (PG). PG is used in this study because it is a good candidate for studying the interaction of peptides and proteins with phospholipid bilayers [31–33]. Consequently, the binary mixed bilayers of the two acidic phospholipids were used to see if Sap C has a preference to one acidic phospholipid over another. In addition, the molecular miscibility of the two phospholipids (PG/PS) in the mixed binary lipid bilayers will be discussed [34].

2. Materials and methods

2.1. Materials

All synthetic phospholipids such as dioleoylphosphatidylglycerol (DOPG), dioleoyl-phosphatidylserine (DOPS) and distearoyl-d70-phosphatidylglycerol (DSPG-d70) were purchased from Avati Polar Lipids (Alabaster, AL). Sodium acetate (anhydrous) was purchased from (Fisher Scientific). Prior to use, the

1 10 20 30 40 50 60 70 80

SDVYCEVCEFLVKEVTKLIDNNKTEKEILDAFDKMCSKLPKSLSEECQEVVDTYGSSILSLSILLEEVSPELVCSMLHLCSG

Fig. 1. The amino acid sequence of Sap C.

phospholipids were dissolved in chloroform and stored at −20 °C. Chloroform and 2,2,2 triflouroethanol (TFE) were purchased from Sigma-Aldrich (Milwaukee, WI).

2.2. Sap C preparation and purification

The recombinant Sap C was overexpressed in *Escherichia coli* cells using an isopropyl-1-thio-β-D-galactopyranoside-induction pET system [9]. Sap C was expressed with a His6 tag and purified on a nickel column. After elution, Sap C was dialyzed and lyophilized. Dried Sap C was dissolved in 0.1% trifluoroacetic acid and further purified via an HPLC C4 reverse-phase column. The column was washed with 0.1% trifluoroacetic acid for 10 min, and a linear (0–100%) gradient of acetonitrile was established over a period of 60 min. The major Sap C peak was collected and lyophilized and the correct MW was determined using MALDI-TOF (data not shown). Protein concentrations were determined as previously described [35].

2.3. NMR sample preparation

The binary mixed lipid bilayers (DOPG and DOPS) containing 1 mol% and 3 mol% of Sap C were prepared using a slightly modified protocol from the Lorigan lab [20]. The phospholipids were dissolved in chloroform and mixed in a small test tube to give a final molar ratio of (5:3, DOPG: DOPS). The phospholipids in the test tube (total of 80 mg) were dried using a steady steam of N_2 gas for about 20 min. Then, the test tube was placed in a vacuum dessicator overnight to remove any residual solvents. The following day, Sap C was dissolved in 3 mL of TFE and added to the test tube. Next, the sample was dried using N_2 gas. The peptide/lipid mixture was resuspended in 190 μL of 0.1 M acetate buffer (the pH of the buffer was adjusted to 4.70 using HCl) by heating in a water bath at 50 °C along with slight frequent sample agitation to avoid frothing of the mixture. After the phospholipids mixture was completely dissolved, the sample was transferred to a NMR sample tube. DSPG-d70 (3 mg) was added to the samples when performing the ^{2}H NMR experiments.

2.4. NMR spectroscopy

^{2}H and ^{31}P NMR spectra were recorded on a Bruker Avance 500-MHz WB solid-state NMR spectrometer using a Bruker 4 mm triple resonance CP-MAS probe (Bruker, Billerica, MA). ^{2}H NMR spectra were collected at a frequency of 76.77 MHz. A quadrupolar echo pulse sequence was employed using quadrature detection with complete phase cycling of the pulse pairs [36]. The 90° pulse length was 3.8 μs, the interpulse delay was 40 μs, the recycle delay was 0.5 s, and the spectral width was set to 100 kHz. A total of 20,480 transients was averaged for each spectrum and processed using 100 Hz line broadening. The ^{31}P NMR spectra were recorded with ^{1}H decoupling using a 4 μs π/2 for ^{31}P and a 4-s recycle delay. For the ^{31}P NMR spectra, 512 scans were taken and the free induction delay was processed using 100 Hz of line broadening. The spectral width was set to 300 ppm.

