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Solid-state NMR study of membrane interactions of the pore-forming cytolysin, equinatoxin II

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ABSTRACT

Equinatoxin II (EqtII) is a pore-forming protein from *Actinia equina* that lyses red blood cell and model membranes. Lysis is dependent on the presence of sphingomyelin (SM) and is greatest for vesicles composed of equimolar SM and phosphatidylcholine (PC). Since SM and cholesterol (Chol) interact strongly, forming domains or "rafts" in PC membranes, ^{31}P and ^{2}H solid-state NMR were used to investigate changes in the lipid order and bilayer morphology of multilamellar vesicles comprised of different ratios of dimyristoylphosphatidylcholine (DMPC), SM and Chol following addition of EqtII. The toxin affects the phase transition temperature of the lipid acyl chains, causes formation of small vesicle type structures with increasing temperature, and changes the T_2 relaxation time of the phospholipid headgroup, with a tendency to order the liquid disordered phases and disorder the more ordered lipid phases. The solid-state NMR results indicate that Chol stabilizes the DMPC bilayer in the presence of EqtII but leads to greater disruption when SM is in the bilayer. This supports the proposal that EqtII is more lytic when both SM and Chol are present as a consequence of the formation of domain boundaries between liquid ordered and disordered phases in lipid bilayers leading to membrane disruption.

1. Introduction

Equinatoxin II (EqtII) is a 179 residue protein from the Mediterranean sea anemone *Actinia equina* that forms pores in red blood cells and is a member of the actinoporin class of pore-forming toxins [1]. Actinoporins are a class of eukaryotic pore-forming toxins found exclusively in sea anemones and, like bacterial toxins, are proteins that act by the formation of pores in the targeted membrane. Pore formation by EqtII occurs via a multi-step mechanism, which includes membrane binding, protein oligomerization and insertion of the N-terminus [2]. The initial binding step, which is relatively non-specific, involves interaction between the lipid membrane and a cluster of exposed aromatic residues. In the presence of sphingomyelin (SM), EqtII undergoes a second binding step where it inserts more deeply into the membrane and is anchored by the N-terminal region [3]. During pore formation the N-terminal region appears to remain helical, increasing from ~15 residues in native EqtII to ~22 residues in the membrane associated N-terminal peptide [4], penetrating the membrane at an angle of approximately 21° to the bilayer normal [2]. A specific lipid interaction with SM has been proposed to drive EqtII binding [5]. In addition, EqtII has been shown to associate at domain boundaries between ordered and disordered phases, as seen in SM/phosphatidylcholine/cholesterol monolayers, and this association may promote oligomerization in membranes [6].

Although the structural changes of EqtII upon membrane binding and oligomerization have not been characterised in detail, in order to understand the mechanism of pore formation and cytotoxicity the effect of the toxin on phospholipid bilayers has been examined [7,8]. Earlier solid-state NMR studies from our group [7] reported the effect of the addition of EqtII (at 1:1000 or 1:250) on dimyristoylphosphatidylcholine (DMPC) and DMPC:SM (10:1) bilayers, which show only minimal lysis. EqtII affected the lipid dynamics and resulted in disruption of the mixed phospholipid bilayer and formation of small vesicles (visualized by electron microscopy) that produced an isotropic peak in the NMR spectra. By contrast, electron micrographs of EqtII (at 50:1) in palmitoyloleoylphosphatidylcholine (POPC):SM (2:1) bilayers appeared to show toroidal pores [8]. These different effects could be due to differences in membrane fluidity or order between the DMPC and POPC bilayers or domain formation at higher SM content. We now report a solid-state NMR study of the effect of the toxin on DMPC bilayers with higher SM content.

Abbreviations: $CDCl_3$, deuterated-chloroform; Chol, Cholesterol; CSA, chemical shift anisotropy; DMPC, dimyristoylphosphatidylcholine; EqtII, equinatoxin II; MAS, magic angle spinning NMR; MLV, multilamellar vesicles; POPC, palmitoyloleoylphosphatidylcholine; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electophoresis; SM, sphingomyelin

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Solid-state NMR is able to determine the phase, order and mobility of membrane lipids [9]. The effect of EqtII on deuterated phospholipid bilayers was probed further by ^{31}P and ^{2}H solid-state NMR spectroscopy. ^{31}P NMR is a sensitive reporter of changes in the lipid–water interface environment and phospholipid dynamics and phase [10]. Similarly, ^{2}H NMR of chain perdeuterated lipids gives insight into changes in chain order and dynamics [11]. Changes in lipid dynamics can be probed by NMR relaxation times, primarily the transverse relaxation time (T_2), which reports on low-frequency motions; and the spin-lattice or longitudinal relaxation time, (T_1), which reports on higher-frequency motions on the μs to ns timescale [12].

Since recent studies using fluorescence confocal microscopy have shown that EqtII tends to accumulate in the phase boundaries between liquid ordered and disordered phases [13], we have investigated the effect of EqtII on the order and phase of mixed DMPC, SM and cholesterol (Chol) bilayers over a range of temperatures. The following lipid compositions were chosen: DMPC:SM (1:1), in which EqtII activity is optimal, DMPC:SM:Chol (1:1:1) for bilayers likely to produce phase separation or domain formation and, therefore, phase boundaries, and DMPC:Chol (1:1) and (2:1) to determine the effect of cholesterol and SM. Understanding the effect of EqtII on mixed lipid bilayers may lead to better understanding of the lytic process by other pore-forming toxins.

2. Materials and methods

2.1. Equinatoxin II and lipids

EqtII was expressed in *Escherichia coli* BL21(DE3) strain and isolated from bacterial cytoplasm, as described previously by Anderluh et al. [14]. Brain sphingomyelin (SM) and chain perdeuterated dimyristoylphosphatidylcholine (DMPC-d_{54}) from Avanti Polar Lipids (Alabaster, USA) and cholesterol (Chol) from Sigma (St Louis, USA) were used as shipped without further purification. Deuterated chloroform ($CDCl_3$) was obtained from Sigma (St Louis, USA).

2.2. Sample preparation of hydrated dispersions

Lipids were dissolved in chloroform/methanol (3:1, v/v) and combined at the appropriate molar ratio to produce 10 mg of lipid per sample. The solvents were removed by rotary evaporation and the sample pumped under a high vacuum overnight. The samples were hydrated to a total volume of 200 μL with de-ionized water or with EqtII in water to give the desired toxin:lipid ratio. Dilute HCl or NaOH was used to adjust the pH to 5.5 or 7.0. The following lipid molar ratios were prepared: DMPC:SM (1:1), DMPC:Chol (1:1), DMPC:Chol (2:1) and DMPC:SM:Chol (1:1:1); and EqtII/lipid molar concentrations of both 1:1100 and 1:250 were examined. The hydrated sample was vortexed and put through at least four freeze–thaw cycles, involving rapid freezing in liquid nitrogen and equilibrating in warm water at 50 °C, to produce multilamellar vesicle (MLV) suspensions. The sample was then centrifuged in a bench-top centrifuge (15000 rpm for 30 min at 4 °C) to produce a lipid pellet and 150 μL of excess water was removed. The lipid pellet was loaded directly into 5 mm NMR tubes for wideline NMR experiments. After acquisition of the static NMR spectra, the 50 μL samples were again centrifuged and ~30 μL of clear supernatant removed. The hydrated lipid pellets were transferred to 5 mm MAS rotors for ^{31}P MAS NMR experiments, including T_2 determination.

2.3. SDS-polyacrylamide gel

The amount of EqtII in the supernatant was estimated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), by comparison of band intensity against a standard 1.0 mg/mL EqtII sample. SDS-PAGE gels were prepared as described previously [15] and visualized using Coomassie Blue stain.

After SDS-PAGE was used to determine the concentration of soluble EqtII in the supernatant, the remainder of the supernatant was lyophilized, weighed and re-suspended in $CDCl_3/CD_3OH$. High-resolution ^{31}P NMR spectra were obtained to determine the ratio of lipids in the supernatant.

2.4. NMR experiments

2.4.1. Solid-state NMR

Static ^{31}P and ^{2}H NMR and ^{31}P magic angle spinning (MAS) NMR experiments were carried out on a Varian (Palo Alto, USA) Inova 300 NMR spectrometer equipped with a Doty (Doty Scientific Inc., Columbia, USA) MAS probe operating at 121.5 and 46.1 MHz for ^{31}P and ^{2}H, respectively. Wideline variable temperature spectra were acquired at 5 °C intervals between 25 and 45 °C. MAS spectra and transverse relaxation (T_2) experiments were acquired at 25 °C and 45 °C. ^{31}P resonances were referenced externally to phosphoric acid at 0 ppm.

^{2}H spectra were obtained using a quadrupole or solid echo sequence ($\pi/2$–τ–$\pi/2$–τ–acquire) [16], with inter-pulse delay τ of 40 μs, 250 kHz spectral width, 90° pulse length of 5 μs, a recycle time of 0.5 s, and 58,000–100,000 transients. A line broadening of 100 Hz was applied. The quadrupole splitting, $\Delta\nu_Q$, observed from each deuterated methylene segment of the lipid acyl chains is a sensitive probe for detecting changes in the overall order of the phospholipid chains and is related to the order of the C-D bond, S_{CD},

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

where the quantity in brackets is the deuterium quadrupole coupling constant, equal to 167 kHz for C–D bonds [17]. Thus S_{CD} can be determined from the observed splitting.

^{31}P NMR was employed to gain information on the local order and conformation in the phosphate region of the polar headgroup [18], as well as the lipid polymorphic phase [19]. ^{31}P static NMR spectra were acquired with a standard spin-echo pulse sequence ($\pi/2$–τ–π–τ–acquire) and proton decoupling, a spectral width of 62.5 kHz, a $\pi/2$ pulse of 4 μs, τ delay of 0.1 ms, a recycle time of 4.0 s and 7500–14000 transients. A line broadening of 100 Hz was applied. ^{31}P MAS spectra of the MLV dispersions were obtained in a 5 mm rotor spun at 7 kHz under the same conditions but using a spectral width of 15 kHz. A line broadening of 10 Hz was applied and 2500–3000 transients were acquired.

2.4.2. Determination of ^{31}P T_2 relaxation times

The ^{31}P relaxation time, T_2, is dominated by slow motions, primarily the correlation time for diffusion of the phospholipid in the membrane bilayer. In MLV, correlation times for phospholipid reorientation range between 8 and 40 ms [20, 21]. Spectra were acquired using a spin echo pulse sequence ($\pi/2$–τ–π–τ–acquire) with τ delays arrayed incrementally between 0.1 and 400 ms. For each array, 5–15 τ values were acquired and 300–800 transients were averaged. At least two experiments were done, each using a different array of τ values. Where possible the peak intensities were determined using the Varian software package, VNMR, but if a peak was under the VNMR threshold the intensity was measured and entered manually. Peak intensity versus twice the τ delay was plotted, and either a single or double exponential curve fitted using Excel (Microsoft). The T_2 values obtained for each experiment were averaged and the range between these values reported.

2.4.3. Solution NMR spectroscopy

Solution spectra were acquired on a Varian Inova 400 NMR, using a 5 mm broad band probe, operating at 162 and 100 MHz for ^{31}P and

^{13}C, respectively. 1D ^{31}P (and ^{1}H or ^{13}C) NMR spectra of the MLV supernatant were obtained after freeze-drying the supernatant and dissolving in $CDCl_3/CD_3OH$ to determine the lipid composition of the supernatant. Spectra were acquired at 25 °C with proton decoupling.

3. Results

The effects of EqtII on the ^{31}P chemical shift anisotropy (CSA) of the phospholipid headgroups and the ^{2}H quadrupolar splittings ($\Delta\nu_Q$) of the acyl chains of perdeuterated DMPC in mixed DMPC, SM and Chol bilayers were determined.