2.5. NMR data analysis

DSPG-d70 is used in this work as a ^{2}H NMR probe molecule for the DOPG/DOPS/Sap C system. Powder-pattern ^{2}H NMR spectra of the multilamellar dispersions were numerically deconvoluted (dePaked) using the algorithm of McCabe and Wassall [37,38]. The spectra were deconvoluted such that the bilayer normal was perpendicular with respect to the direction of the static magnetic field. The quadrupolar splittings were directly measured from the dePaked spectra and converted into the S_{CD} order parameter using the following expression [20,39,40]:

$$\Delta\nu_Q^i = 3/4\left(e^2qQ/h\right)S_{CD}^i$$

Where $\Delta\nu_Q^i$ is the quadrupolar splitting for a deuteron attached to the *i*th carbon, e^2qQ/h is the quadrupolar splitting constant (168 kHz for deuterons in C–^{2}H bonds), and S_{CD}^i is the chain order parameter for a deuteron attached to the *i*th carbon of the acyl chain of DSPGd70. The ^{2}H nuclei attached to the terminal methyl carbons were assigned carbon number 17. The remaining ^{2}H assignments were made in decreasing order along the phospholipid acyl chain. Therefore, the corresponding order parameters for the individual C–D methylene groups were directly evaluated from the quadrupolar splittings of the dePaked ^{2}H NMR spectra. The ^{2}H peaks in the NMR spectra were assigned based upon dynamic properties of the individual CD_3 and CD_2 groups. The quadrupolar splitting of the CD_3 methyl groups at the end of the acyl chains are the smallest and closest to 0 kHz because they rotate at the fastest frequency. The ^{2}H attached to the C-16 have the next smallest splitting followed by the ^{2}H attached to the C-15, and so forth along the acyl chain. According to the literature, the quadrupolar splittings of the plateau region can be estimated by

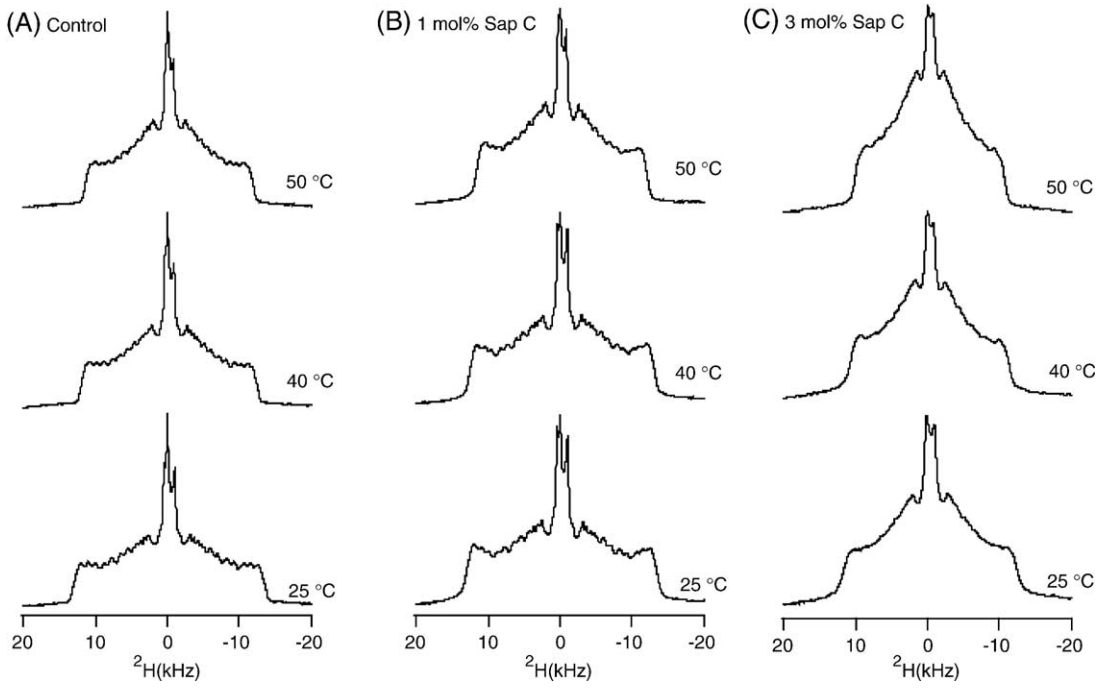

Fig. 2. Temperature-dependent ^{2}H NMR powder-pattern spectra of the DOPG/DOPS system in the absence (A) and in the presence of 1 mol% (B) and 3 mol% (C) Sap C at various temperatures. The temperature at which each spectrum was taken is noted on the right side of that spectrum.

integration of the last broad peak in the ^{2}H NMR spectra [41]. The order parameters calculated for the CD_3 quadrupolar splitting were multiplied by 3 [20,42,43].

The normalized order parameter difference profiles of the DOPG/DOPS/Sap C system were calculated to enable comparison of the relative change in order parameter along the acyl chain upon the addition of the peptide according to equations described in the literature [28]. Simulations of the ^{31}P NMR spectra were carried using the DMFIT software program [44]. Fig. 5 was generated using the MOLMOL software and a G5 Apple Mac computer [45].

3. Results and discussion

3.1. ^{2}H NMR study of sap C interacting with a mixed DOPG: DOPS bilayer

The effect of Sap C on the order and dynamics of the acyl chains of the binary mixed bilayers of DOPG and DOPS was studied using ^{2}H solid-state NMR spectroscopy in the absence and in the presence of 1 mol% and 3 mol% Sap C at various temperatures (Fig. 2). DSPG-d70 is used in this work as ^{2}H NMR probe molecule for the DOPG/DOPS/Sap C system. Clearly, Fig. 2 indicates that the addition of Sap C to the multilamellar dispersions alters the lineshape and the spectral resolution as the concentration of Sap C increases. The loss in spectral resolution is manifested by the disappearance of sharp edges of the peaks especially for 3 mol% Sap C. The changes in spectral resolution of the ^{2}H NMR spectra confirm that Sap C interacts with the acyl chains of the lipid bilayer. The central resonance doublet (splitting $\cong$ 2 kHz) corresponds to the terminal CD_3 groups and the remaining overlapped doublets result from the different CD_2 segments of the acyl chain of the bilayer. Furthermore, Fig. 2 also shows that as the temperature is raised, the spectra retain the overall lineshape in both cases with and without Sap C. However, as the temperature increases, the quadrupolar splittings decrease for all samples with and without the Sap C peptide, indicating that the mobility of the acyl chains increase as the temperature increases. However, at each of the temperatures, the quadrupolar splittings decrease as the peptide concentration increases. Moreover, the reduced quadrupolar splitting is more pronounced for the different CD_2 segments of the acyl chain of the bilayer that are closest to the phospholipid head groups, indicating that the peptide is interacting with the phospholipid head group region and that the peptide is not inserting itself deep into the phospholipid bilayers.

Studies probing the structural and dynamic properties of membrane associated peptides utilizing ^{15}N and ^{2}H solid-state NMR spectroscopy have been reported in the literature [26,28,46,47]. For Sap C, the smoothed segmental C–D bond order parameters (S_{CD}) can be calculated by dePakeing the powder ^{2}H NMR spectra represented in Fig. 2. The S_{CD} order parameters depend upon several averaging modes provided by intramolecular, intermolecular, and collective motions [48–50]. The segmental S_{CD} order parameter describes local orientation or dynamic perturbations of the C–D bond vector from its standard state due to perturbations of phospholipid conformations or dynamics as a result of the addition of Sap C to the lipid bilayer. Fig. 3A reveals a characteristic profile of decreasing order (S_{CD}) with increasing distance from the glycerol backbone for the pure bilayer and for the 1 mol% and 3 mol% Sap C-bound bilayer at 25 °C. In addition, the data indicate that there is more disorder and motion in the first half (C1–C8) than the second half (C9–C18) of the acyl chain upon interaction with 3 mol% of Sap C when compared to the control sample. However, in the order parameter profile of the 1 mol%, Sap C shows a very slight decrease in the acyl chain order when compared to the control sample (absence of the peptide) and this could because at 1 mol%, the Sap C effect on the membrane is too small to be detected by the order parameter profiles. The same trend is observed for the order parameter profiles at various temperatures (data not shown).