3.1. MLV lipid dispersions

The ^{31}P spectra of DMPC:SM (1:1), DMPC:Chol (1:1), DMPC:Chol (2:1) and DMPC:SM:Chol (1:1:1) MLV at 25 and 45 °C are shown in Fig. 1. At 25 °C a single ^{31}P powder pattern was observed from both phospholipid headgroups, DMPC and SM, for all lipid compositions (Fig. 1), indicating that the lipid species are homogeneously mixed in the lipid bilayer. However, the spectrum of DMPC:SM (1:1) was different, with a less well defined 0° edge (near 25 ppm) and a narrower CSA (41 ppm) relative to the cholesterol containing compositions DMPC:Chol (1:1), DMPC:Chol (2:1) DMPC:SM:Chol (1:1), with CSA of 44, 49, and 52 (±1) ppm, respectively. The ^{31}P spectra are consistent with powder distributions of the lipid phosphates with partial motional averaging due to fast axial rotation of the phospholipid molecules, characteristic of lipids in the fluid bilayer phase in dispersions where the average size of the MLV exceeds 1 μm [18].

As the temperature was increased from 25 to 45 °C, the spectral shape remained similar for all cholesterol compositions, with a reduction in width of ~3 ppm due to the increased averaging of the ^{31}P CSA (Fig. 1). The increase in intensity at the 90° edge of the CSA (~−15 ppm) at 45 °C (Fig. 1) indicates that the MLV are aligning in the magnetic field and the reduced CSA indicates an increase in headgroup motion [22].

The small peak at 0 ppm evident in the spectra of DMPC:SM (1:1) at 45 °C (Fig. 1) indicates the formation of a separate phase where the phospholipids are undergoing faster, almost isotropic, motions, which results in almost complete averaging of the ^{31}P CSA. The formation of this peak at 0 ppm suggests that at higher temperatures the DMPC:SM (1:1) bilayer is less stable and, therefore, possibly more prone to EqtII disruption than the DMPC:Chol (1:1) and (2:1) and DMPC:SM:Chol (1:1:1) dispersions.

The ^{2}H spectra of deuterated DMPC in the mixed lipid MLV are shown in Fig. 2. The spectra of DMPC:Chol (1:1), DMPC:Chol (2:1) and DMPC:SM:Chol (1:1:1) at 25 °C are characterized by quadrupole splittings of 55, 55 and 57 kHz, respectively, from the most ordered CD_2 nearest the glycerol backbone. The quadrupolar splitting due to each deuterium label down the acyl chain is directly proportional to the order parameter S_{CD} for that carbon-deuterium bond [22], with the inner-most ^{2}H splitting of the spectrum assigned to the terminal methyl group, being the most mobile or disordered group of the lipid acyl chain.

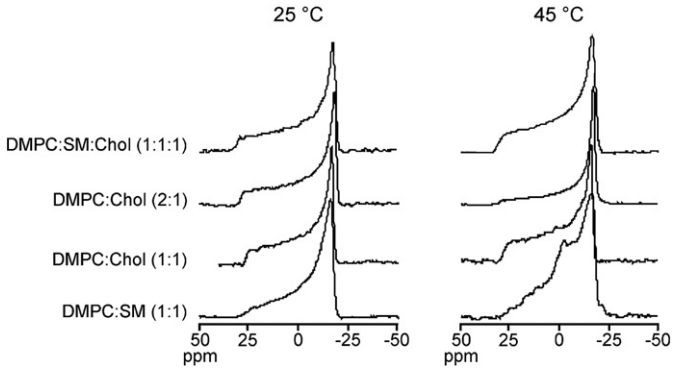

Fig. 1. ^{31}P NMR spectra of MLV dispersions at 25 °C and 45 °C.

The ^{2}H spectrum of DMPC:SM (1:1) at 25 °C (Fig. 2) is different from that of MLV containing cholesterol. The powder pattern of DMPC:SM (1:1) is more rounded and typical of phospholipids in a mixed phase, with a narrower quadrupolar splitting of 34 kHz compared to ~55 kHz for the cholesterol compositions. The more fluid acyl chains were more disordered in the absence of cholesterol compared to the liquid ordered phase of cholesterol-phospholipid bilayers [23], as seen in Fig. 2. An increase in temperature to 45 °C resulted in a small change in the ^{2}H spectra of the cholesterol-containing MLV dispersions. A reduction in the quadrupolar splitting was evident, from 55, 55 and 57 kHz at 25 °C, to 53, 47 and 48 kHz at 45 °C, for DMPC:Chol (1:1), DMPC:Chol (2:1), and DMPC:SM:Chol (1:1:1), respectively, indicating a decrease in the acyl chain order. However, the DMPC:SM (1:1) powder spectrum (Fig. 2) is now characteristic of a fluid bilayer with a quadrupole splitting of 26 kHz, i.e., in the absence of cholesterol the lipid acyl chains experience a higher degree of disorder. The gel to fluid phase transition occurred between 30 and 35 °C (spectra not shown), which is roughly midway between that of DMPC (22 °C) and SM (42 °C) and, together with the ^{2}H powder spectra in Fig. 2, suggests that the lipids are homogeneously distributed.

3.2 DMPC:SM (1:1) in the presence of EqtII

The ^{31}P spectra of the DMPC:SM (1:1) MLV dispersions in the presence of EqtII, are shown in Fig. 3. At an EqtII/lipid ratio of 1:1100 mol/mol, several changes to the ^{31}P spectra were observed when compared to the spectra without toxin (Fig. 1), including the formation of an additional narrow peak at 0 ppm, which broadened at higher temperatures, a decrease in the width of the CSA and a loss of definition at its edges. The lipid CSA was 41 and 42 ppm without EqtII; 50 and 34 ppm for EqtII:lipid (1:1100); and 48 ppm and ~30 ppm for EqtII:lipid (1:250) at 25 and 45 °C, respectively (Table 1). Opposite effects of EqtII were seen at the two temperatures: at 25 °C the addition of the toxin resulted in a restriction of the lipid headgroups whereas an increase in temperature resulted in an accentuated reduction of the ^{31}P CSA in the presence of EqtII. The exact width of the CSA could not be determined because of a loss of definition in the CSA at higher temperature and could result from changes in T_2 [17], as discussed later. The observed changes in the ^{31}P spectra indicate that the toxin produces an increase in disorder of the lipid headgroups and a decrease in size of the MLV at higher temperatures [7].