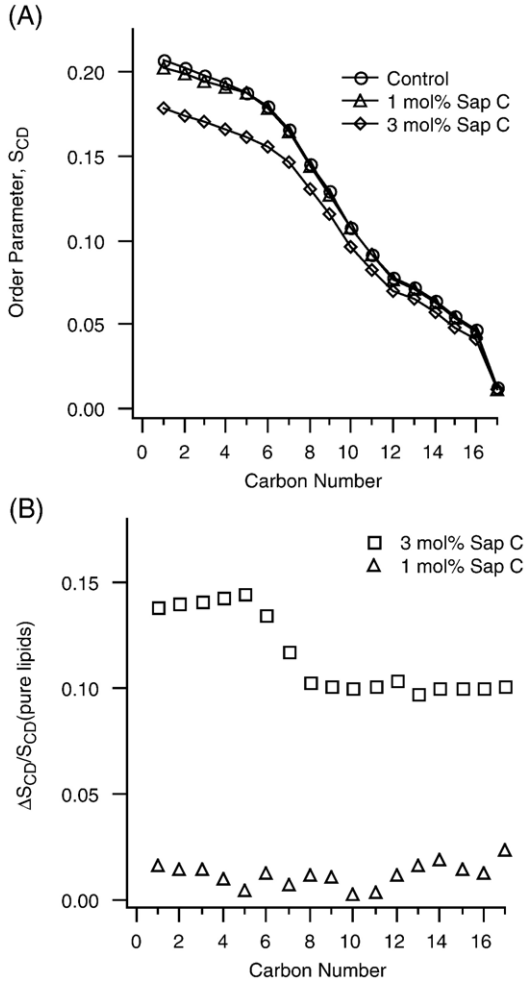

Fig. 3. (A) The smoothed acyl chains orientational order S_{CD} profiles calculated from the dePaked ^{2}H NMR of (Fig. 2) of the DOPG and DOPS mixed phospholipid bilayers at 25 °C. The circles, triangles and diamonds represent the control, 1 mol% Sap C and 3 mol% Sap C samples, respectively. (B) Normalized ^{2}H NMR order parameter difference profiles upon addition of 1 mol% and 3 mol% Sap C to DOPG and DOPS mixed phospholipid bilayers (DOPG: DOPS) at 25 °C.

In Fig. 3B, the normalized order parameter difference profiles were calculated to enable comparison of the relative change in order parameter along the acyl chain of the binary mixed bilayers of DOPG and DOPS upon the addition of 1 and 3 mol% Sap C at 25 °C. Interestingly, the normalized order parameter profiles confirms the conclusion that, relative to the

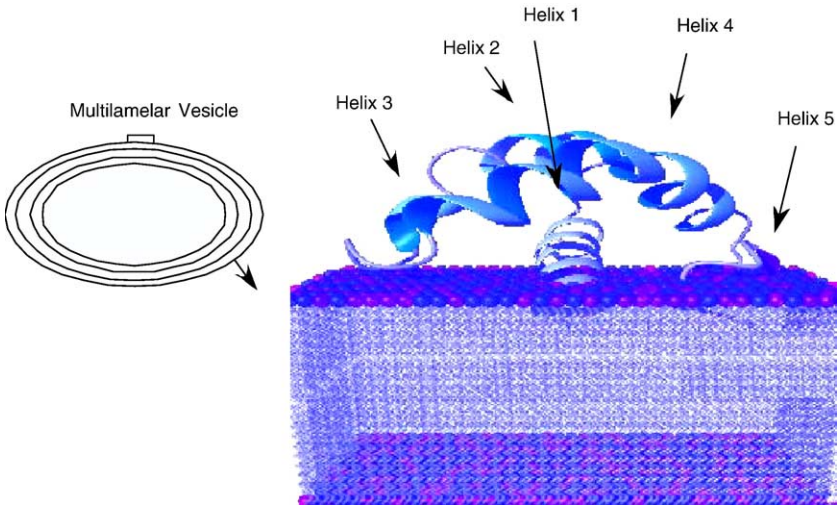

Fig. 4. The previously proposed model of Sap C interacting with MLVs [1]. Helices 1 and 5 of the C molecule are partially embedded into the phospholipid bilayer membranes and the middle region of Sap C is exposed to the aqueous phase [1]. The three-dimensional structure of Sap C is obtained from the published solution NMR study [14].

control sample, there is more disorder and motion in the upper part than the lower part of the acyl chain upon interaction with 3 mol% of Sap C. In addition, in the normalized order parameter difference profile of 1 mol%, Sap C shows a very slight relative decrease in the acyl chain order when compared to the control sample (absence of the peptide) over the entire acyl chain length.

The data suggest that the magnitude that Sap C affects the membrane is concentration dependent. In addition, at 3 mol% Sap C is not inserting deep into the binary mixed membrane, and that the interaction is affecting mainly the first half of the acyl chain (C1–C8) of the acidic phospholipids.