The isotropic peak at 0 ppm is indicative of almost complete averaging of the CSA, most likely due to the formation of smaller vesicles around 100 nm in diameter [24]. At higher temperatures this peak grew in intensity, indicating an increase in the proportion of more mobile structures relative to the bulk lipid. Further investigation of the isotropic peak indicated that there was some residual phosphate buffer present, but this was only a sharp minor component at 0 ppm and the increases in intensity with temperature are due to EqtII interactions. Broadening of the ^{31}P resonance at 0 ppm at higher temperatures (Fig. 3) suggests that two spectral components may be merging. This may be due to contributions from a multi-component system with a range of different sized MLV dispersions between 100 and 2000 nm [25] resulting in a broad range of incompletely averaged CSA. The effect was reversible when the MLV dispersion was frozen, suggesting that the small vesicles formed in the more fluid phase can reform into larger bilayer structures.

^{2}H NMR spectra of the deuterated DMPC in DMPC:SM (1:1) with EqtII at a 1:1100 and 1:250 toxin/lipid ratios at 25 and 45 °C are shown in Fig. 3 also. These spectra are very different from those obtained for the DMPC:SM (1:1) lipid bilayers in the absence of EqtII

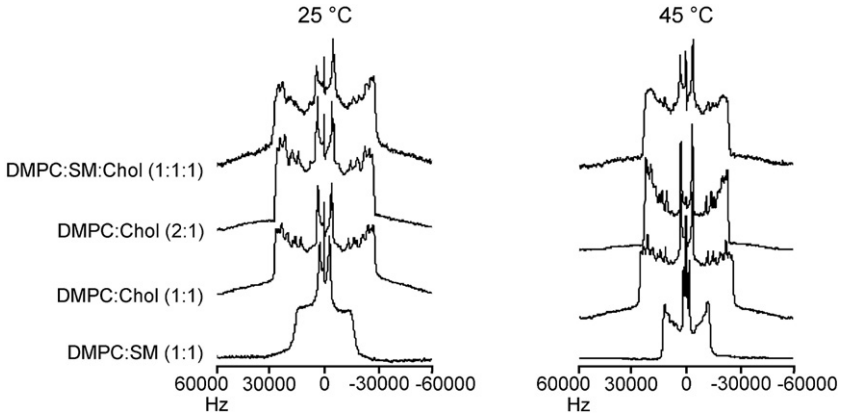

Fig. 2. ^{2}H NMR spectra of MLV dispersions at 25 °C and 45 °C.

(Fig. 2). For both the toxin/lipid ratios, the ^{2}H NMR spectra appear to be a mixture of components. The spectrum below 35 °C is dominated by a broad isotropic peak at 0 ppm overlaid on a very broad bilayer spectrum typical of gel phase lipids. Above 35 °C, the isotropic peak persisted with a decrease in width, but the underlying spectrum was characteristic of CD_2 quadrupolar splittings of lipids in a liquid crystalline phase, typical of fluid phase MLV superimposed with spectra from a range of smaller vesicles. The MLV splittings were similar for both EqtII ratios, with 27 kHz at 1:1100 and 28 kHz at 1:250 (Table 2). The similarity of the ^{2}H spectra at both toxin/lipid ratios, suggests that the higher concentration of EqtII does not result in greater disruption of the lipid acyl chains.

These results differ from the ^{31}P spectra which showed a greater increase of the isotropic peak at ~0 ppm with the higher EqtII ratio, presumably as a consequence of the different timescales of the two NMR interactions. The ^{31}P CSA is of order 6 kHz, whereas the 90° edge of the quadrupolar splitting is of order 50 kHz, so faster isotropic tumbling is needed to average the ^{2}H spectra.

3.3. DMPC:Chol (1:1) and (2:1) in the presence of EqtII

^{31}P spectra of DMPC:Chol (2:1) with EqtII are shown in Fig. 4. The addition of EqtII at both toxin/lipid ratios resulted in similar two-component spectra for both DMPC:Chol (1:1) and (2:1). A narrow peak at 0 ppm characteristic of an isotropic phase was overlaid on a ^{31}P CSA spectrum typical of lipids in a fluid bilayer phase, although the ^{31}P CSA spectra were slightly broadened/rounded relative to the same lipid composition without EqtII (Fig. 1). This may be due to a change in the T_2 relaxation rate of the lipid headgroups, which is discussed below. The isotropic peak, which did not increase with temperature but did with EqtII concentration, showed a contribution from the phosphate buffer, as discussed in the MAS section below. Note that the ^{31}P signal was less intense as half of the lipid composition is Chol.

The addition of EqtII had no significant effect on the width of the ^{31}P CSA of the bilayer lipids, with a small decrease at 1:1100 and no change at 1:250 (Table 1), indicating that addition of EqtII resulted in a negligible change in the disorder of the lipid headgroup when cholesterol was present. Increasing the temperature to 45 °C had a small effect on the width of the ^{31}P CSA, which decreased ~3 ppm with the addition of EqtII, demonstrating that in the presence of cholesterol at the higher temperature there was no increased disruption of the lipids by the toxin.

^{2}H NMR spectra of deuterated DMPC in DMPC:Chol (2:1) following addition of EqtII are shown in Fig. 4. The ^{2}H spectra also appeared to arise primarily from two components, with two narrow splittings around 0 Hz, superimposed on a broader ^{2}H powder pattern, characteristic of ordered lipids in a fluid bilayer for both DMPC:Chol (1:1) and (2:1). The splitting, presumably due to the CD_3 group at the end of the lipid acyl chain, decreased with temperature by 24% and 13% at EqtII:lipid ratios of 1:1100 and 1:250, respectively, compared to that at 25 °C (Table 3, Fig. 5). The methyl splitting for the DMPC:Chol (2:1) showed a greater decrease, with ~45 % for 1:1100 and ~27% for 1:250 EqtII:lipid (Table 3), possibly due to phase separated lipid induced by the toxin. The addition of EqtII to DMPC:Chol resulted in an increase in the quadrupolar splitting for EqtII:lipid (1:1100), but a negligible change for EqtII:lipid (1:250) (Table 2). The increase in lipid acyl order bestowed by the toxin at lower concentrations was not seen at higher concentrations, possibly due to formation of toxin

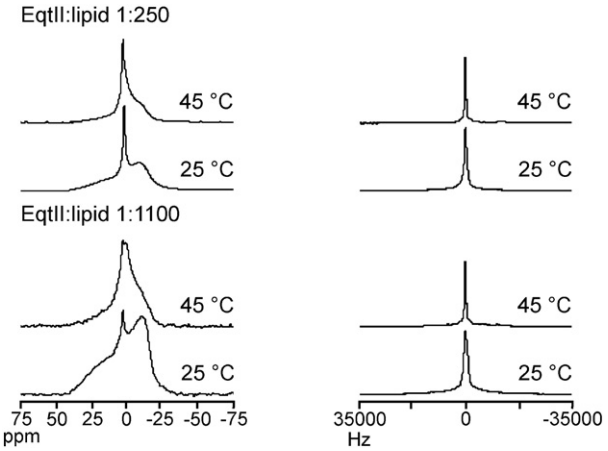

Fig. 3. NMR spectra of DMPC:SM (1:1) MLV dispersions in the presence of EqtII at 25 °C and 45 °C. (Left): ^{31}P spectra, and (Right) ^{2}H spectra.

Table 1
^{31}P CSA (in ppm)[a] of MLV with 1:1100 and 1:250 EqtII:lipid.

Lipid composition	EqtII:Lipid	25 °C	35 °C	45 °C
DMPC:SM (1:1)	Lipid only	41	46	42
DMPC:SM (1:1)	1:1100	50	44	34
DMPC:SM (1:1)	1:250	48	40	n/d
DMPC:Chol (1:1)	Lipid only	44	45	45
DMPC:Chol (1:1)	1:1100	42	43	41
DMPC:Chol (1:1)	1:250	44	44	n/d
DMPC:Chol (2:1)	Lipid only	49	48	48
DMPC:Chol (2:1)	1:1100	46	43	43
DMPC:Chol (2:1)	1:250	48	n/d	45
DMPC:SM:Chol (1:1:1)	Lipid only	52	49	49
DMPC:SM:Chol (1:1:1)	1:1100	46	45	46
DMPC:SM:Chol (1:1:1)	1:250	49	48	n/d

n/d—CSA width not determined due to loss of edge definition for DMPC:SM (1:1) and dominance of large isotropic peak for other compositions.
[a] Uncertainty in CSA ±1 ppm.

Table 2
^{2}H quadrupolar splitting (in kHz) of 90° edge for CD_2 of MLV with 1:1100 and 1:250 EqtII:lipid.

Lipid composition	EqtII:Lipid	25 °C	35 °C	45 °C
DMPC:SM (1:1)	Lipid only	34	29	27
DMPC:SM (1:1)	1:1100	n/d	n/d	27
DMPC:SM (1:1)	1:250	n/d	31	28
DMPC:Chol (1:1)	Lipid only	55	54	53
DMPC:Chol (1:1)	1:1100	59	55	54
DMPC:Chol (1:1)	1:250	56	54	53
DMPC:Chol (2:1)	Lipid only	55	52	47
DMPC:Chol (2:1)	1:1100	57	55	52
DMPC:Chol (2:1)	1:250	55	53	48
DMPC:SM:Chol (1:1:1)	Lipid only	57	53	48
DMPC:SM:Chol (1:1:1)	1:1100	56	54	53
DMPC:SM:Chol (1:1:1)	1:250	56	55	51

Uncertainty ~ ±1 kHz.
n/d—^{2}H splitting values not determined due to lipids in gel phase resulting in a very broad line shape.

Table 3
CD_3 splittings (kHz) for MLV with 1:1100 and 1:250 EqtII:lipid based on the two innermost peaks either side of the large central peak(s) as shown in Fig. 5.

Lipid composition	EqtII:Lipid	25 °C	30 °C	35 °C	40 °C	45 °C
DMPC:SM (1:1)	Lipid only	5.2	4.3	3.8	3.3	3.0
DMPC:SM (1:1)	1:1100	–	–	–	–	–
DMPC:SM (1:1)	1:250	–	–	–	–	–
DMPC:Chol (1:1)	Lipid only	7.2	7.0	6.7	6.6	6.5
DMPC:Chol (1:1)	1:1100	3.8	3.7	3.6	2.9	2.7
	central[a]	1.0	1.0	1.0	0.8	0.7
DMPC:Chol (1:1)	1:250	3.9	3.8	3.6	3.5	3.3
	central	1.0	1.0	1.0	1.0	0.9
DMPC:Chol (2:1)	Lipid only	8.1	7.7	7.2	6.8	6.2
DMPC:Chol (2:1)	1:1100	4.2	4.1	3.9	3.6	3.6
	central	1.0	1.0	1.0	1.0	1.0
DMPC:Chol (2:1)	1:250	5.8	5.6	5.3	4.9	4.6
	central	1.2	1.2	1.2	1.0	1.0
DMPC:SM:Chol (1:1:1)	Lipid only	9.0	8.8	8.7	8.2	7.4
DMPC:SM:Chol (1:1:1)	1:1100	4.9	4.7	4.4	4.2	4.1
	central	1.0	1.0	1.0	0.9	1.0
DMPC:SM:Chol (1:1:1)	1:250	5.6	5.3	5.2	4.6	4.3
	central	1.2	1.2	1.1	1.0	1.0

Uncertainty ~ ±0.1 kHz.
(–) ^{2}H splitting values not determined due presence of a large isotropic peak.
[a] Central ^{2}H splitting (kHz) labelled (b) in Fig. 5.

domains or phase separation, and was more obvious at the higher DMPC concentration DMPC:Chol (2:1). Increasing the temperature did not greatly affect the spectra, but resulted in a decrease in the ^{2}H quadrupolar splitting similar to that seen without EqtII. Upon addition of EqtII, the methyl splitting decreased significantly (Table 3), possibly due to phase separation whereas the inner splitting ~1 kHz (Table 3) is due to the formation of small vesicles.