The addition of 3 mol% Sap C to our binary mixed bilayer indicates that the acyl chains near the head groups are more disordered respectively than the hydrophobic core when compared to the control sample. In other words, Sap C is not behaving similar to the in-plane partially inserted peptides like the antimicrobial peptide ovispirin. For ovispirin, the ^{2}H quadrupolar splittings are significantly reduced over the entire chain length of predeuterated phospholipids upon peptide binding [47]. In addition, the antimicrobial peptide LL-37 perturbs the hydrophobic core of lipid bilayers by its in-plane insertion into the hydrophobic/hydrophilic interface of the bilayer [28]. Insertion of the LL-37 peptide is sensitive to the bilayer composition. The insertion depth of the in-plane LL-37 peptide controls the order of the acyl chains. Under certain insertion depth, LL-37 is able to increase the disorder in the lower half of the acyl chain more than the upper half

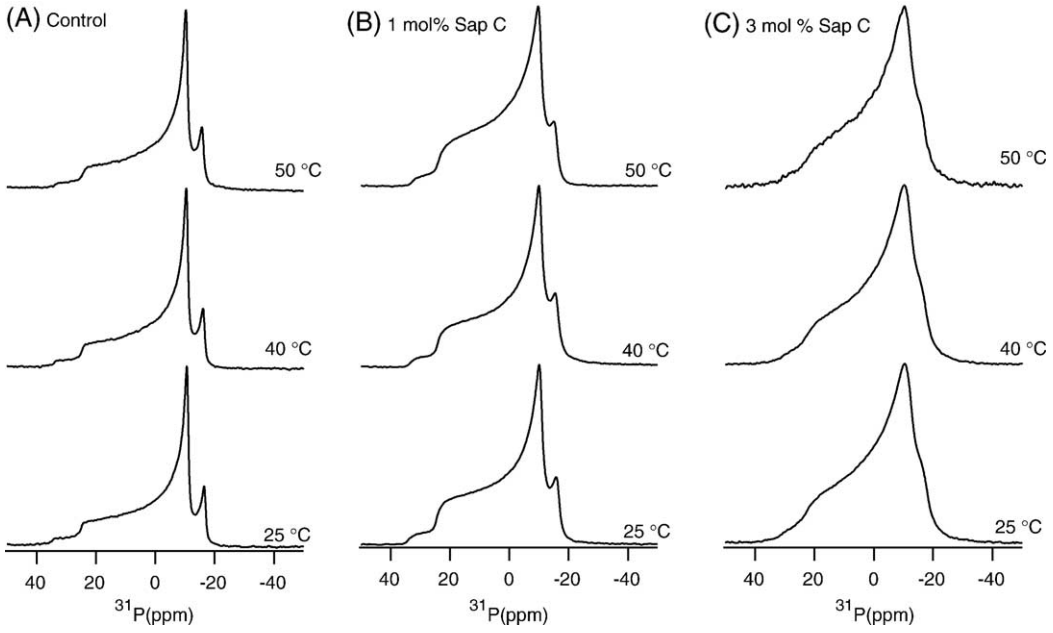

Fig. 5. The ^{31}P NMR spectra of the DOPG and DOPS mixed phospholipid bilayers (DOPG: DOPS, 5:3) investigated at different temperatures. (A) In the absence of Sap C (B) 1 mol% Sap C with respect to the lipids. (C) 3 mol% Sap C with respect to the lipids.

of it because of its effect on the chain packing. However, at 3 mol%, Sap C is not behaving similarly and it mainly alters the first part of the acyl chains suggesting that Sap C does not have an in-plane partial insertion similar to that of LL37. However, this is not the case for all antimicrobial peptides. The surface antimicrobial peptide, magainin, has been reported to associate with the lipid head groups without significantly disturbing lipid acyl chain packing leading to the conclusion that magainin does not penetrate deeply into the acyl chain region [51,52]. Magainin lies on the surface of the bilayer and does not insert deeply into the membrane. This type of membrane distribution is called the carpet mechanism [52]. In this mechanism, the peptide first binds and spreads at the membrane surface at low concentrations like a carpet. Secondly, when the peptide concentration reaches a threshold, the bilayer will be micellized and break into smaller subunits [52]. It has been reported that, even at higher peptide concentrations, magainin associates with the head groups without significantly disturbing lipid acyl chains packing [51–53]. Similar to magainin, our data also show that 1 mol% Sap C does not significantly decrease the disorder of the entire acyl chain suggesting that the peptide could be lying on the surface. However, at 3 mol% concentration, Sap C has the ability to increase the disorder on the upper part of the acyl chain and not alter the lower part of the acyl chain when compared to the control. Thus, suggesting that the Sap C insertion mechanism is different than magainin.