Comparison of the ^{31}P and ^{2}H spectra showed that at the higher EqtII concentration, the spectra were more similar to those in the absence of EqtII. However, at the lower EqtII concentration, the ^{31}P spectra decreased slightly in width and the ^{2}H spectra increased slightly in width, which suggests disordering of the headgroups and ordering of the chains. Also, the presence of narrow peaks in both the ^{31}P and ^{2}H spectra upon addition of EqtII suggests some small vesicle formation, although not to the extent as when SM is present, which is consistent with Chol preserving bilayer stability [26,27].

3.4. DMPC:SM:Chol (1:1:1) in the presence of EqtII

^{31}P NMR spectra of DMPC:SM:Chol (1:1:1) following addition of EqtII are shown in Fig. 6. Again the spectra consist of two components, a broad ^{31}P CSA characteristic of a fluid bilayer phase in conjunction with a narrow peak at 0 ppm indicative of an isotropic phase and a significant component due to residual phosphate buffer. At both EqtII concentrations the ^{31}P CSA becomes rounded as the temperature is increased from 25 to 45 °C, which could indicate an increase in the T_2 relaxation rate as discussed below.

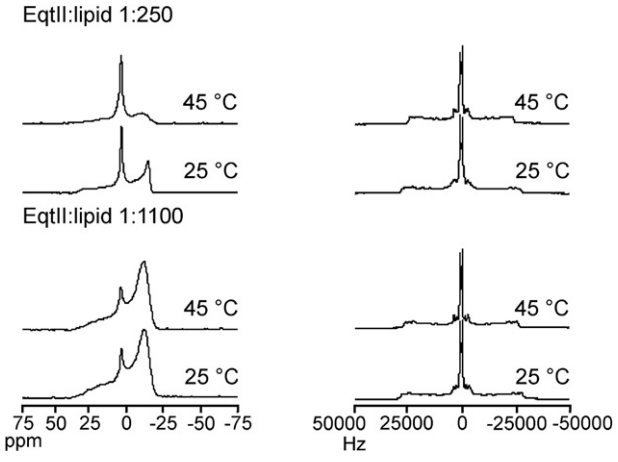

Fig. 4. NMR spectra of DMPC:Chol (2:1) MLV dispersions in the presence of EqtII at 25 °C and 45 °C. (Left): ^{31}P spectra, and (Right) ^{2}H spectra.

The width of the CSA is reduced from 52 ppm without EqtII to 46 ppm for EqtII:lipid (1:1100) but further addition of EqtII to 1:250 gave a CSA of 49 ppm at 25 °C (Table 1). In the absence of EqtII the temperature increase reduced the CSA by 3 ppm, whereas for EqtII:lipid (1:1100) the decrease was negligible. At the higher EqtII concentration there was a loss of definition in the CSA edge, which resulted in the width being difficult to determine at higher temperature and suggests formation of smaller vesicles as the temperature increased for both toxin concentrations.

^{2}H NMR spectra of DMPC:SM:Chol (1:1:1) MLV dispersions following addition of EqtII are shown in Fig. 6. The spectra are similar, with two narrow peaks around 0 Hz overlaid on a broader ^{2}H powder pattern from the intact fluid bilayer. There was a small increase in the isotropic phase when the EqtII concentration was increased. The methyl splittings decreased by ~45% (Table 3), possibly from domain formation induced by the addition of EqtII, and the methyl splitting decreased by ~20% at 45 °C (Table 3). The addition of EqtII had a negligible effect on the width of the ^{2}H bilayer powder pattern at 25 °C, with a splitting of 57 kHz without EqtII and 56 (±1) kHz for both EqtII concentrations (Table 2). The effect of temperature was less, with a 9 kHz decrease seen for lipid alone, now only 3 and 5 kHz (±1 kHz) for EqtII:lipid (1:1100) and (1:250), respectively. This may be due to domain formation induced by EqtII, which could result in enrichment or depletion of one or more of the lipids, resulting in a less fluid bilayer at higher temperatures.

3.5. ^{31}P MAS spectra

MAS NMR of the bilayers at a sufficiently high-speed results in the reduction of the broad CSA to a narrow isotropic component and

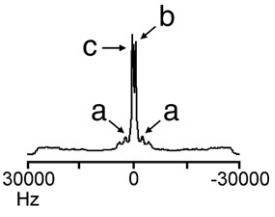

Fig. 5. ^{2}H spectra of DMPC:SM:Chol (1:1:1) with EqtII:lipid (1:1100) at 25 °C showing the a) CD_3 splittings, b) central ^{2}H splitting, and c) ^2H_2O peak.

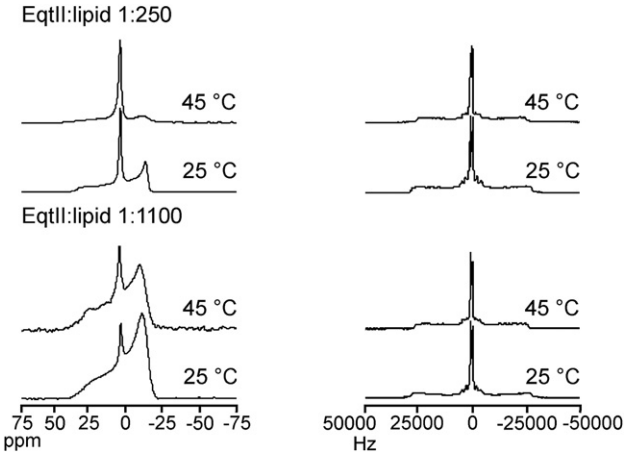

Fig. 6. NMR spectra of DMPC:SM:Chol (1:1:1) MLV dispersions in the presence of EqtII. (Left): ^{31}P spectra, and (Right) ^{2}H spectra.