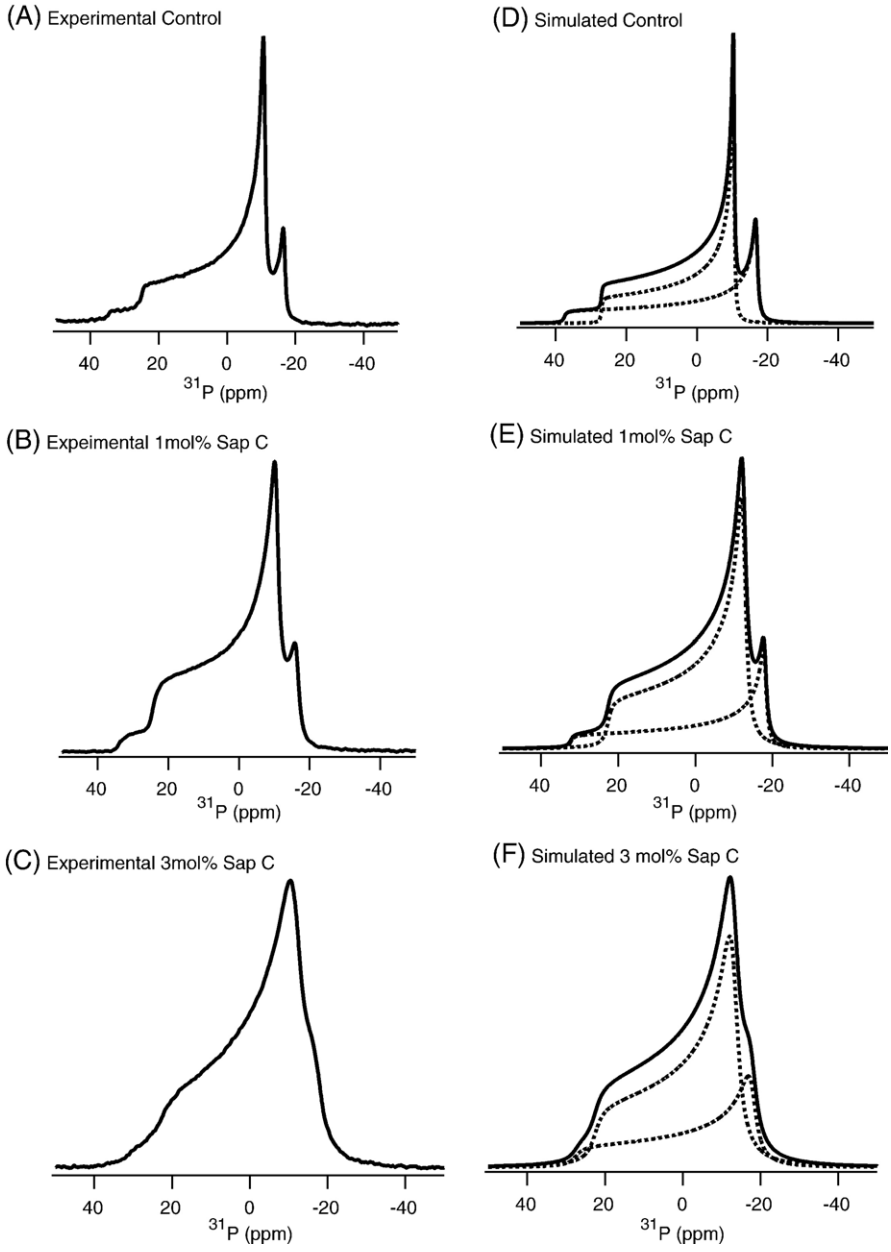

Fig. 6. The experimental and the dotted-line simulated ^{31}P NMR spectra of the mixed DOPG and DOPS bilayers at 25 °C. (A) and (D) in the absence of Sap C. (B) and (E) in the presence of 1 mol% Sap C. (C) and (F) in the presence of 3 mol% Sap C.