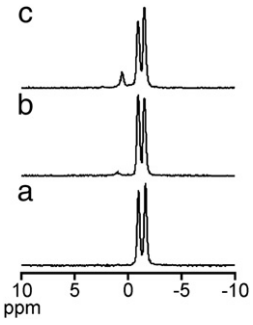

Fig. 7. ^{31}P MAS spectra of DMPC:SM:Chol (1:1:1) MLV dispersions at 25 °C with EqtII: lipid ratio (a) nil, (b) 1:1100, and (c) 1:250.

resolution of different phospholipid resonances [28,9]. MAS was used to examine the differential effect of EqtII on ^{31}P T_2 relaxation times of DMPC and SM. Representative ^{31}P MAS NMR spectra are shown of DMPC:SM:Chol (1:1:1) MLV dispersions (Fig. 7). Two resonances were seen without EqtII, whereas three peaks were evident at EqtII: lipid ratios of 1:1100 and 1:250. The two peaks at −1.0 and −1.5 ppm are due to the SM and DMPC lipid resonances, respectively. Although the two phospholipid peaks are of approximately the same intensity, differences in height are due to differences in width and, therefore, the T_2, the transverse relaxation time. The third, smaller peak at ~0.5 ppm, which increased with EqtII concentration, arises from residual phosphate buffer.

3.6. Effect of EqtII on ^{31}P transverse (T_2) relaxation times

Changes in the ^{31}P T_2 relaxation time are an indication of how low-frequency motions of the lipid are affected by the toxin. The T_2 values at 25 and 45 °C (Table 4) were calculated from the MAS ^{31}P intensity as a function of echo delay and fitted well with a single exponential. Note that at 45 °C both phospholipids would be above the gel–fluid phase transition temperature, whereas at 25 °C only DMPC is above the transition temperature.

The presence of cholesterol increased T_2 (Table 4), which was also evident in the reduced linewidth and higher signal intensity of the cholesterol containing samples. Cholesterol is known to order phospholipid bilayers, producing a liquid ordered phase [29]. The effect of EqtII (1:250) was dependent on the lipid composition and the temperature. The T_2 of DMPC in DMPC:Chol (1:1) without EqtII was 39 ms at 25 °C and 33 ms at 45 °C. The value of T_2 is longer for MLV with Chol than for DMPC:SM as a result of the ordering effect of cholesterol. Upon addition of EqtII to DMPC:Chol (1:1) the T_2 at both temperatures was reduced, but not very significantly: 35 ms at 25 °C and 28 ms at 45 °C. For DMPC:Chol (2:1), T_2 values without EqtII were less: 23 and 17 ms at 25 and 45 °C, respectively; and with EqtII were 31 ms and 20 ms at 25 and 45 °C, respectively. The toxin appeared to have modest effect on the more ordered DMPC:Chol (1:1) and to cause ordering by decreasing the intensity of low-frequency motions for DMPC:Chol (2:1).

The ^{31}P T_2 values of DMPC:SM:Chol (1:1:1) were ~21 and ~22 ms at 25 and 45 °C, respectively. For EqtII (1:250) there was a small increase in T_2 to ~23 ms at 25 °C (Table 4). However, with EqtII at 45 °C there was a more significant decrease in T_2 to ~17 ms. This suggests a slight ordering effect by the toxin at lower temperature (below the gel–fluid phase transition of SM, ~42 °C) and an increase in slower motions above the SM phase transition temperature. The EqtII reduced T_2 at 45 °C to a greater extent, possibly due to an increase in SM mobility above its phase transition temperature.

The DMPC and SM peaks in the DMPC:SM (1:1) sample were not resolved and, therefore, only one T_2 value is given. When EqtII (1:250) was added the phospholipid resonances could be resolved, which is an indication of different lipid environments for SM and DMPC. The T_2 decreased from 6.6 ms to 4.4 and 5.0 ms at 25 °C for SM and DMPC, respectively, and increased from 3.3 ms to ~4.5 ms at 45 °C with EqtII. The toxin appeared to increase the intensity of low-frequency motions at the lower temperature and to reduce the sensitivity of T_2 to temperature for the DMPC:SM bilayers.

3.7. Estimation of EqtII and lipid concentrations

The composition of the supernatant of mixed phospholipid multilayer samples was investigated to determine if EqtII preferentially removes lipid from the bilayer. ^{31}P NMR spectra of the supernatant of the MLV samples dissolved in $CDCl_3/CD_3OH$ (90:10, v/v) were obtained to determine if EqtII preferentially removed a specific lipid from the bilayer. DMPC:SM:Chol (1:1:1) and DMPC:SM (1:1) with EqtII (1100:1) showed similar peak intensities at −1.0 and −2.0 ppm for SM and DMPC, respectively, indicating that EqtII did not preferentially remove either lipid from the bilayer, despite the higher affinity EqtII has for SM [30].

Table 4
^{31}P MAS T_2 relaxation time (ms)[a] of DMPC and SM in MLV with and without EqtII (1:250).