In the literature, it has been proposed that only helix 1 and 5 of Sap C are partially inserted perpendicularly in such a way that allows the positively charged Lys residues to interact with the negatively charged acidic phospholipids [1]. Fluorescence quenching of Trp-Sap C using spin-labeled PCs have indicated that Sap C was embedded in the membrane to a depth of ~5 carbon bond lengths [1]. Our data agree well with these findings, which suggest that, the amino and the carboxyl termini of Sap C are not inserting deep into the phospholipids when anchoring the phospholipid membranes. Taking together our current data and previously proposed data from the literature, the interaction of Sap C with DOPS/DOPG mixed bilayers is illustrated in Fig. 4. In this structural model, only helix 1 and 5 are partially inserted leaving the rest of the peptide either spread out on the surface of the bilayer or suspended in the aqueous solution. The three-dimensional structure of Sap C displayed in Fig. 4 was obtained from the solution NMR structure in the literature and is available online in the Protein Data Bank [14].

Our data indicate that at 3 mol% Sap C, the peptide is affecting the disorder of the acyl chain near the head group more than the acyl chain of the hydrophobic core. This unique behavior at 3 mol% Sap C indicates that increasing the peptide concentration perturbs mainly the first half of the acyl chains.

3.2. ^{31}P NMR study of sap C interacting with DOPG and DOPS binary mixed bilayers

The static ^{31}P NMR spectra of the binary mixed lipid bilayers (molar ratio 5:3, DOPG:DOPS) prepared in the absence and the presence of 1 mol% and 3 mol% of Sap C are shown in Fig. 5. Simulations of the corresponding ^{31}P NMR spectra are displayed in Fig. 6. The ^{31}P powder pattern NMR spectra were recorded at temperatures ranging from 25 °C to 50 °C. The motionally averaged powder pattern spectra are characteristic of phospholipid bilayers (multilamellar vesicles, MLVs) in the liquid crystalline phase (Lα) and are expected for DOPG and DOPS at a temperature well above the different chain melting point transition temperatures of −18 °C and −11 °C, respectively [54]. The static ^{31}P NMR spectra for the control (Fig. 5A) show the presence of two different spectral components and each component has a different ^{31}P chemical shift anisotropy (CSA; is equal $\sigma_{33}-\sigma_{11}$) due to the presence of two different phospholipids in the binary mixed phospholipid bilayers. Moreover, Fig. 5A shows that as the temperature increases, the CSA of the two components decrease, indicating that the motion of the phospholipid head groups (axial rotation) increases with temperature. At 50 °C, the CSA was 2 ppm lower than at 25 °C for both components (see Table 1). In order to better understand the binary mixed bilayers (DOPG/DOPS), we studied the ^{31}P NMR spectra (Supplementary materials, Fig. S1) of DOPS and DOPG phospholipid bilayers separately. The difference in the CSA spectral widths between pure DOPG and DOPS bilayers is about 12 ppm. Also, our data indicate that the CSA of the static ^{31}P NMR spectra of pure DOPG and DOPS at pH=7.0 is very close to the CSA value at pH=4.7 (see Table 1). The CSA value of the first DOPG component in the binary mixed bilayers is 35 ppm at 25 °C and 33 ppm at 50 °C and it is the same as the CSA value of the pure DOPG bilayers (See spectral simulation in Figs. 6A and D, and values in Table 1). In addition, the CSA of the second DOPS component in the binary mixed bilayers is 49 ppm at 25 °C and 47 ppm at 50 °C and it is the same as the pure DOPS bilayers at pH 4.7 using acetate buffer (See values in Table 1). The superposition of the two axially symmetric powder patterns suggests that the conformation of the phosphate groups of both DOPS and DOPG have not changed when they where mixed together. The intermolecular interactions and microscopic miscibility of different phospholipids has been discussed in the literature [34]. Some phospholipid binary mixtures try to adapt to form a uniform bilayer structure with an averaged CSA value (in between the two CSA of each of the individual phospholipid components) upon mixing. This adaptation results from strong interpolar-headgroup interactions including hydrogen bonding among these molecules [34]. In contrast, other phospholipids are microscopically immiscible, and do not possess the ability for molecular adaptation and have weak interpolarheadgroup interactions [34]. The DOPG/DOPS binary mixed bilayers in this Sap C NMR study fall into this second category.

Table 1
The chemical shift anisotropy of different phospholipid MLVs with and without Sap C under pH 4.7 and 7.0 at 25 °C and 50 °C

(A) Mixed binary bilayer

Sample	CSA of DOPG component		CSA of DOPS component	
	25 °C	50 °C	25 °C	50 °C
Control (pH=4.7)	35 ppm	33 ppm	49 ppm	47 ppm
1 mol% Sap C (pH=4.7)	34 ppm	32 ppm	48 ppm	46 ppm
3 mol% Sap C (pH=4.7)	32 ppm	30 ppm	46 ppm	44 ppm

(B) Pure phospholipid bilayer

Sample	CSA values	
	25 °C	50 °C
Pure DOPG (pH=4.7)	35 ppm	33 ppm
Pure DOPG (pH=7.0)	36 ppm	34 ppm
Pure DOPS (pH=4.7)	49 ppm	47 ppm
Pure DOPS (pH=7.0)	50 ppm	48 ppm

The spectral simulations were carried out as explained in the materials and methods section. The static ^{31}P NMR spectra for the 1 mol% Sap C (Figs. 5B and 6B) show the presence of the same two components with slightly different CSA values (see the CSA values in Table 1). The CSA values of the two components of the 1 mol% Sap C sample are less than that of the control, indicating that Sap C is interacting with the head group region of the phospholipids and increasing the motion of the phospholipid head groups. Additionally, the lineshapes of the static ^{31}P NMR spectra for the 1 mol% Sap C are broader than the control sample (no Sap C) spectra for both components and this broadening is strong evidence of interaction between Sap C and the two acidic phospholipids in the binary mixed phospholipid bilayers. The static ^{31}P NMR spectra for the 3 mol% Sap C (Fig. 5C) show that the two

components are less distinct but still exist. For the 3 mol% Sap C (see the CSA values in Table 1), the average CSA value of the first component (DOPG) is 31±1 ppm over 25–50 °C temperature range, and the average CSA of the second component (DOPS) is 45±1 ppm over the same temperature range. Moreover, for the 3 mol% Sap C, the ^{31}P lineshape is broader than in the 1 mol% Sap C spectrum, indicating that the head group is more perturbed by the addition of Sap C at a higher concentration. The CSA values of the two components of the 3 mol% Sap C (Figs. 6C and F) sample are 3 ppm less than the CSA values of the control, indicating that increasing the Sap C concentration increases its interaction with the head group region of both phospholipids and increases the motion (axial rotation) of the phospholipid head groups. Additionally, Sap C interacts similarly with the two negatively charged phospholipids and has no preference to DOPS over DOPG.

Some fusogenic peptides have the ability to induce phase transitions in the lipid bilayer from lamellar to non-lamellar lipid arrangements (non-bilayer structures, where isotropic motion occurs) [55,56]. However, no isotropic motion was observed in the 1 mol% and 3 mol% Sap C samples. It has been reported that some other fusogenic peptides have not shown an isotropic peak upon peptide addition [29]. Therefore, Sap C does not have the ability to induce significant phase transitions in the lipid bilayer from lamellar to non-lamellar lipid arrangements under the conditions presented in this ^{31}P NMR study.

In conclusion, the S_{CD} order profiles calculated from the dePaked ^{2}H NMR spectra of DSPG-d70 indicate that the 3 mol% Sap C is not inserting deep into the DOPG and DOPS binary mixed bilayers and that the interaction is mainly perturbing the first 8 carbons of the acyl chain of the acidic phospholipids. ^{31}P NMR spectra for the binary mixed DOPG and DOPS bilayers show that the difference in the CSA values for DOPG and DOPS is about 12 ppm and that the two phospholipids are microscopically immiscible. For the 3 mol% Sap C sample, the ^{31}P NMR spectral simulations indicate that the CSA values for both phospholipids are 3 ppm less than the control sample (without Sap C) and that spectral broadening for both phospholipids is higher than the control sample. Thus, indicating that Sap C interacts similarly with DOPG and DOPS in the binary mixed bilayers. The solid-state NMR data in this paper support the structural model of Sap C proposed in the literature (Fig. 4).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbamem.2005.09.014.

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