Lipid composition	EqtII:Lipid	25 °C		45 °C	
		SM	DMPC	SM	DMPC
DMPC:SM (1:1)	Lipid only	6.6 (±0.3)		3.3 (±0.2)	
DMPC:SM (1:1)	1:250	4.4 (±0.1)	5.0 (±0.1)	4.5 (±0.1)	4.4 (±0.2)
DMPC:Chol (1:1)	Lipid only	n/a	38.7 (±3.7)	n/a	32.6 (±3.8)
DMPC:Chol (1:1)	1:250	n/a	35.3 (±0.4)	n/a	28.4 (±0.4)
DMPC:Chol (2:1)	Lipid only	n/a	22.8 (±1.1)	n/a	17.1 (±0.5)
DMPC:Chol (2:1)	1:250	n/a	31.3 (±0.1)	n/a	20.4 (±0.4)
DMPC:SM:Chol (1:1:1)	Lipid only	21.1 (±0.1)	21.6 (±0.1)	22.5 (±0.2)	20.8 (±0.8)
DMPC:SM:Chol (1:1:1)	1:250	23.6 (±1.3)	22.8 (±0.2)	17.5 (±0.1)	17.1 (±0.3)

[a] The errors are based on the range over at least two experiments.

The amount of toxin in the supernatant of a DMPC:SM with EqtII (250:1) prior to and following the static solid-state experiments was determined using SDS-PAGE by comparison of band intensity against a standard 1.0 mg/mL EqtII sample. The 100 μL removed immediately after sample preparation was less concentrated in EqtII (~30 μg/ml) than the "supernatant" following NMR spectral acquisition (~120 μg/ml). Almost all the EqtII (~6.3 mg/ml) appeared to be with the MLV and there was no evidence of EqtII being preferentially removed from the MLV.

4. Discussion

A comparison of ^{31}P and ^{2}H NMR of lipid systems in the absence of EqtII shows that the compositions containing cholesterol were more ordered than DMPC:SM (1:1) dispersions and less affected by temperature. At 25 °C, the ^{31}P CSA and ^{2}H quadrupolar splittings for the cholesterol containing lipid dispersions are representative of phospholipids in the liquid ordered bilayer phase, typical of biological membrane. The DMPC:SM (1:1) dispersions, however, were in a gel phase at 25 °C and at higher temperature a small isotropic peak at 0 ppm was evident, indicating the formation of a more disordered phase and suggesting a more fragile bilayer.

The addition of EqtII resulted in the formation of a significant isotropic peak in the ^{31}P and ^{2}H spectra, which was higher in intensity than previously reported for DMPC MLV with only 10% SM [7]. The CSA underneath the isotropic peak showed little change in width upon addition of EqtII for samples containing Chol but led to a significant reduction for DMPC:SM (1:1). The isotropic peak increased in intensity and broadened with temperature, and the effect was reversible. This suggests that the change in lipid morphology was reversible and, therefore, that the possible formation of vesicles and budding on the MLV surface was also reversible. Similar ^{31}P results were seen using total lipid extracts of red blood cells and heart (data not shown).

Generally, the addition of EqtII had little effect on the width of the quadrupolar splitting of the ^{2}H powder pattern except for DMPC:Chol (1:1), which showed a 4 kHz increase in width at the lower EqtII concentration. This increase in splitting also correlates with the decrease in ^{31}P T_2, as though the toxin was ordering the bilayer and increasing the phospholipid correlation time. In the absence of EqtII the ^{2}H quadrupolar splitting decreased with increasing temperature, but this effect was reduced in the presence of EqtII, suggesting that EqtII may increase the phase transition temperature and alter the packing of the lipids.

The effect of EqtII (1:250) on the phospholipid headgroup dynamics was investigated through the change in the transverse relaxation times (T_2). Cholesterol ordered the bilayer and increased T_2, leading to well resolved spectra, whereas the DMPC:SM bilayers had broader lines which were better resolved in the presence of EqtII. The addition of EqtII to the DMPC:SM (1:1) was found to 'round out' the spectra, a change which can be associated with the reduction in T_2 at 25 °C, for the SM and DMPC peaks. However, at 45 °C the effect was opposite upon addition of EqtII, possibly due to an increase in the production of small, highly curved lipid structures as seen in earlier studies with DMPC and 10% SM [7]. The T_2 of DMPC:SM:Chol (1:1:1) was affected by the addition of EqtII in an opposite manner to DMPC:SM (1:1), indicating an increase in low-frequency motions at the higher temperature in the presence of cholesterol, likely due to a liquid disordered phase [31].

EqtII binds preferentially to the SM-enriched liquid ordered over the liquid disordered phase and the presence of a domain interface is required to form pores [13]. This would suggest that EqtII may be in a pore conformation in the case of DMPC:SM (1:1) and DMPC:SM:Chol (1:1:1) MLV, in which lipid domains are able to form [32] and be lysed by EqtII [33]. The ^{2}H methylene and methyl splittings also indicate phase separation in that, when the toxin is present, the bilayer splittings resemble more those of a phospholipid bilayer without Chol. For the liquid ordered bilayers, EqtII led to the formation of smaller vesicles but in the case of the liquid disordered phase POPC:SM (2:1), EqtII appeared to form toroidal pores [8]. Although in the latter the toxin concentration was higher (50:1), EqtII was added to already formed liposomes whereas in the present study EqtII would be on both sides of the bilayer. This could lead to the formation of small vesicles in the DMPC bilayer in the presence of the toxin, which refuse with the larger bilayers, especially with lipids in the liquid ordered phase [34].

The ability to form small vesicles in MLV correlates with the lytic activity of the toxin in unilamellar vesicles. Permeabilisation of small unilamellar vesicles by EqtII [33] has shown that DMPC:SM:Chol (1:1:1) reaches a higher maximum lysis than DMPC:SM (1:1), which previously was thought to be the most lytic composition. The solid-state NMR results indicate that, although the bilayer phase appeared to be more stable with cholesterol in the presence of EqtII, when SM is present greater bilayer disruption occurred. EqtII may be more lytic due to the formation of domain boundaries when both SM and Chol are present in lipid bilayers. In summary, EqtII affects the phase transition temperature of the lipid acyl chains, induces formation of small vesicle type structures in liquid ordered bilayers with increasing temperature and changes the dynamics of the phospholipid headgroup. These bilayer disruptions may indirectly relate to the formation of pores in red blood cell membranes, which occurs at much lower EqtII concentrations.

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